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Bioscience Biotechnology Research Communications
VOLUME-15 NUMBER-1 (JAN-FEB-MAR 2022)

REVIEWS AND CASE REPORTS

RESEARCH ARTICLES

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Bioscience Biotechnology Research Communications

Open Access International Journal

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Editors Communique

Have we tamed the coronavirus? May be yes,
as pandemics do not die, they can only be faded !

Science and technology has made it possible, in the shortest span of time, it has shown that with firm determination and international cooperation, we can win over the onslaughts of even the worst of the pandemics. COVID-19 is perhaps fading over now, due to our coordinated efforts worldwide. Though we have lost millions, in the two year period, partly due to the mishandling of the viral attacks and somewhat by our own follies and carelessness. Anyway lessons learnt from the past, always make us more stronger and determined. Let us now not relax and work on a better mode, as all is still not well yet. The almost taming of the virus and its cousins have indicated some of the concealed failures, on which we have to focus now. We have to be more vigilant, and even a bit of laxity can spoil the good work done. On societal and governmental parts, utmost care and caution is required on a long term basis.

On behalf of Bioscience Biotechnology Research Communications, we falter at words to express our deep sense of solitude and grief on the catastrophic events of the world wide pandemic, spanning over two years now. We pray for the strength to bear this universal calamity and come up with long lasting fortitude to eradicate it soon.

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Quality publication is one of the ways to keep science alive, and good journals have a leading role to play in shaping science for humanity! As teachers, we have great responsibilities, we have to advocate our students to accomplish and show them the path to test their mettle in hard times to excel, especially in the post COVID 19 era. Science and its advocates will rise more to the occasion and will soon provide succor to the already grief stricken humanity.

Sharique A. Ali, PhD
Editor-in-Chief

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Durable Resistance to Rice Blast Mediated by Race-nonspecific Genes in Rice: A Mini Review

Kazutoshi Okuno

Formerly: Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, 305-8577 Japan Present address: 2069-6 Kayada, Yachiyo, 276-0043 Japan

ABSTRACT

Rice blast is a major biotic constraint of rice worldwide, that causes a serious threat to rice production. Genetic improvement of blast resistance is one of important objectives in rice breeding programs. Race-specific resistance genes (R-genes) confer complete resistance to rice blast fungus, but a breakdown of resistance mediated by R-genes has been frequently caused by new races of blast pathogen. To avoid the risk of genetic vulnerability, the use of race-nonspecific resistance has been concentrated in Japanese upland rice varieties whose resistance has been maintained for a long time. However, linkage drag between genes underlying race-nonspecific resistance and undesired traits has hindered its use. Among QTLs detected, a single recessive resistance gene, pi21 was identified by map-based cloning and characterized. The use of pi21 has improved durable resistance in rice breeding programs in Japan. Three QTLs conferring race-nonspecific resistance to rice blast were detected on chromosomes 4 and 12. Among them, a single recessive gene, pi21 was isolated by positional cloning and characterized. This allele has been used for genetic improvement of durable blast resistance through DNA marker-assisted selection in rice breeding programs in Japan and Africa Rice Center.

KEY WORDS: RICE BLAST RESISTANCE, RACE-SPECIFIC, RACE-NONSPECIFIC, QTLS, POSITIONAL CLONING.

INTRODUCTION

Rice blast caused by the fungus Magnaporthe grisea (*Pyricularia oryzae*) is a major destructive disease in rice cropping areas around the world. More than 100 resistance genes to rice blast have been identified and more than 30 of them have been cloned (Ashkani et al., 2016). Race-specific R genes encode nucleotide-binding site (NBS) leucine-rich repeat (LRR) proteins that interact with pathogen effectors and trigger defense reactions according to the gene-for-gene model of recognition. R-genes dramatically enhance blast resistance and result in stable rice production, but their extensive use poses a serious risk of emerging new races of the blast pathogen and the quick breakdown of resistance which is defined as genetic vulnerability. On the other hand, race-nonspecific resistance is quantitatively controlled by multiple quantitative trait loci (QTLs) or genes and is concentrated on the durability of resistance to rice blast, (Okuno and Fukuoka 2020, Maria et al 2021).

Mapping and map-based cloning of QTLs for race-nonspecific resistance: Two resistance QTLs on chromosome 4 and one on chromosome 12 were firstly identified using Japanese resistant rice variety (Fukuoka & Okuno 2001; Fukuoka et al. 2012). Each QTL explained from 13.7% to 45.7% of the total phenotypic variation. One of QTLs on chromosome 4 was inherited as a single recessive gene and was designated pi21. This gene encodes a protein with a putative heavy-metal-binding domain and a proline-rich region (Fukuoka 2001). Comparison of sequences between resistant and susceptible varieties identified 21- and 48-bp deletions in the resistant variety. Transgenic complementation testing confirmed that a loss-of-function mutation in Pi21 improves resistance to rice blast.

Asian cultivated rice (*Oryza sativa* L.) has 12 haplotypes determined by insertion/deletion variations at three sites in the proline-rich region, which is presumed to be involved in protein–protein interactions in multicellular organisms. Inoculation testing using a series of backcrossed lines carrying each of the Pi21 haplotypes in the same genetic background indicated that only the line carrying the haplotype of a resistant variety showed improved the resistance to rice blast. The results suggest that the two deletions in the resistance pi21 allele are optimal to cause the loss of function, which increases the resistance to rice blast.

Durable resistance to rice blast mediated by pi21: Slow induction of defense by pi21 contributes to pathogen control

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without penalty on yield. The pi21 allele is effective against diverse fungus races, so the use of pi21 might not be a strong driving force for changes in the structure of pathogen populations. The durability of resistance mediated by pi21 may be confirmed by prolonged resistance of varieties under natural field conditions.

Monitoring of newly released varieties carrying pi21 will provide further evidence to confirm or disprove the durability of resistance mediated by pi21. Besides, this allele alone may not be sufficient to control blast disease under high disease pressure. Two breeding approaches are proposed to increase the durability of resistance to rice blast in rice, i) the use of multiline varieties carrying different resistance genes and ii) combining multiple resistance genes in the same genotype (Fukuoka & Okuno 2019).

Pyramiding race-nonspecific resistance genes for sustainable control of rice blast: Pyramiding multiple resistance QTL alleles is considered to additively enhance race-nonspecific resistance (Fukuoka et al., 2015). However, knowledge of the impact of QTL pyramiding on the robustness of plant defense in rice is limited. A more important observation is that a QTL pyramid improves the stability of resistance; the coefficient of variation of lesion area across field tests in the line carrying four resistance QTL alleles was smaller than those in lines with only one or two. The study demonstrated the importance of pyramiding of minor QTL alleles for strengthening the durability of resistance, even if the effect of each QTL allele is sensitive to the environment.

CONCLUSION

Three QTLs conferring race-nonspecific resistance to rice blast were detected on chromosomes 4 and 12. Among them, a single recessive gene, pi21 was isolated by positional cloning and characterized. This allele has been used for genetic improvement of durable blast resistance through DNA marker-assisted selection in rice breeding programs in Japan and Africa Rice Center.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

Conflict of Interest: Author declare no conflicts of interests to disclose.

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A Systematic Review of Epidemiolocal, and Time-Trend Prevalence of Obesity-Related Comorbidities and their Health Effects in Saudi Arabia

Abdullah Almilaibary*

Assistant Professor of Community Medicine & Epidemiology, Family and Community Medicine Department, Faculty of Medicine, Albaha University Alaqiq, 65779-77388, Saudi Arabia.

ABSTRACT

There has been significant westernization in Saudi Arabia and is one of the countries with the highest prevalence rates of overweight and obesity. According to the study, obesity has become a substantial cause of concern in the country, whereby 70% of people experienced the problem. Obesity was associated with obstructive sleep apnea and osteoarthritis. A systematic and comprehensive search of the selected keywords on PubMed, Medline, and Saudi Digital Library (SDL) database was conducted between November 2020 and January 2021. The result of our study suggests that the prevalence of adult and childhood obesity in Saudi Arabia is extremely high and currently it is undergoing a fast-growing rate of obesity crisis. Genetic factors, reduced physical activity, and high caloric intake contribute to its prevalence. Because of its association with other cardiovascular diseases, it is regarded as a significant matter of concern in Saudi Arabia. Obesity currently considered an epidemic and therefore, is a major public health concern.

KEY WORDS: OBESITY, COMORBIDITIES, HEALTH EFFECTS, DIETARY HABIT.

INTRODUCTION

Obesity has been regarded as a significant global health concern and is characterized by excessive body fat that poses a risk to an individual's health. Obesity is currently associated with many health conditions that can affect the individuals' quality of life, impose stress on the healthcare system and impose an economic burden on the country (Althumiri, Nora A et al. 2021).

Obesity is a risk factor as well as a cause of other co-morbidities, including diabetes, cardiovascular disorders, cancers, and other illnesses, leading to diseases and mortality in some situations. There has been a high prevalence of obesity globally throughout the last decade (Memish et al., 2014). Saudi Arabia currently is among top countries with the highest prevalence rates of overweight and obesity, about 40% of the adults were found overweight, while about a quarter were found obese. (DeNicola et al., 2015).

It is argued that there is inadequate research on obesity including its related diseases in the Kingdom of Saudi Arabia (KSA), and this has therefore paused difficulties in government efforts in evaluating and controlling the trend of obesity in the country (El Nashar et al., 2017). Therefore, it is necessary to review the emerging issues and form a basis for future analysis and projections of the prevalence and the incidence of adult obesity.

METHODOLOGY

A systematic and comprehensive search of the selected keywords on PubMed, Medline, and Saudi Digital Library (SDL) database was conducted between November 2020 and January 2021. The search included only those articles that were published in English. The characteristics defined by the Participant, Intervention, Comparison, Outcome (PICO) system and a series of comprehensive electronic searches and filters were applied. Randomized control trials, pre-post (controlled before-after), interrupted time-series studies and cohort and cross-sectional research were included in this systematic review. However, case reports and reviews that were not published in peer - reviewed articles were not included in this study.

RESULTS

Memish and colleagues conducted a national survey about obesity and the associated risk factors in KSA. The research was conducted through interviews of about 10,000 individuals, aged 15 and above, to collect information on

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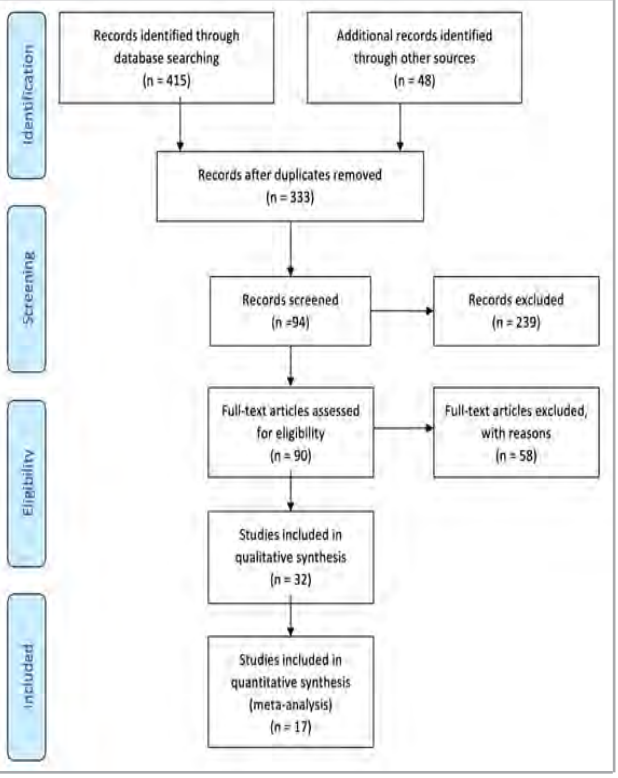
their social and demographic features, physical activities, dietary and health-related habits, accessibility to health services, and respondents' chronic diseases. The interviews to determine the factors were computer-assisted personal interviews. Of the total respondents 28.7% were found obese, that is, BMI higher than 30 kg/m2, which is more common in women than men. Amongst men, obesity was found to be related to the dietary pattern, physical activity, diabetes, hypercholesterolemia, hypertension, and marital status. Amongst the female obesity is linked to a positive history of chronic illnesses, hypertension marital status and education (Memish et.al. 2014).

Figure 1: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram.



A recent study (Horaib et al., 2013) discovered that, the obesity may be a problem brought about by unhealthy dietary habits, lack of physical exercise, consumption of unhealthy food, and the increased psychological stress. Horaib et al. examined the relationship between obesity-associated gene (FTO), fat mass, obesity (BMI), glucose, and other metabolic related traits in Saudi population. It is important to note that the FTO gene's effect can be a factor that could influence the success of efforts to curb obesity, for example, lifestyle interventions (physical activity/ dietary habits).

On the other hand, this gene performance may be influenced by environmental or lifestyle factors. At that the FTO can impact on heightened food consumption and craving for high-calorie foods bringing about a lifestyle; this with factors such as age put into consideration whereby it mainly occurs between 7 and 20 years hence obesity due to FTO's is most common in childhood and adolescents seen through SNPs (Kalantari et al.,2016). The study data is that of 186 female university students, whereby one third have higher glucose levels than usual while a tenth is not obese. However, 50% of the "T" allele students have FTO which is heterozygous.

Understand lifestyle factors and their contribution to obesity among the youth is vital. Data collected from 1400 female and 2906 male secondary school students ascertained this fact. Study participants were living in Al-Khobar, Riyadh and Jeddah, KSA (Al Hazzaa et al., 2014).

Obesity and overweight among males and females have a prevalence of 43.6% and 34.8%, respectively. This is based on anthropometric measurements such as BMI, dietary habits, and physical exercise. Obese adolescents are found to be less active compared to non-obese adolescents. It was found that obesity is significantly inversely proportional to the level of physical activity, vegetable intake, the consumption of sweetened beverages and frequency of meals consumed (Al Mohaimeed et al., 2015).

A study (Elbadawi et al., 2015) to address obesity in school going children in Saudi Arabia, consisting of primary school children aged 6-10 years, show that the average Basal Mass Index is high, while the average body fat is relatively lower in females than males. Therefore, the obesity prevalence is higher in females than in males. The result of our study suggests that the prevalence of adult and childhood obesity in Saudi Arabia is extremely high and currently it is undergoing a fast-growing rate of obesity crisis. Genetic factors, reduced physical activity, and high caloric intake contribute to its prevalence. Because of its association with other cardiovascular diseases, it is regarded as a significant matter of concern in Saudi Arabia.

DISCUSSION

The studies also highlight the association between obesity and diabetes, hypertension, and hypercholesterolemia and what can be termed as a gap that has led to the problem's escalation is the lack of deep understanding and consideration of the risk factors and their relationship. As earlier mentioned, the increase in weight is a contributive aspect of genetics, individual behaviors, and environmental or social-economic factors. The result indicates that obesity is a significant economic burden in Saudi Arabia (Malkin et. al. 2022). Some of the points to be considered in individual behaviors are dietary tendencies; this is directly related to body weight outcomes due to caloric intake. The other individual behavior factor is physical activity. Environmental factors are the neighborhood characteristics regarding healthy foods environments compared to environments with restaurants and fast-food joints, presence of recreational facilities to encourage physical activity, etc.

Another study to determine the prevalence of obesity and overweight among clients attending health centers in Southwestern region of the kingdom, in the year 2019 showed that obesity has become a significant health issue of

this 21st century since it has significantly contributed to the occurrence of cardiovascular conditions in most third world countries. The study examined 1681 adult patients, and from the findings of the study, the prevalence of obesity and overweight was 27.6% and 38.3% respectively prevalence of hypertension was highly associated with obesity. An outstanding finding was that the prevalence of overweight and obesity was linked to a higher monthly income. Therefore, according to the study, obesity and overweight are major public health concerns that require interventions in a bottom-up approach, starting with community level and primary prevention programs (Al-Qahtani,2019).

Over the last few years, the overweight and obesity rates of have increased mainly due to consumption of food with high caloric content and a decrease in physical activity. Weight management, characterized by increased physical exercise brings forth better health outcomes for obese individuals. Increased physical exercise for obese individuals increases physical fitness and decreases the risk for the development of eating disorders such as bulimia nervosa and anorexia nervosa, which contribute to psychosocial effects. The study pointed out that there is limited research on the incidence and prevalence of obesity and overweight at the primordial, community-level (Al-Qahtani,2019).

An array of factors that contributed to the rising incidence of obesity, in broader terms, were classified as environmental, behavioral, and biological factors. Behavioral factors included high-fat consumption and sedentary lifestyles. The study also linked obesity with high socioeconomic status. People with a high socioeconomic status spent less energy in daily activities; thus, energy intake was more than body requirements. The study concluded that obesity prevalence differed among various age groups and occupations. The risk factors included family history and dietary patterns. It also emphasized that its vital to create awareness on obesity and overweight and formulate strategies to address these disorders (M Alqarni, 2016).

A study focusing on the prevalence of overweight and obesity among children between two to twenty years demonstrated the epidemiological features indicating that, eastern Saudi Arabia had the highest obesity prevalence rate. The risk factors were higher parental education and a sedentary lifestyle. It was concluded that there is an increasing rate of obesity in childhood in Saudi Arabia which necessitates immediate intervention. There is a need to address the cultural factors that are associated with the high rates of obesity in the country (Hammad et al., 2017). Drastic changes have occurred in the last four decades, which is notable from the significant rising prevalence and occasions of diseases related to a sedentary lifestyle, including ischemic heart diseases, metabolic disorders, and hypertension (Al Daghri et al., 2011).

Obesity and overweight are the two most common causes of coronary artery disease, especially in the female. KSA conducted a national health survey by obtaining data among over 15,000 households between the ages of 30-70 (Ahmed et al., 2014); the researchers reported the prevalence of obesity and overweight at 35.5% and 36.9% respectively. Females are considered more obesity, while males are significantly more overweight. Another risk factor that resulted from this is the rise of diabetes cases. This is mainly the rise of type two diabetes amongst individuals of 45 years and above if they are found to be overweight or obese. Moreover, excess weight gain amongst young adults may present as a significant risk for diabetes (Reilly, 2011).

Cancer is another factor associated with obesity. Statistics site that an estimated 6% of all cancers are attributed to obesity. The percentage goes up to 7% in women and is about 4% in men (Elkum et al., 2014). Obesity in women thus has been understood to cause breast cancer. A study was conducted to determine this consisted of other different causal factors put in place; in this respect, therefore, the selected sample consisted of illiterate, employed or married women who were obese. Furthermore, in the study, age was also a consideration conducted between women of 22-75 years. According to the results stage, II tumors stood at 56.7%, while stage III tumors were 38.4% (Elkum et al., 2014). According to a study conducted on school-going children to determine the impact of obesity on physical, social, and emotional behaviors, 46% of obese children showed increased stress levels, which interfered with their interaction with other kids (Al-Agha et al., 2016).

With all those outlined, additional research on the interventions put in place, their responses, and impacts resulting from the interventions. It is essential to conduct this on diverse individuals and with individuals with different characteristics. Genetics, individual behaviors, environmental or social-economic should be deeply examined and discussed since they form the critical aspects of most challenges. Other research gaps that could be conducted are on the relationships between the above factors and their expediting overweight and obesity prevalence in the Kingdom of Saudi Arabia.

CONCLUSION

As obesity becomes more common, so do the risk factors, especially in Saudi Arabia, where it is more prevalent than it is worldwide. Obesity treatment typically involves one of three treatment modalities, with psychosocial therapies receiving much less attention. Public health experts, practitioners, and policymakers are urged to apply results to speed up the decrease in the incidence of obesity throughout Saudi Arabia. Obesity currently considered an epidemic and therefore, is a major public health concern.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

Conflict of Interest: Author declare no conflicts of interests to disclose.

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Population Status, Distribution, Threats and Conservation of Blackbuck *Antilope cervicapra* in South Asia: An Updated Literature Review

Rabia Tahir,[1] Fozia Afzal,[2] Samra,[3] Anabat Bin Sohail,[4] Sobia Abid[2] and Muhammad Wasim Tasleem2*

[1]College of Animal Science and Technology, Sichuan Agricultural University, Ya'an, China
[2]Department of Zoology, The Islamia University of Bahawalpur, Bahawalpur, Pakistan
[3]Department of Zoology, University of Agriculture Faisalabad, Faisalabad, Pakistan
[4]College of Resources, Sichuan Agricultural University, Ya'an, China

ABSTRACT

Blackbuck, *Antilope cervicapra*, is a diurnal ungulate species with distinct sexual dimorphism and spellbinding beauty. Male blackbuck has mesmeric beauty with its unique darker coat, showing the increased intensity of color with age. Blackbuck is endemic to Pakistan, Nepal, India and Bangladesh but now its population is reduced to a few areas. Threats such as hunting, stress, habitat loss, diseases, poaching, road accidents, habitat fragmentation, interspecific competition, predation pressure etc., have reduced the population size of blackbuck to a threatened level. Therefore, different conservation strategies are underway to increment its count for improvement of faunal diversity, tourism development and dispersal of the local culture in South Asia. Captive breeding of species is the most efficacious conservation strategy in South Asia so far. Furthermore, various rules and regulations along with strategies like hormone-mediated conservation by injections of prostaglandin and artificial insemination are assisting the species by increasing its birth rate. Genetic studies, introduction to non-endemic but suitable habitat and religious affiliation of communities also contributed to blackbuck conservation. Current conservation practices are helping to conserve the blackbuck but are associated with a few concerns also, thereby proper management, planning, monitoring of conservation practices is required. Population size, distribution range, threats confronted by species, conservation practices and recommendations have been discussed in this article, which will help in advancement of work in this area.

KEY WORDS: BLACKBUCK, CAPTIVE BREEDING, CONSERVATION STRATEGIES, CONSERVATION STATUS, DISTRIBUTION RANGE.

INTRODUCTION

Blackbuck, (*Antilope cervicapra*) (Linnaeus, 1758) is native to Pakistan, India, Nepal and Bangladesh with presence of some individuals in UAE, Argentina, USA and Texas (Wright and Glaze 1988; Mallon and Kingswood 2001; Long 2003). It is the single existing member of genus *Antilope* (Ranjitsinh, 1989). Fossils of species are found in the Siwaliks Hills of Pakistan (Lydekker 1878; Pilgrim 1937; Pilgrim 1939; Khan et al. 2006; Chauhan 2008). Species are diurnal ungulate with distinct sexual dimorphism and spellbinding beauty (Van der Geer 2008; Mahato et al. 2010; Saluja et al. 2012; Sheikh and Molur 2014). Males have whorled horns up to 79cm which are absent in females. The color of male progressively turns into darkish with age, tawny to intense brown or black. Female and young ones are yellow at their front and rear. Chin and undersides of legs and chest are white in both sexes. Eyes encircled by a white ring (Sheikh and Molur 2004). The body length of species ranged from 100-150cm with the tail length 10-17cm and body weight for the male ranged between 20-57kg and of female 19-33kg (Roberts 1997b; Sheikh and Molur 2004). From a biological point of view, Blackbuck is part of nature so we require conserving it to maintain the beauty and biodiversity of nature. In addition, it helps in tourism development in the country. Tourists, researchers and animal lovers may have an interest in it so it will aid the dissemination of culture. Humans have interest in its hunting due to its delicious meat, which is exported for subsistence, and trade (Woodford 1995; Nocon 1999; Sheikh and Molur

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2004). Species performs a negative role by damaging the crops, mainly sorghum and millet that induce the farmers to kill them (Jhala 1993; Nocon 1999; Behera and Mohanta 2019; Meena et al. 2020).

Open grassland, dry thorn scrub, scrubland, and sparsely forested area, as well as agricultural edges, where it is sometimes spotted feeding in fields, are all habitats for this species as indicated in table 1. Because blackbuck require water on a daily basis, distribution is limited to locations where surface water is available for the most of the year. Blackbuck are grazers by nature, but they browse when grasses are few, forcing them to rely more on leaf litter, flowers, and fruits. They are mostly sedentary, however during the summer they may travel greater distances in search of water and food (Tahir et al. 2021).

Table 1. Habitat preferences of Blackbuck (IUCN, 2017)

Habitat	Season	Suitability	Major Importance
Forest (Subtropical/ Tropical Dry)	Resident	Marginal	-
Grassland (Subtropical/ Tropical Dry)	Resident	Suitable	Yes
Desert (Desert – Hot)	Resident	Marginal	-
Artificial / Terrestrial (Arable Land)	Resident	Suitable	No
Artificial / Terrestrial (Pastureland)	Resident	Suitable	No

Blackbuck's spatial detectability and density distribution rise significantly with grassland size, habitat openness, and grass biomass, but drop significantly with Prosopis cover, shrub cover, proportion of woodland, and distance to water, demonstrating their negative effect on the blackbuck. Furthermore, Prosopis cover reduces the area of grassland, habitat openness and grass productivity, all of which are important positive predictors of blackbuck density distribution. Thus, alien invasive species has a deleterious impact on the native blackbuck population. This highlights the importance of eradicating or regulating invasive species like *Prosopis juliflora* in order to save the endemic blackbuck in the long run (Rathore 2017; Rajput et al. 2019; Arandhara et al. 2021). Constant monitoring of blackbuck sociality will aid in understanding of population distribution, formulation and implementation of successful conservation strategies for this rare species (Jyoti 2021).

As case study of Hisar region of India, where 1715 blackbucks observed the mean group size of 13.19 and 29.66 mean crowding value during 2017-2019 (Jyoti 2021). Likewise, 7134 blackbucks observed at Odisha, India with herd size of 19.49 varying with seasons and mean group size for blackbuck was 13.84 with crowding value of 31.31 at Haryana, India for 941 blackbuck members (Debata 2017; Rai 2019). Although blackbuck have vanished from many regions due to habitat loss from agricultural usage and hunting, they are reappearing in several protected areas in South Asia and Vishnoi-dominated areas in Rajasthan and Haryana at India (Rahmani 2001; Jyoti 2021).

Converting dense scrub and woodland to grassland and agriculture expands the amount of appropriate habitat available. Due to excessive hunting, blackbuck numbers reduced over the twentieth century, and while they are now protected, some blackbucks are still shot illegally (Mallon and Kingswood 2001). In South Asian countries, blackbuck is legally protected and can be found in a number of protected places. Likewise, species is also protected by different laws established in different south Asian countries (Sheikh and Molur 2004; Jyoti 2021).

Threats such as hunting, stress, habitat loss, diseases, poaching, road accidents, habitat fragmentation, interspecific competition, predation pressure etc., have reduced the population size of blackbuck to threatened level. Hormone-mediated conservation, genetic studies and introduction to non-endemic but suitable habitat also contributed to blackbuck conservation. Current conservation practices are helping to conserve the blackbuck but associated with few concerns also, thereby proper management, planning, monitoring of conservation practices is required. Thereby, distribution range, population size, conservation status, threats to blackbuck, conservation struggles and recommendations are accounted in the text. Review article will act as limelight for taking further steps for conservation of blackbuck and focus over research lack as identified.

Distribution of Blackbuck: Previously, Blackbuck could be found practically everywhere on the Indian subcontinent at south of the Himalayas. But now, their range shrank, and they are now observed to get extinct in Bangladesh and Pakistan. Blackbuck has been introduced to Texas and Argentina and found extant there currently as indicated in Figure 1 (IUCN 2017). Blackbuck have been found in the Terai and adjacent areas of foothills in Nepal (Wegge 1997). Species also found at single small, isolated location of Blackbuck Conservation Area (BCA), Nepal at grassy areas having semi-arid environment (Mallon and Kingswood 2001; Bashistha et al. 2012).

Other protected areas enlisted in text where blackbuck is existing according to literature studied. Pakistan is occupying almost 195 species of mammals from 10 orders (Roberts 1997; Roberts 2005). 44 mammalian species recognized as Critically Endangered or Near Threatened. While others found extinct in region and data deficient (Khattak et al. 2021). Among recorded species of Antelopes in Pakistan are Nilgai, Blackbuck, Chinkara or Indian Gazelle and Goitered Gazelle (Roberts 1977a). In Pakistan, Blackbuck has been existed at an altitude of 100-200 m (Sheikh and Molur 2004). Species was found in Bahawalpur and Fort Abbas at the northern area of the Cholistan Desert, Punjab at the

border with India (Roberts 1977a). Blackbuck was found under captive breeding at the Lal Suhanra National Park, Bahawalpur; Manglot Wildlife Park, Nowshera and Togh Mangara Safari Park, Kohat (Khattak et al. 2021) (Figure 2). But species get regionally extinct in its native distribution now and only found in captive conditions. Protected areas occupying blackbuck in Pakistan are enlisted in text (Sheikh and Molur 2004; Khattak et al. 2021).

Figure 1: Distribution range of *Antilope cervicapra* in South Asia (Source: IUCN, 2017 and modified by authors according to recent literature).

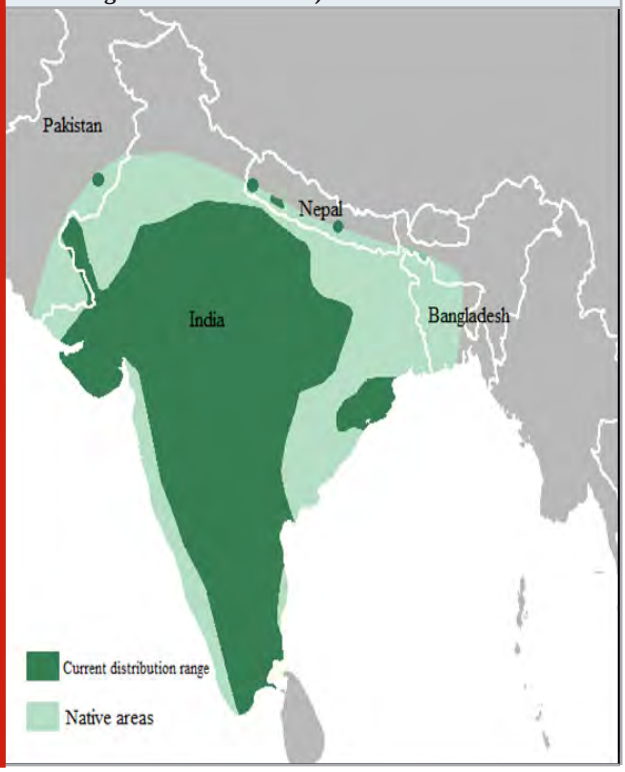

Figure 2: Male Blackbuck at Togh Mangara Safari Park, Kohat (Khattak et al. 2021)

Blackbuck population found once at forest regions, grassy sites and agricultural lands of the Bangladesh. But species now reckoned as 'Extinct' as there has been no site found in the country where antelopes were introduced again (Akonda 1997; IUCN 2017). Recent data indicated the no blackbuck population at Bangladesh which need to be focused for reintroduction of species at its possible breeding sites. Six species of antelopes including Blackbuck observed in India (Mallon and Kingswood 2001). During 1970's and 1980's, there has an increment in count of species due to many rehabilitation projects (Ranjitsinh 1982; Rahmani 1989; Rahmani 1991; Rahmani and Sankaran 1991).

Many sites where blackbuck conserved was observed such as Thar Desert National Park, sites at ganjivaripall, Velavadar National Park, Gujarat, Great Indian Bustard Wildlife Sanctuary, Maharashtra and a lot of other sites listed in article. Blackbuck is found in 15 states of India including 19 districts of Rajasthan, where more than 30,000 blackbucks observed in 2016 (Rahmani 1991; Srinivasulu and Nagulu 2002; Sharma et al. 2003; Saran and Meena 2018). Blackbuck evidenced to exist at the grassy plains, forest areas and agricultural land of India (IUCN 2017; Meena and Chourasia 2017; Meena et al. 2020). At the Sir Bani Yas, location of UAE, major assemblage of freely moving antelopes including Blackbuck was noticed at semi-arid regions. UAE was not included in native distribution of species but maybe possible site for species living (Mallon and Kingswood 2001; Meena et al. 2020).

Conservation Status of Blackbuck: Conservation status of Blackbuck at a global level was assessed as "Vulnerable (VU)" during 1994-1996. While from 2003-2008, conservation status found as 'Near Threatened (NT)' (Prater 1971; Suwal and Verheugt 1995; Khanal et al. 2002; Ernest 2003; Wiegl 2005; Baral and Shah 2008; Bhatta 2008; Mallon 2008). But according to last recent assessment carried out during 2016, conservation status of Blackbuck at a global level was ascertained as "Least concern (LC)" under category of ver 3.1 (IUCN 2017). On the basis of Conservation Assessment and Management Plan Workshop 2003, Blackbuck has been given the status of 'Extinct in the wild' in Pakistan (Sheikh and Molur 2004). But according to last recent assessment carried out during 2016, conservation status of Blackbuck in Pakistan was ascertained as "Extinct (EX)". Now many members of Blackbuck species are observed at the Lal Suhanra National Park (LSNP) which are being flourished successfully at their breeding center (Meena et al. 2020).

But species found regionally extinct in its distribution area at Pakistan. Population size need to be focused by the researcher to assess the recent population of Blackbuck in Pakistan within protected areas (IUCN 2017). Status of Blackbuck in Nepal was ascertained as 'Critically Endangered' few years ago (Jnawali et al. 2011). But according to last recent assessment carried out during 2016, species is found extant in Nepal. Likewise, conservation status of Blackbuck in India has figured out as 'Near Threatened' few years back but now status of Blackbuck in India was ascertained as extent (Asif and Modse 2016; IUCN 2017). Conservation status of Blackbuck at Bangladesh found "Extinct" according to estimation during 2016. There seems to be no site in Bangladesh where

Blackbuck could be reintroduced under captivity since most of the potential habitat has been deteriorated (Mallon and Kingswood 2001; IUCN 2017; Meena et al. 2020).

Population Size of Blackbuck: Population trend assessed as "unknown" according to study in 2016 given by IUCN. While 35000 mature individuals of Blackbuck counted during this assessment at global level. Blackbuck are still widespread and prolific in certain locations at South Asia, are increasing in many protected areas, and are becoming an agricultural pest in others, despite their range and numbers declining over the last century (IUCN 2017; Behera and Mohanta 2019; Meena et al. 2020). Another recent study of 2021 has shown about 50,000 individuals of Blackbuck at global level (Zhongming et al. 2021). General range of Blackbuck has shrunk as a result of habitat loss, but this has been partially offset by the conversion of dense scrub and woodland to agriculture, which has resulted in the creation of more appropriate, open habitats. In spite of adaptability to many geographic areas, Blackbucks are increasingly under threat by growth of human population, domestic cattle expansion and economic development (Tahir et al. 2021). No quantitative statistics identified for population trends of Blackbuck currently, however even if species is reducing in general, there is little evidence that it is declining over nineteen years for three generations of species, which is near to threshold for Vulnerable under criterion A (IUCN 2017; Zhongming et al. 2021).

Four million members of Blackbuck speculated to exist a century ago but in 1947, that number reduced upto the 80,000 members. Blackbuck in India observed to increase from 24,000 to 50, 000 members of Blackbuck with 35000 mature blackbucks during 2000. Maximum count of species observed at Rajasthan state in Punjab, Madhya Pradesh, Gujarat and Maharashtra then (Rahmani 2001). At Nepal, 200 Blackbucks were observed during 2012 (Bashistha et al. 2012). Blackbuck were introduced in South America and Argentina. members observed were 8600 at Argentina while at USA, there were 35000 individuals of blackbuck (Mallon and Kingswood 2001). Blackbuck still found extant at both of these sites (IUCN 2017; Zhongming et al. 2021).

There has been no systematic census, hence there are no reliable population estimates for the present population size. It is, nevertheless, still common and numerous in many areas. The species has evolved to the edges of agricultural land, and data suggests that clearing scrub and woodland benefits it by providing adequate habitat. In some locations, the Blackbuck population has grown to the point where it has become an agricultural nuisance, though not on the same magnitude as Nilgai (Tahir et al. 2021). So current population size needs to be estimated.

Threats To Blackbuck: For the most part, threats to Blackbuck are anthropogenic in nature. Of import, threats to the species are as what is listed next:

Hunting: Hunting for subsistence and trade by humans outside and in protected areas has threatened the species (Schaller 1967; Macdonald 1984; Sheikh and Molur 2004). Earlier, 4 million blackbucks were present in India and were hunted by Maharajas using tamed Cheetah (*Acinonyx jubatus venaticus*). The decision of the government in 1996 to allow shooting of Nilgai as crop pest has led to increase in illegal hunting of Blackbuck in areas where both species have common habitat (Mallon and Kingswood 2001). This threat is also indicated by the IUCN (2017) as indicated in table 2 (Isvaran 2007; Tahir et al. 2021).

Table 2. Threats faced by the Blackbuck under different regions (IUCN, 2017)

Threat	Timing	Scope	Severity	Impact Score
Agriculture and aquaculture (Livestock farming and ranching-Small-holder grazing,	Ongoing	Majority (50-90%)	Slow, Significant declines	Medium Impact: 6
ranching or farming)	Stresses: Ecosystem stress by ecosystem change and degradation			
Biological resource use (Hunting & trapping terrestrial	Ongoing	Minority (50%)	No decline	Low Impact: 4
animals-International use,	Stresses: Species mortality			
Persecution/control)				

Diseases: Various disorders are affecting the blackbuck. Among these diseases, dystocia is the main disease, which is disorder in female confronting the difficulty in giving birth maybe due to increase in level of epinephrine hormone (Roberts 1971; Fraser 2010; Riaz and Aleem 2012). Parasitic infestations are one of the most serious hazards to a small population of wildlife, and it is especially prevalent in captive populations in small enclosures (Khanal and Chalise 2011a; Tahir et al. 2021).

Endoparasites like *Haemonchus cortortus, Trichostrongylus axei* etc and ectoparasites like *Hyalomma anatolicum, Psoroptes cuniculi, Amblyomma americanum* have affected the species, with extreme cases leading to death (Kreis 1935; Rewell 1948; Rewell 1951; Jansen 1959; Singh and Pande 1963; Patnaik 1964; Wetzel and Fortmeyer 1965; Thornton et al. 1973; Cole et al. 1984; Flach and Sewell 1987; Wright and Glaze 1988; Mertins et al. 1992; Prakash et al. 2015). *Listeria monocytogenesis* the main bacteria that bear on the species (Krüger 1963; Webb and Rebar 1987). Protozoas like *Trypanosoma cruzi* has also impacted over the population of Blackbuck species (Schmidt et al. 1981; Tahir et al. 2021).

Paramphistomum, Strongyles, *Ascaris* and *Coccidia* were most prevalent parasites of animals grazing in Blackbuck habitat and protected areas (Khanal and Chalise 2011). Chaudhary and Maharjan (2017) found *Entamoeba* and *Eimeria among protozoans, Paramphistomum* and *Fasciola* among trematodes, *Moniezia* among cestodes, and *Trichostrongylus, Ascaris, Haemonchus, Strongyloides, Bunostomum, Trichuris* and *Oxyuris* among nematodes in Blackbuck. Although no Blackbucks were found to be infested in study by Rita and Khanal (2019). But chances of parasite and disease transmission were seen to be higher due to the Blackbuck's strong relationship, nutritional and habitat overlap with that of Spotted deer and Monkeys. Study by Pant and Joshi (2019) indicated the *Eimeria, Strongyloides* sp. and *Strongyle* sp in blackbuck owing to the parasite transference from livestock, (Chaudhary and Maharjan 2017; Rita and Khanal 2019; Pant and Joshi 2019; Tahir et al. 2021).

Fracture is a leading cause of death in free-ranging ruminants when it comes to non-infectious diseases. Tibial fracture is the most common long bone fracture in small ruminants. After femur, radius and ulna, tibial fractures are third most common form of long bone fracture, accounting for 21% of all long bone fractures. Tibial diaphyseal fractures are responsible for 75-81% of all tibial fractures. Treatment of severe injuries and fractures in non-domestic animals is difficult due to difficulties in restraint and wound dressing. It's also tough to keep an animal under control during an examination (Tahir et al. 2021). Study by Singh et al. (2019) shown that blackbuck was stabilised with use of analgesics and hydration treatment. Pre-anaesthetic agent xylazene 0.2 mg/ kg body weight I/m and local anaesthetic agent inj Xylocaine epidurally were used in next day to perform amputation.

Immobilization and surgical intervention should be carefully monitored and conducted under strict veterinarian supervision, since they can result in dangerous and short-term behavioural changes (Singh et al. 2019). Treatment of traumatic injuries in non-domestic animals is also difficult because to difficulties in restraint and wound dressing. Antibiotic sensitivity test showed that ciprofloxacin had the highest sensitivity, followed by amoxicillin + Salabactam, amoxycillin, amoxicillin and cefotaxime (Kumari et al. 2017; Singh et al. 2019; Tahir et al. 2021).

Competitive behavior: Apart from parasite infestations and diseases, competitive behavior among different species in wild and captive conditions also impact over species. Increased competition for food by Blackbuck with spotted deer and monkeys has been identified as one of the Blackbuck's significant concerns at Mrigasthali Enclosure at Pashupatinath Area of Nepal. Blackbucks were consistently on the losing end of both exploitative and interference type competitions for the ingestion of supplemental food. Various animals, particularly ungulates, have been observed to engage in interspecific aggressive interactions (Hanzlikova et al. 2014; Rita and Khanal 2019). Also in wild habitats, excessive densities of competition such as feral cattle have an impact on the Blackbuck's health and survival (Khanal 2006; Khanal and Chalise 2011b; Baskaran et al. 2016; Prashanth et al. 2016; Rita and Khanal 2019; Tahir et al. 2021).

Stress: Stress hormones as glucocorticoids produced by stimuli as new environment, vehicles, social stress and aggressiveness, human interference, and predators, as observed in study by Terio et al. (1999) and Wielebnowski et al. (2002). Stress induced by captivity, which led to health and behavioral alterations (Nemat et al. 2013). A large number of zoo visitors have affected the behavior and adrenocortical secretions in Blackbuck (Rajagopal et al. 2011). To forbid the crop damage; Blackbucks have been trammeled in their habitat in India (Haryana district) as a captive condition. Species have confronted stress there owing to chase by farmers and killing assail by predatory dogs outside the fence, which has abridged its breeding rate (Chauhan 1990; Joseph 2011).

Small and isolated populations of Blackbuck mainly in captivity has confronted the genetic troubles like stress caused by inbreeding, homozygosity and environment (Purvis et al. 2000; Jnawali 2011). Fecal cortisol was observed in range of 0.18-2.62 ng/mg in blackbuck residing at the Rajiv Gandhi Zoological Park, Pune at India. Number of visitors and temperature humidity index (THI) impacted over the cortisol amount mainly during winter season but not in October heat. Whereas, stress level was not linked to the sex category of blackbuck. Management of Blackbuck population under captive condition will be aided by studying stress response of blackbuck to design the captive facilitation for effective conservation (Nikhil 2020).

Figure 3: Blackbuck resting under shady area at Lal Suhanra National Park, Breeding centre for Blackbucks, Pakistan (Imran, 2011)

Habitat loss: Human interference for agricultural intentions by livestock and farming are deteriorating the habitat of species (Schaller 1967; Macdonald 1984; Oza 1988; Sheikh and Molur 2004; Mahato et al. 2010; Jnawali 2011). Threat level by agricultural practices is indicated in table 2. Agricultural uses, human interruptions in habitat, deforestation and economic melioration have imperiled the species by deterioration of its habitat (Macdonald 1984; Sheikh and Molur 2004; Mahato et al. 2010). Population increments in South Asian country like Pakistan (at a rate of around 3%) has coerced the policy makers to orient the attention towards the feeding of an ever-growing population, which has deteriorated the blackbuck' habitat

(Cade 1988; Rahbeck 1993; Snyder et al. 1996; Komers and Curman 2000). Eco transformation and accelerating count of livestock have also pressurized the species (Mahato et al. 2010; Nikhil 2020).

Predation Pressure: Predation to blackbuck observed as threat to endangered blackbuck at many places like case reported at Marwar region of India where feral dogs cause decline in blackbuck population in rainy season (Meena and Jaipal 2020). Soft and slippery surface by rainy season cause difficult run by Blackbuck so making easy capture of infants and adults as well. Feral dogs prey heavily on calves, particularly during the breeding season. Wolf (*Canis lupus pallipes*) and golden jackal (*Canis aureus*) are also observed as main predators of infants (Meena et al. 2017). Leopard predation, hyena attacks, and stray dog attacks were also the main threats to Blackbuck inside BCA, Nepal (Gyawali et al. 2020).

Other factors: Human blackbuck conflict cause the significant threat to survival of blackbuck. Thereby, illegal use of naked electric wires with 220V current by farmers around the crop area has also impelled death of blackbuck (Chauhan 1990). Accidents and pollution are also affecting the blackbuck population size (Schaller 1967; Macdonald 1984; Sheikh and Molur 2004; Meena and Chourasia 2018). Habitat degradation, illegal poaching, road accidents, cattle overgrazing, and wildlife crime has already reduced the blackbuck population to limited site at its endemic area as case study observed at Marwar region. Another major threat is habitat fragmentation by roads construction. Road accident cause great mortality mainly to infants (Meena et al. 2017; Meena and Jaipal 2020).

Blackbuck Conservation Struggles

Rules and regulations: Different rules and regulations were designed to implement for protection of blackbuck in different countries of South Asia. Blackbucks have dislodged from Schedule III of Protected animals and birds to Schedule II by Punjab Parks and Wildlife Department, Pakistan, which has countenanced the private sectors to raise them (Ali et al. 2011). The species have given highest protection status under National Park and Wildlife Conservation Act 2029-1973 in Nepal (Jnawali 2011). Blackbuck protected in India under Schedule-I of the Indian Wildlife Protection Act WLPA, 1972. All these regulations help in conservation of blackbuck (Kankane 2014).

Captive breeding: Captive breeding has become an important aspect of conservation around the world as a result of increasing human pressure on the environment. Extinction rates are rising 100-1000 times faster than natural rates as a result of manmade activity, wiping out 150 species in a single day (Ahmed 2007). Humans have shortened, taken over and modified natural ecosystems to the point where many species' survival is now dependent on captive breeding. Captive breeding is a broad word that refers to a variety of situations ranging from the laboratory to animals kept in close confinement (such as a zoo's indoor enclosure) to semi-free wandering (outdoor enclosures).

Successful breeding, population increase and potential translocation are all significant goals of keeping wild animals in captivity. However, due to inadequate management, inhospitable climatic conditions, competition with other co-housed species, illnesses and other factors, many species have less behavioral flexibility and fail to maintain a healthy population in captivity (Rita and Khanal 2019). One study by Rao (2011) indicated that Blackbucks are one of the most significant creatures in the zoo's collection, and they breed well when provided good care. When given sufficient protection, a well-balanced diet, and treatment, the Blackbuck population explodes as seen at Kanpur Zoological Park, India. Thereby, Blackbuck also conserved by captive breeding at different areas as listed (Rao 2011; Khattak et al. 2021).

Lal Suhanra National Park, Pakistan: Lal Suhanra National Park (LSNP), 35km east of Bahawalpur, Punjab, includes desert, forest and wetland. Area of LSNP was suggested firstly in 1966 as a good place for breeding Blackbuck and important wetland area (Mountfort and Poore 1967). By the groovy plan for LSNP, a system of partition has been proposed, by which the entire Cholistan Desert has been integrated within the wild zone in which exploitation has not permitted. Enclosures have established for the Blackbuck breeding program to which entrance is prohibited (Masud 1980). Blackbuck shown at Lal Suhanra National Park in Figure 3 (Khattak et al. 2021).

Blackbuck reintroduction programme: It has commenced in April 1970 with an initial consignment of ten animals (seven females and three males) from a Texas ranch to small enclosure of Lal Suhanra Sanctuary, Pakistan under the aegis of the Worldwide Fund for Nature (WWF) and Government of Punjab and more species have anticipated to be introduced into the vicinal larger fenced enclosure of 518.4 ha (Mirza and Waiz 1973; Schaller 1975; Aleem 1978; Sheikh 1982; Ahmad 1983; Ranjitsinh 1989; Mallon and Kingswood 2001). Survival of young has turned out to be depleted so in 1980 five more females and one male have added to the collection by Cophenhagen Zoo. By that time, number of species has increased to 48 by 1982. Another breeding center has launched in a separate area in 1982 with Blackbucks from Cophenhagen Zoo and Western Plains Zoo, New South Wales (Aleem 1978; Sheikh 1982; Ahmad 1983). Captive breeding plan at LSNP proved successful at that time but there is lack of data about the recent population of Blackbuck at Lal Suhanra National Park so it needs to be measured by wildlife researcher (Khattak et al. 2021).

Karachi Zoo and Safari Park, Karachi, Pakistan: Species have thrived successfully in captivity as reckoning delineated: in Karachi Zoo, 14 in 2009, 16 in 2010, 17 in 2011 and 18 in 2012; in the Safari Park, 44 in 2009, 48 in 2010, 57 in 2011 and 65 in 2012 (Khan et al. 2014). Estimation for current population at this site is still noy available thereby it needs to be calculated through proper research design along with factors impacting over the population of Blackbuck so it can be conserved properly (Khattak et al. 2021).

Kalabagh Game Reserve, Pakistan: Species have introduced in Kalabagh Game Reserve as a part of national endeavors for the conservation of species in Pakistan. But there is lack of last and current population size of blackbuck at this site (Khattak et al. 2021).

Kirthar National Park, Pakistan: It is in Southwest Sindh in the Kirthar Mountain range near Karachi. Fifteen Blackbucks from the USA have been introduced in Khar Wildlife Breeding Centre of Kirthar National Park, in October 1984. It has contrived to get the species to the park, but the most preferred habitat of species has overdriven by the human beings (Mirza 1973; Mallon and Kingswood 2001). Increment in count of species in this national park as an aftermath has engendered the enclosure to be deficient for the species. Therefore, an earlier king of the UAE, late Sheikh Zayed Bin Sultan Al Nahyan, has donated the large enclosure of 700 acres confining the Hub Dam and mountainous terrain (Khattak et al. 2021).

Other protected areas for Blackbuck in Pakistan: Thirteen breeding centers in Punjab, Pakistan have established to maintain the count of species under captivity (Mallon and Kingswood 2001). Species have conserved at the private farms by conservationists in Sindh province, including the Tando Muhammad Khan, Nawabshah, Khangharah, Ghotki and New Jatoi (Amar 2011). Blackbuck was found under captive breeding at the Manglot Wildlife Park, Nowshera and Togh Mangara Safari Park, Kohat at KPK (Mallon and Kingswood 2001; Khattak et al. 2021).

Blackbuck Conservation Area, Nepal: Blackbucks have placed in a single isolated and small size location (16km²) of Blackbuck Conservation Area at Bardiya district of Nepal which has left them jeopardize to different threats. Action Plan for Conservation (2016-2020) for blackbuck in Shuklaphanta Reserve, Nepal has referred by the Department of National Parks and Wildlife Conservation (Prater 1971; Suwal and Verheught 1995; Mallon and Kingswood 2001; Khanal et al. 2002; Ernst 2003; Weigal 2005; Mallon 2008; Baral and Shah 2008; Bhatta 2008; DNPWC 2016; Khattak et al. 2021).

Mrigasthali Enclosure, Pashupatinath Area, Nepal: Since 2004, Blackbucks have been kept in Mrigasthali enclosure near Pashupatinath Temple in Kathmandu, in a semi-captive situation with food provided by the Pashupati Area Development Trust management. Total of 20 Blackbucks were put into the Mrigasthali enclosure. About 150 Spotted deer (Axis axis), some Barking deer (Muntiacus muntjak), and about 400 Rhesus monkeys (*Macaca mulatta*) occupy the enclosure with blackbucks. But their population has dropped dramatically according to study in 2016. Population size was checked in 2016 for previous 15 years whereas behavioral pattern was also analyzed from April to July, 2016. By 2010, the population had grown to over 54 animals, but then unfortunately, more than a third of them died within a few months of 2014. Following that, Blackbuck population tried to recover and in 2016 only four individuals was found.

This study found that the population has dropped dramatically since the emergence of foot-and-mouth disease in 2014, putting the remaining species at risk of extinction. The diurnal activity pattern and time budgets of the surviving individuals are markedly different from those of wild populations; in particular, they spend less time feeding and more time sleeping. Despite the cooler climate in open areas, fierce competition for food and space with spotted deer and monkeys, lesser behavioral flexibility among species, anthropogenic disturbances, stochasticity due to the small population size, and other factors were seen as major threats to Blackbuck in enclosure (Rita and Khanal 2019; Khattak et al. 2021).

Other protected areas at Nepal: By 10 September 2020, once-extinct Blackbuck *Antilope cervicapra* recovered to population of 9 to 234 in Krishnasaar Conservation Area, Khairapur and 28 to 115 in Shuklaphanta National Park, Hirapur Phanta. Government of Nepal designated the Krishnasaar Conservation Area in Khairapur as a special protected area with electric fencing to ensure the survival of the introduced population. To develop a free-roaming wild population, a second colony was established in Hirapur Phanta in Shuklaphanta National Park. Effective management interventions (population, habitat, and health) combined with active stakeholder participation, institutionalization and extension of specific protected area dedicated to blackbuck conservation marked the growth of the species' last remaining population in Nepal's seminatural habitat (BK and Awasthi 2018; Bist et al. 2021).

Protected areas for Blackbuck in India: Vallanad Blackbuck Sanctuary, India has isolated small natural hill having scrub forest, a place for Blackbuck habitation (Joseph 2011). Other noteworthy protected areas for Blackbuck are Velavadar National Park, Gujarat; Point Calimere Wildlife Sanctuary, Tamil Nadu; Ranebennur Wildlife Sanctuary, Karnataka; Great Indian Bustard Wildlife Sanctuary, Maharashtra; Kanpur Zoological Park, Uttar Pardesh; Nehru Zoological Park, Hyderabad; Guda-Vishnonian and Taal Chhapar Blackbuck Sanctuary, Rajasthan; Rajiv Gandhi Zoological Park and Wildlife Research Centre, Pune; Balipadar- Bhetnoi blackbuck reserve, Ganjam; Conservation and Breeding Centre of Arignar Anna Zoological Park, Tamil Nadu; Sathyamangalam tiger reserve, Tamil Nadu; Basur Amruth Mahal Kaval Conservation Reserve, Chikamagaluru and Guindy National Park, Chennai (Rahmani 1991; Bagchi et al. 2003; Sontakke et al. 2009; Rao 2011; Joseph 2011; Sagar and Antoney 2017; Das et al. 2018; Rajagopal et al. 2018; Rajput et al. 2019). It has hinted that pilot projects on translocation of Blackbuck to sites of earlier habitat, culling, and evaluation of threat of a sport-hunting programme should be carried out (Mallon and Kingswood 2001; Khatri et al. 2021).

Hormone-mediated conservation: Hormonal level conservation by oestrus synchronisation and non-surgical AI technology studied to be successful for the conservation and population management of blackbuck. Evaluation of blackbuck ejaculates and testosterone concentrations, as well as the possibility of short-term semen storage at cold,

suggested that AI technology could be used to improve genetic breeding and conservation of blackbuck. Two of five inseminated blackbuck females achieved successful pregnancies after receiving Norgestomet ear implants and i.m. administration of pregnant mare's serum gonadotropin (PMSG), although both had twin pregnancies that were delivered prematurely. However, two doses of prostaglandin 11 days apart found efficient for synchronizing oestrus in blackbuck. In oestrous-synchronised animals, transcervical AI resulted in successful pregnancies in four of six inseminated females (67 percent) and delivery of three live fawns following the second prostaglandin injection after 72-96 hour. This research show how AI technology could be used to help save endangered ungulates (Sontakke et al. 2009; Khatri et al. 2021).

Genetic analysis: Distribution and patterns of intraspecies genetic variation are critical for developing effective conservation measures (Avise 2000). Thereby, genetic diversity measurement was carried out for blackbuck along-with phylogenetic analysis at South India. Sequencing of mitochondrial DNA of cytochrome b for 120, cytochrome c oxidase subunit-1 (COI) for 137, and control region for 137 fecal pellets from eleven different locations in southern India for phylogenetic and genetic diversity analyses of blackbuck populations among different distribution ranges in southern India. The genetic structure of three mitochondrial markers, control region, cytochrome b, control region and COI area, was investigated separately and in combination. Control region had a larger haplotype diversity and nucleotide diversity than cytochrome b and COI, with 0.969 and 0.047, respectively (Bhaskar et al. 2021).

Several unique haplotype groups were detected within blackbuck using Bayesian phylogeny and a MJ network based on the control region and combined dataset (105 sequences), however no clusters were identified using the cytochrome b and COI phylogenetic analyses. The combined data set's molecular variance analysis found 52% genetic variation within the population. With examination of the combined dataset in each population and study of each marker separately in the overall population, mismatch distribution analysis revealed that blackbuck populations underwent extensive alterations. These findings show that due to habitat fragmentation, blackbuck populations in different geographic regions have diverse population structures. These findings give preliminary genetic data for monitoring, maintaining, and reintroducing wild blackbuck populations in their natural habitat in Southern India (Bhaskar et al. 2021).

Another similar study reported by Kumar et al. (2017) for assessment DNA barcoding for blackbuck using COI region. Likewise genetic study for blackbuck was done by De et al. (2021) at Kaimoor Wildlife Sanctuary of India. In this study, panel of five microsatellite markers was suggested for blackbuck identification and monitoring of its population. Along with it seven additional markers given for genetics studies for blackbuck conservation. Few other genetic studies observed by Jana and Karanth (2019) and Abbas et al. (2020). Further studies also required at other areas endemic for blackbucks so it can be reintroduced into its natural habitats (De et al. 2021).

Introduction in non-endemic but suitable habitat: Blackbucks have brought to Texas (Willard 1995) during 1932, where count in 1974 estimated a population of 7,339 Blackbuck (Ranjitsinh 1989). So, by introduction in Texas Blackbuck show increment in number (Mirza and Waiz 1973). These Blackbucks are the posterity of 35 Blackbucks gifted to Texas in 1940 by the late amir of Bahawalpur (Aleem 1978; Sheikh 1982; Ahmad 1983). Blackbuck were also introduced in Argentina and United states. Members observed were 8600 at Argentina while at USA, there were 35000 individuals of blackbuck (Mallon and Kingswood 2001). Blackbuck still found extant at both of these sites (IUCN 2017; De et al. 2021).

Religious association: Blackbuck in Thar Desert of India has given protection owing to religious affiliation so their intervention in human vicinage and agricultural locality has granted. Vishnoi Community also sets up in Rajasthan has given protection to Blackbuck under their precepts (Rahmani 1990a; Rahmani 1990b; Rahmani 1991; Mohapatra 2014; Kankane 2014; Sinha and Singh 2020). The protected status of species has gained ground at public level publicity by the case of Salman Khan (India's leading film star) to which he was sentenced imprisonment of five years for the killing of two black bucks and several endangered Chinkara. Actuation for arrest has done by extreme protests on behalf of the Vishnoi ethnic group (on whose area the hunting had occurred), which consider the animals and trees sacred as reported by the Times of India in January 2017. Orans have created by native communities in Thar desert of Rajasthan for conservation of blackbuck as it is propitious habitat for species (Kankane 2013). Survey was carried out from March to December, 2017 at Dhansu and Dobhi village of Haryana district, India. As an agricultural pest, Blackbuck found to reduce in number by 46% at Dhansu village and 51% at Dobhi village. But at Dhansu village, 59% people of Vishnoi community involved in survey agreed for protection of Blackbuck. While at Dobhi village, 18% agreed to protect the blackbuck (Rai 2018; De et al. 2021).

Recommendations: Recommended suggestions to fructify the conservation program are as follows: blackbuck demands a great deal of research on survival, breeding, behavioral aspects, selection, and availability of food, which is under continuance (Mirza and Waiz 1973; Sheikh and Molur 2004). Blackbucks are capable of subsisting in mixed agricultural areas, so the transition of scrubland and forest into grassland and cropland may do well to Blackbuck (Mallon and Kingswood 2001). For meliorated conditions in protected areas proffers are given as: to observe the animal behavior in captivity to contrive an ideal enclosure. Data of the behavior and biology of the animals in the form of pamphlets and guidebooks, etc. should be circularized to the visitors and students.

A guidance map should be available at the entry point. Safari and Zoo came to grip by linking its management with

conservation organizations. Animal conservation programs should be organized which will invigorate visitants and educates to take part in such endeavors. Among the observed reasons of deaths, major is the consumption of scraps and shopping bags etc. thrown by visitants. Some visitors present unhealthy food to the animals causing the flu, tuberculosis and lung infection, etc. so it should be averted. Green area should be created to boost the oxygen level in Safari. Species should be placed in couplet form to generate natural and social behavior among them. The pond should be accessible to animals for cleanliness (De et al. 2021).

Increased numbers of visitors induce stress in species, which should be grappled. High rainfall drives lots of water in the enclosures. Therefore, there should be few high ground terrains where animals may take shelter (Khan et al. 2014). Breeding sites at zoological park should be modifies for blackbuck by creating hidden watching sites so the stress created by visitor's contact will not impact over the population of blackbuck. Impacts created by visitors like movement, mocking, sounds or any physical harm should be reduced by the taking effective measures in the zoo. Continuous health assessment and physiological studies should be carried out for checking the reproductive potential of blackbuck under captivity. Effective husbandry practices along with blackbuck conservation plans should be created for safety of blackbuck (Nikhil 2020).

Eradication of *Prosopis juliflora*, appropriate management for improvement of a blackbuck habitat and indigenous floral species have favorable consequences on high density blackbuck populations. Removal of Prosopis has allowed for more canopy opening, which has resulted in increased grass growth as shown by Rajput et al. (2019). Good habitat state of open land for blackbuck has been ensured by moderate cover and grass density. To combat the Prosopis juliflora invasion, robust modern controlling measures such as mechanical eradication, prescribed burning, and chemical control are being recommended. Furthermore, sustainable management to guarantee ecological balance and livelihood enhancement of local people will be community-based *Prosopis juliflora* eradication within protected areas. Thereby, *Prosopis Juliflora* eradication has a favorable impact on blackbuck population and will provide as a baseline data foundation for invasive habitat management through appropriate management plan within protected areas (Rajput et al. 2019). Constant monitoring of blackbuck sociality will aid in understanding of population distribution, formulation and implementation of successful conservation strategies for this rare species (Jyoti 2021).

CONCLUSION

The findings of the present review has shown that animals are resplendence of nature and Blackbuck is a wondrous example of it, discoursed in the text. Unique darker coat of species gives it hypnotic beauty and attraction. Species are confronting different threats by the hand of human beings. Imperilments encountered by species include stress, hunting, poaching, diseases, habitat loss, habitat fragmentation, agricultural practices, illegal killing, human population explosion, road accidents, noise and pollution. Because of all these threats, various conservation strategies were planned and, on the way, to conserve blackbuck. Rules and regulations, captive breeding, hormone mediated conservation, artificial insemination and religious affiliation are the efforts being carried out for its conservation. Captive breeding is concluded as most expedient one in contemporary circumstances, owing to the fact that its natural habitat is being deteriorated by man in most of the places.

Captive breeding is underway in various sites of Pakistan like in Lal Suhanra National Park, Bahawalpur; Karachi Zoo and Safari Park, Karachi; Kalabagh Game Reserve; Kirthar National Park etc. Lal Suhanra National Park, Bahawalpur, Pakistan renders the peachy habitat for species with desert, forest and wetland as its part. So, it is suggested as a groovy site for conservation by captive breeding of Blackbuck to emendate its count as Cholistan is the native place for species. Karachi Zoo and Safari Park, Karachi, Pakistan is also good site for breeding of species. Likewise, protected areas at Nepal and India are also enlisted in article. In addition to all of these practices, further maneuvers and plans are necessitated for the conservation of species. Recommendations are also given in text for blackbuck conservation in its endemic area and protected sites. Research lack was also identified at each level which should be focused for the proper management and monitoring of population size, distribution and conservation of species.

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The Gut Microbiome and Their Alterations in Parkinsons Patients: Recent Literature Based Review

Aminah Al-Lohibi,[1,2] Hanaa Al-Lohibi,[3] Raed Albiheyri[1] and Ahmed Bahieldin[1,4]

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia,

[2]Department of Genetic, Faculty of Sciences, King Abdulaziz University, Rabigh, Saudi Arabia,

[3]College of Computer Science and Engineering, Jeddah University, Saudi Arabia,

[4]Departmentj of Genetics, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

ABSTRACT

The microbiome and the host have complex hormonal, metabolic, neurological, and immunological associations. In regulating many physiological processes this molecule cross-speech is critical. Changes in gut microbiome composition or function can have profound negative or positive consequences for the host. Cohort studies comparing well-healthy, diseased patients' gut microbiome profiles found a relationship between many conditions and one individual's intestinal microbiome. Dysbiosis is often referred to as a change in the microbiome linked to a disease. In most cases, determining whether dysbiosis is a reason or disease action is difficult, and further research (e.g., intervention and longitudinal strategies) is needed to establish cause-effect. Another significant discovery is that no two people, even identical twins, have the same microbiome. In reality, the gut microbiome profiles of healthy people of similar age and demographic are significantly different. Our attempts to define what a "healthy" microbiome has so far failed. A "Healthy" stomach is assumed to have high levels of taxonomic variety (richness), as well as the lack of harmful species. Alterations in gut microbiota are associated to Parkinson's disease, while the functional importance of these changes is uncertain. A lot of attention has recently been paid to faecal metabolomics, which provides a functional readout of microbial activity.

KEY WORDS: PARKINSON'S, DISEASE; MICROBIOTA, INTESTINAL. MICROBIOTA.

INTRODUCTION

Microorganisms are responsible for essential fermentation mechanisms, as well as for infectious diseases "germs theory". These incidents have tainted the public's opinion of microbes. Because of their association with diseases and food spots, microorganisms were commonly regarded as antagonistic throughout much of the twentieth century. In the next change, it will take another century before we realize the major roles microbes have in each day life. Advances in DNA sequencing technology have made it routine in research institutions all around the world in the previous decade. Scientists may identify and describe huge microbial ecosystems in and on corpses by using next-generation sequencing (NGs) technology (dubbed human microbiome). Our human microbiome contained 1,000 distinct species of prokaryotes, archaea, eukaryotes, and viruses, according to NGs measurements. In addition, both microbial and human cells make up a typical human individual (Sender et al., 2016).

Microbial genes amount in the human microbiome may signify the phylogenetic variety and vast metabolization potential of the human microbiome. The human microbiome has roughly three million (non-redundant) genes, despite the fact that human genome has approximately 20,000 genes. Babies are inoculated with their first microbiota upon delivery. According to studies, different delivery strategies (e.g., vaginal vs. caesarian) result in distinct microbiota patterns in the kid. Infants were traditionally thought to be sterile in pregnancy, but germs were detected in nearly a third of placental samples in 2013 research. Infant's gut microbiota constituents are affected by their diet (Qin et al., 2010; Stout et al., 2013; Goedert et al., 2014).

Human breast milk, for example, contains oligosaccharides, which a child cannot understand but which may be digested by particular bacteria in the gut. As a result, human breast milk evolved to support the gut microbiome of both the child and the infant. Early development of the baby gut microbiome is critical for the creation and operation of the

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adaptive and innate immune systems. The gut microbiome is thought to make up around 75% of the immune cells in the body, and there's growing evidence that it's where autoimmune illnesses like inflammatory bowel disease start and are controlled (Furness et al., 1999; Frese, 2017; DeWeerdt et al., 2018).

In developed countries, the prevalence of allergy and autoimmune illnesses has increased considerably during the previous four decades. According to "hygiene hypothesis" or "microbial exposure hypothesis," this increase is due to developing countries' raising sanitary lifestyles. The relationship between human microbiome and its effect on immune system growth and function has been carefully investigated in two recent publications, "Missing Microbes" and "Dirt is Good," both published by notable academics. Human microbiome has been an underestimated and understudied target for new illness detection and treatment options until recently. Irritable bowel syndrome, colorectal cancer, chronic idiopathic constipation, and obesity are linked to a shift in gut microbiota (Okada et al., 2010; Isolauri, 2012; Russel, 2013; Khan, et al., 2014; Christodoulides et al., 2016; Gilbert et al., 2017; Roca-Saavedra et al., 2018).

Obesity is a complex condition with numerous causes, one of which may be linked to gut microbiome contents. The transplantation of faeces from obese and non-obese twins into mice was one of the most impressive gut microbiomes works produced by obesity research. Following that, the mice were served a high-fat diet; those who received the lean microbiome stayed that way, whereas those who received the obese microbiome gained weight. In comparison to genetics and other modifiable risk factors, dietary modifications are thought to have a five-fold effect on gut microbiota compositions (Zhang et al., 2010; Ridaura et al., 2013; Jeffery et al., 2013; Duncan et al., 2013; Roca-Saavedra et al., 2018).

Though short-term dietary modifications resulted in transitory changes in gut microbiota composition, long-term dietary alterations resulted in significant alterations. It's been difficult to fully comprehend or monitor human diets to judge the effect of their daily food, which is why animal models and small feeding trials, as well as supplementation studies, have been used extensively. Dietary diversity and food quality are crucial predictors of gut microbiome composition; with higher-quality meals leading in a 47 percent more diverse and likely healthier gut microbiota (Walker et al., 2011; O'Connor et al., 2014; Holscher, 2017).

This is particularly true of plant-based diets, which contain a wide variety of dietary fibres; the more fibres available, the more diversified the microbiota grows. Frailty, inflammation, and poor health outcomes have been linked to elders' lack of food diversity and consistency since moving into residential care (Claesson et al., 2012; Perez Martinez et al., 2014; Holscher, 2017; Roca-Saavedra et al., 2018). A microbial culture is a group of microorganisms living together. Multi-species assemblages of (micro) organisms that interact in a continuous habitat are more aptly described as microbial communities. The microbiome is the microbial community in a well-developed ecosystem with different physio-chemical features which result in the establishment of distinct ecological niches. On the other hand, there have been plethoras of microbiome descriptions published. A community of commensal, symbiotic, and pathogenic microorganisms residing inside a body area or other habitat is referred to as a microbiom. Marchesi and Ravel described genomes, microbial (and viral) gene expression patterns, and proteomes in a particular habitat, as well as the biotic and abiotic components that were present at the time (Whipps et al., 1988; Lederberg et al., 2001; Konopka, 2009; Marchesi, 2015; Berg et al., 2020).

The microbiome is a living, breathing micro-ecosystem that evolves over time and across multiple scales. Due to technological limitations, particularly in examining non-cultivable bacteria of interest and a lack of population-scale data displaying microbiota compositions and activities, the properties of the human microbiome and host–microbiota interactions were essentially unknown until recent decades (Berg et al., 2020). Meanwhile, advances in sequencing technology and large-scale sequence-based microbiome projects, such as the Human Microbiome Project (HMP) consortium funded by the National Institutes of Health (NIH) and the Meta HIT (Metagenomics of the Human Intestinal Tract) consortium funded by the European Commission, have ushered in a new era of sequencing-based microbiome research. These large-scale studies aim to characterize the human microbiome and its activities in health and illness, with Meta HIT focused exclusively on the gut microbiome. To taxonomically characterize microbial communities, 16S ribosomal RNA (rRNA) was sequenced. To taxonomically characterize microbial communities, 16S ribosomal RNA (rRNA) was sequenced.

To taxonomically characterize microbial communities, 16S ribosomal RNA (rRNA) was sequenced. To taxonomically characterize microbial communities, 16S ribosomal RNA (rRNA) was sequenced. A type of RNA present in ribosomes is 16S ribosomal RNA. Whole genome shotgun (WGS) metagenomic sequencing of body-site specific whole community DNA, followed by reference genome mapping, metagenomic assembly, gene cataloguing, and metabolic reconstruction, as described by Methé et al. (2012); and 16S ribosomal RNA (rRNA) sequencing to taxonomically characterise microbiota communities, to facilitate maximum capture of organismal and functional information (The Human Microbiome Project Consortium, 2012).

Human Microbiome and Its Functions: The human microbiome is unparalleled in its diversity and quantity. Bacteria are the most common members of this group, having the highest density in the gut, especially the colon. Bacteroidetes and Firmicutes are the most prevalent bacterial phyla, followed by *Actinobacteria, Proteobacteria, Verrucomicrobia,* and *Fusobacteria*. A "heart" human microbiome has been established in most computational analyses in most human subjects tested, that described by a collection of common genes present in certain habitat (as

skin, oral mucosa, stomach, and vagina) (Huttenhower et al., 2012; Le Chatelier et al., 2013, Ding et al., 2014; Ding et al., 2014; Ursell et al., 2012; Lloyd-Price et al., 2017).

Microbiome affects human physiology in ways that go beyond gut function, inspite the fact that the human GI tract has most diverse microbial community. This is accomplished by a range of immunologic, homeostatic, and metabolic processes; however, due to the enormous number of microbial species, distinguishing which strain is responsible for each trait can be challenging. The fact that germ-free (GF) animals born and housed without exposure to microbes exhibit a number of systemic abnormalities, including an underdeveloped immune system and gastrointestinal tract, highlights the significance of microbiota-host interactions. After recolonization, some of these shortcomings, such as better gut immunity, can be recovered in GF animals (Umesaki et al.,1999; Smith et al., 2007; Smith et al., 2007).

The gut microbiota is required for the production, regulation, and differentiation of intestinal epithelium in the small and large intestines, as well as modulating GI motility and enhancing normal ENS development, regulates the integrity and fortification of mucosal barrier, and stimulates angiogenesis. Microbiota's close association with the host resulted in the establishment of a variety of molecular pathways that allow host's defense system to learn to accept commensal population while continuing to function normally. Both innate and adaptive responses are affected and programmed by microbiota. Innate immune system, for example, has evolved a system of protein receptors that recognize common microbe-associated molecular patterns (MAMPS), as bacterial cell wall components (lipopolysaccharide and peptidoglycan) and flagellin, which are similar across bacterial species (Hooper et al., 2001; Anitha et al., 2012, Brun et al., 2013, Collins et al., 2014; Goto et al., 2014; Thaiss et al., 2016).

Defense initial line against invading pathogens is the quality of this receptor family, which contains transmembrane proteins. Because the intestinal mucosa is closely related to ENS, TLRs are vital for gut homeostasis and neurochemical communication with it. In mice, knocking down TLR2 or TLR4 disrupts the morphological and functional integrity of the intestinal mucosa, changes gut movement, and decreases myenteric neurons numbers and neurotrophic factor output. Gut microbiota has an impact on B cells ability to generate and secrete IgA. Connections could be mutualistic, communalistic, or pathogenic. The genomic materials of organisms (microbiota) that live in a specific location of the human body make up the human microbiome (Anitha et al., 2012, Brun et al., 2013; Furusawa et al., 2013; Wesemann et al., 2013; Kawasaket al., 2014; Berg et al., 2020).

Microorganisms can be found in a variety of locations, including the skin, mucosa, gastrointestinal system, respiratory tract, urogenital tract, and mammary gland. They create a distinct, dynamic ecosystem that adapts to the niche's particular environmental conditions. Following birthing, a long-term association (symbiosis) develops between human body and its native microbiota. For total enjoyment and health, these connections are necessary. Co-evolution has allowed microbiota species to actively adapt to their habitats and live in niches within the human body (Grice et al., 2011, Yilmaz et al., 2014; Whitesid et al., 2015).

These species are known as body parts because of their biological roles, which cause a wide range of changes from conception to death. As a result of the host's influences, the human microbiome is always changing. At any one time, age, diet, lifestyle, hormonal swings, inherited genes, and underlying disease all have an effect on the human microbiome. Dysbiosis, on the other hand, is a shift in human microbiota composition that can cause life-threatening disorders. The importance of a healthy microbiome in maintaining good health can't be overstated. The stomach is where the human microbiome is most dense. These animals are essential for human health preservation and maintenance. Changes in the immunological environment have been linked to a dysbiotic gut flora in previous researches on human microbiome project (Whitesideet al., 2015).

Dybiosis has also been linked to life-threatening illnesses like cancer, bowel inflammatory disorders, cardiovascular diseases, and antibiotic-resistant bacterial infections. Mutualistic, communalistic, or pathogenic connections are all possible. The human microbiome is made up of the genomic material of species (microbiota) that live in a specific area of the human body. Microorganisms can be found on the skin, mucosa, respiratory tract, gastrointestinal system, urogenital tract, and mammary gland, among other areas in the body. They establish a dynamic and unique ecosystem that responds to the niche's unique environmental conditions. Microbiota species have actively adjusted to their special circumstances and exist in niches inside human body due to coevolution (Whiteside et al., 2014; Hoeppli et al., 2015; Pascal et al., 2018).

Due to their biological activity, these species are recognized as part of body, resulting in a variety of alterations from conception to death. In response to host cues, the human microbiome is continually developing. Healthy microbiome is essential for maintaining good health. The stomach is where the human microbiome is most dense. These animals play an essential role in human health preservation and maintenance. According to prior researches on human microbiome project, alteration in immunological environment can be associated with dysbiotic gut flora. Dybiosis has been related to life-threatening health conditions such as cardiovascular diseases, cancer, bowel inflammatory disorders, and resistance bacterial infections, all of which are associated to antibiotic resistance (Morgan et al., 2012; Whiteside et al., 2014; Pascalet al., 2018; Ogunrinola et al., 2020).

Microbiome and the Gut-Brain Axis: Changes in gut integrity and microbial development can have an impact on brain function. Gut–brain axis is a two-way communication system that communicates between CNS and gut through neuronal, endocrine, and immunological signals. Vagal or spinal innervation is supposed to transmit neural

knowledge. Microbiota deliver multiple signals to the CNS and ENS, either directly or indirectly, through the creation of neurotransmitters or neurochemical-like precursors in ENS. Bifidobacterium infant is boosted tryptophan levels in the circulation, which is a precursor to serotonin and SCFAs also enhance the serotonin formation by intestinal cells (Desbonnet et al., 2010, Yano et al., 2015, Dinan et al., 2017; Miraglia et al., 2019).

Control microglia maturation in the CNS, influence ENS activity via G-protein coupled receptors like GPR41 and GPR43, and mediate epigenetic change via histone deacetylation. Microbiome of the human stomach has multiple effects on brain health. Lipopolysaccharides, for example, are structural bacterial components that stimulate innate immune system in a low-grade tonic manner. Systemic and/or CNS inflammation is caused by excessive stimulation from bacterial dysbiosis, small intestine bacterial overgrowth, or enhanced intestinal permeability. Adaptive immune system failure is caused by bacterial proteins reacting with human antigens. D-lactic acid and ammonia are neurotoxic metabolites produced by bacterial enzymes. Short-chain fatty acids, which are beneficial metabolites, may be neurotoxic (Venteret al., 2001; Qin et al., 2010; Soret et al., 2010; Nohr et al., 2013; Surjyadipta, 2013; Jacquemin, 2014; Erny et al., 2015).

Human-like hormones and neurotransmitters can be created by gut microbes. Microbial growth and pathogenicity are affected by bacterial receptors for these hormones. Gut bacteria excite afferent neurons in the ENS, prompting the vagus nerve to transmit messages to the brain. The architecture of sleep and the stress reactivity of the hypothalamic-pituitary-adrenal axis are both influenced by gut microorganisms via these several pathways. They have an impact on memory, mood, and cognition, and are important in the treatment of alcoholism, chronic fatigue syndrome, fibromyalgia, and restless legs syndrome (Hooper, 2004; Galland, 2014).

Gut Microbiota and Central Nervous System Disorders: Human cells account for nearly half of all cells and 1% of all unique genes in our body, with rest originating from microorganisms such as archaea, bacteria, fungus, and viruses. These bacteria make up the human microbiota, with the majority colonizing the gut. The identification of these bacteria and the examination of their contribution to neurological health has been made possible by recent technology developments, open access data repositories, and the use of high throughput sequencing. Alterations in gut microbiota associated with neurological illness risk, activity, and progression, according to new research. Despite substantial clinical and biological research, many neurological illnesses' aetiology, development, and effective therapy remain unknown. The aetiology of these diseases is complex.

The gut bacteria and CNS interact in a variety of ways, according to numerous researches (Sampson et al., 2015). These bidirectional interactions make up gut microbiota–brain axis (Bauer et al., 2016, Dinan, 2017). The first step in identifying whether and how the gut microbiota–brain axis influences human neurological illnesses is to conduct epidemiological investigations (Tremlett et al., 2017). Alzheimer's disease, multiple sclerosis, autism spectrum disorder, Parkinson's disease, and stroke have all been linked to the microbiome as a potential risk factor. Changed microbial composition contributes to the pathogenesis of such disorders, according to cross-sectional clinical research (Cryan et al., 2020).

Basic Microbiome Methodology: Until the 1990s, the majority of gut microbiology research was done using culture, staining, and microscopy. Many anaerobic microbes could not be cultivated or investigated because growth media and circumstances preferred fast-growing, aerobic microbes with DNA sequencing advent, this changed. The 16S bacterial ribosomal RNA (rRNA) gene sequencing method (hereinafter 16S) gained a lot of traction quickly (Matsuki et al., 2002). Conserved sections of this gene are utilized to make broad-spectrum polymerase chain reaction (PCR) primers that can amplify hypervariable areas that are rapidly developing across a wide range of bacteria. By comparing the amplified hypervariable area sequences to a curated library of fully sequenced bacterial 16S genes, the sequences can be taxonomically identified (Louis et al., 2007; Hiergeist et al., 2015).

Although 16S is still the most extensively used approach for describing bacterial communities in research, it has a number of drawbacks. For starters, taxonomic classifications are widely employed to classify microorganisms. Second, because many species within the hypervariable region under study have the same sequence, sequence classification is frequently confined to genus value. Using data from Human Microbiome Project, tools like PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) can infer potential functional pathways from 16S results, but 16S analysis is susceptible to primer bias and does not provide direct information about gut microbe function or potential interactions with host physiology (Langille et al., 2013; Srinivasan et al., 2015; Pollock et al., 2018).

Factors Infusing the Gut Microbiome: A variety of factors, beginning at birth, influence gut microbial makeup and metabolic capacities, as measured by various methods. The embryonic intestine has extremely few bacteria, if any, because the womb is normally sterile. Microbial colonisation begins at birth, and the method of delivery (vaginal, Cesarean, and newborn feeding) has a significant impact. Improved sanitation, immunisation, the eradication of enteropathogens, and exposure to antibiotics and nonantibiotic treatments are all factors that affect the commensal, or native, microbiota. Throughout one's life, one's diet has effect on microbial compositions of one's body (Penders et al., 2006; David et al., 2014; Perez-Muoz et al., 2017; Maier et al., 2018).

Parkinson's disease: Parkinson's disease (PD) is commonest neurological condition, second only to Alzheimer's disease in terms of prevalence. Clinically, it is distinguished by parkinsonism (rigidity, rest tremor, bradycinesia, and postural instability) and pathologically by

neuronal loss in the nigra and elsewhere, which is linked to ubiquinone protein deposits in neuronal cytoplasm (Lewy organisms) and protein-like threading inclusions within neuritis. Parkinson's disease strikes 0.1 percent of those aged 65 to 69, and 1-3 percent of those aged 80 and up. Other illnesses with significant parkinsonian symptoms and signs, such as postencephalitic, drug-induced, and arteriosclerotic Parkinsonism, may be mistaken for Parkinson's disease until an autopsy confirms the diagnosis. About six million people around world suffer from Parkinson's disease (Nussbaum et al., 2014, Armstrong et al., 2020).

Beyond the classic view of Parkinson's disease as a movement disorder, it has become obvious that non-motor symptoms such cognitive impairment, autonomic dysfunction, sleep issues, depression, and hyposmia are all part of the disease and contribute considerably to the total burden. Parkinson's disease strikes men twice as often as it does women in most regions. There was no gender split or perhaps a female excess in a few of villages, including one in Japan. The male majority could be explained by the protective effect of female sex hormones, a sex-related genetic mechanism, or sex-specific disparities in risk factor exposure in the environment, as well as health-care discrepancies (Van Den Eeden et al., 2003; Poewe et al., 2017).

Causes and Genetics of Parkinson's disease: People frequently question, "Why?" after receiving a Parkinson's diagnosis. Parkinson's disease has no recognized etiology for most people ("idiopathic"). Parkinson's disease triggered by a mix of causes, according to researchers. If there existed a continuum with hereditary causes on one end and environmental causes on the other, people with Parkinson's disease would tumble all over the place. Some cases may be genetically determined, while others may be more impacted by environmental factors. Aging is also a factor. Researchers may be able to develop medicines to slow or perhaps prevent the disease if they learn more about what's causing it. Genetics, according to researchers, accounts for roughly 30% of Parkinson's risk. Only around 10% of this risk can be explained by existing genetic linkages, implying that more Parkinson's genes have yet to be uncovered. Studies discovered a number of causal Parkinson's genes (GBA, LRRK2, PRKN, SNCA) in the last decade, where genetic abnormalities dramatically enhance one's risk of developing the disease. Other factors must play a role because not everyone with these genetic abnormalities develops Parkinson's disease,(Khan & Ali 2017, 2018, and Ali and Khan 2021).

Environment and Aging: Other factors linked to elevated risk of Parkinson's disease. Head injuries and pesticide exposure are two of them. After consuming drugs infected with a toxin called MPTP, a group of heroin addicts in California developed a form of Parkinson's disease in the early 1980s.Smoking and coffee usage have been related to lower incidence of Parkinson's disease in numerous studies. Parkinson's disease is most commonly caused by old age. Researchers predict that by 2040, the number of persons with Parkinson's disease will have doubled due to an ageing population. Scientists believe that as we age, our cells become more vulnerable to degeneration. Furthermore, the expression of our genes may alter with time, potentially triggering a cascade of biological processes that leads to Parkinson's disease (Ali and Khan 2021).

Microbiome and Parkinson's disease: Parkinson's disease is a neurodegenerative disease that causes both nonmotor and motor symptoms. Nonmotor symptoms of Parkinson's disease often appear years before motor symptoms. Pathophysiologic abnormalities in the gastrointestinal system, as well as the ENS and CNS, are hypothesized to be linked to dysbiosis of the typical gut microbiome. These alterations are thought to lead to the death of dopaminergic neurons by a variety of mechanisms, including the release of neurotoxins into the bloodstream, a decrease in the production of neuroprotective substances, and an increase in inflammatory and autoimmune responses (Shulman et al., 2011; Elfil et al., 2020).

Intracellular deposition of aggregated -synuclein, which leads to neuronal cell death and inflammation, has long been thought to constitute its pathogenic characteristic PD is now understood to be a multi-systemic disease that affects both the CNS and the PNS and results in a variety of non-motor symptoms like gastroparesis and constipation. Due to the early involvement of the gastrointestinal system, which commonly precedes motor symptoms by years, changes in gut microbiota composition were studied in relation to PD pathogenesis. The hypothesis that the gut microbiota has a role in Parkinson's disease and other neurodegenerative diseases is supported by animal research. According to Sampson and colleagues, the microbiota can affect synucleinopathy and neuroinflammation (2016). (Cersosimo et al., 2012; Pellegrini et al., 2018; Keshavarzian et al., 2020).

As a result, the microbiome could be exploited to provide diagnostic markers and as a therapeutic target. Increased gut permeability and inflammation have been linked to reduced gastrointestinal short-chain fatty acid (SCFA) concentrations in PD patients. SCFAs are the byproducts of bacterial fermentation of dietary components, and they are critical for colonic epithelium feeding and maintenance. Reduced SCFA-producing taxa in PD patients leads to low levels of SCFA. The composition of the PD gut microbiota has been studied in over 20 case–control studies. There were over 100 taxa that were found to be varied in abundance between PD patients and controls (Clairembault et al., 2015; Keshavarzian et al., 2015; Ungeret al.,2016; Hill-Burns et al., 2017; Schwiertz et al., 2018; Qian Yang et al., 2018; Pietrucci et al., 2019; Aho et al., 2019).

Several studies have suggested that persons with Parkinson's disease have a different gut microbiota than people without the condition, although the findings are often conflicting, and there is no consensus on which taxa are associated to the disease. Bacteria from the genus Akkermansia and the Verrucomicrobiaceae family were found to be enriched in patients with Parkinson's disease, but bacteria from the Lachnospiraceae family were found to be deficient. The

Lactobacillaceae family has been found to be high in PD in Western cohorts, although this has never been found in Chinese studies (Qianet al., 2018).

Bacteria belonging to the Prevotellaceae family have also shown inconsistent outcomes. Several studies revealed that the abundance of these taxa was dramatically reduced in PD patients when compared to controls, whereas others found no differences in abundance or found that these taxa were enriched in PD patients. Inherent variability of gut microbiota across cultures, lifestyles, and diets, as well as differences in study designs and methods for obtaining and evaluating 16S rRNA-gene amplicon data, could all lead to disparities between studies. To further understand the significance of changes in the intestinal microbiota composition in PD and assess its potential as a biomarker for PD risk, diagnosis, and prognosis, cross-study comparisons and identification of disease-specific modifications are required (Petrov et al., 2017; Heintz-Buschart et al., 2018; Aho et al., 2019; Savva et al., 2021).

The potential role of gut microbiota in PD pathogenesis: As a result of its critical role in human body functioning, the gut microbiota evolved and became an integral component of people's life. Gut flora influences the release of neurotransmitters such as serotonin, dopamine, norepinephrine, gamma-aminobutyric acid, and glutamate, which may have implications for intra-cerebral functions. As a result, gut dysbiosis could play a role in the disruption of brain signalling networks. Bacterial components such lipopolysaccharide and amyloid protein curli increase synuclein aggregation, suggesting gut microbiome role in synucleinopathies like Parkinson's disease. Additionally, in genetically sensitive animals, gram-negative bacteria in the intestines can cause PD-like symptoms.

Helicobacter pylori infection has been linked to more severe motor impairment, decreased brain dopamine levels, impaired levodopa absorption, as well as immunological and inflammatory reactions in Parkinson's disease patients. H. pylori eradication, on the other hand, had no effect on motor function, according to an RCT. Finally, the gut microbiome of people with Parkinson's disease differs from that of healthy people. Despite the fact that results vary widely due to a differences of confounding factors as geographic dispersion, diet, gender, age, BMI, and medication, the findings are encouraging (including catechol-O-methyl transferase inhibitors).

Two new meta-analyses reveal that people with PD have a pro-inflammatory gut dysbiosis, which is defined by low amounts of SCFA-producing bacteria. SCFAs are immunomodulatory and metabolic byproducts of bacterial fermentation. Another factor to consider when studying gut microbiota composition in Parkinson's disease is some bacteria's ability to metabolise levodopa, primarily via the tyrosine decarboxylase pathway, which causes premature conversion of levodopa to dopamine in the small intestine, resulting in decreased levodopa availability and increased levodopa dose required (Metta et al. 2021).

PD and Gastrointestinal Symptoms: Constipation had a positive probability ratio of 2.2 in a probabilistic approach for detecting prodromal PD in patients with non-motor symptomatic signs, according to the Movement Disorders Society. Some researchers hypothesized that early degenerative PD pathology is associated to constipation, or that early PD pathology begins in the intestinal plexus, based on these associations. However, the idea of multifocal disease, or at the very least the complicated interplay that happens years or decades before motor traits manifest as a result of centrally mediated non-motor prodromal traits like RBD and hyposmia, which have a high prodromal PD risk, should be mentioned (Abbott et al., 2001, Dickson et al., 2009, Hawkes et al., 2010; Berg et al., 2015).

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Conventional Medicinal Uses and Chemical Structure of Important Secondary Metabolites in the Genus *Eremostachys*: A Literature Review

Amrendra M Khan[1], Narinder K Agnihotri,[1] Vinay K Singh,[2] Mukesh C Joshi[1] and Krishan Kumar[1]*

[1]Deparment of Chemistry, Motilal Nehru College, University of Delhi, Delhi, India.

[2]Deparment of Chemistry, Sri Aurobindo College, University of Delhi, Delhi, India.

ABSTRACT

Genus *Eremostachys* Bunge is a key medicinal plant grown in Eastern Europe, Central and Western Asia and Middle East. The plants of this genus have numerous secondary metabolites, which exhibit both traditional and pharmacological applications. Eremostachys contains several classes of reactive chemical ingredients such as flavonoids (viz. Apigenin, Luteolin, Loasifolin, Loasin A, Apuleisin, Apigenin and Kaempferol etc), isoflavonoids (viz. Soforanarin B, Loasin B and Vicarin), iridoid glucosides (viz. Shanzhiside, Lamalbide, Lamalbidic acid, Epiloganin, Pulchelloside, Harpagide, Pulchelloside, hamighriprasin, Eremoside, Phloyoside and Barlerin etc.), phenylethanoid glycosides (viz. Verbascoside, Leucosceptoside A, and Echinacoside etc,), acids, hydrocarbons, terpenes, diterpenoids and sterols (viz. Eremostachiin, Phlomisoside II, Stigmasterol, β-Sitosterol, Daucosterol and Oleanolic aicd) etc. These metabolites are well known for their pharmacological applications such as antibacterial, anti-inflammatory, antioxidant, antirheumatic, anti-poisonous, antimalarial, anticancer, antimalarial, antiallergic, antiarthritic and antidepressant etc. Before the identification of chemical constituents, genus *Eremostachys* was used by few countries since ancient viz. by China, Iran, India, Pakistan, Tajakistan and few middle and south Asian countries etc. This genus has been used by people of these region since ancient as analgesic, anti-inflammatory, wound healing, ant-insecticidal, antiparasitic, antiallergic, liver care, joint pain, arthritis, antioxidant, antibacterial, antidepressant, antimalarial, perfumery, detergent, soap, beauty products. In India, E. superba has been used as a food for cattle to increase milk production. In the present review, the important traditional uses of some important species of the genus *Eremostachys* have been briefly discussed due to their availability and affordability. The number of medicinal and pharmacological applications of the plant genus *Eremostachys* are also summarized in the paper.

KEY WORDS: ANTI-INFLAMMATORY; ANTIOXIDANT; DITERPENOID; EREMOSTACHYS; SECONDARY METABOLITES.

INTRODUCTION

Genus *Eremostachys*, known as desert rod, belongs to the family Lamiaceae. Presently, around 80 species of this genus have been documented, which are mainly distributed in Eastern Europe, Central and Western Asia and the Middle East (Harley et al. 2004). However, more than 45 species are distributed only in Azerbaijan, Armenia, Turkey, Iran, and Turkmenistan (Azizian et al. 1982; Hedge et al. 1986). It is an Irano-Turanian genus and majorly distributed in the desert mountains of the Iranica area especially covering Central Asia. However; few species viz. *E. laciniata, E. molucelloides* and *E. vicaryi* expanded their distribution towards Turkey, Pakistan and Afghanistan etc. Overall, genus *Eremostachys* has been represented by 52 taxa of Flora in the USSR (Former Soviet Union); 41 taxa of Flora found in Iranica; 16 species in Iran; 8 species in Pakistan; 5 species in China; 2 taxa of Flora in Palaestina and 1 taxa in Flora Europeae and one critically endangered taxa of the flora is found in Northern Himalaya of Uttarakhand, Himachal Pradesh and Jammu & Kashmir of India (Knorring et al. 1954; De Filipps et al. 1972; Shishkin et al. 1977; Zohary et al. 1978; Azizian et al. 1982; Rechinger et al. 1982; Chowdhary et al.1984; Jain et al; 1984; Radcliffe-Smith et al. 1986 ; Hedge et al. 1990; Li et al. 1994; Rao et al. 1994; The Hindu 10 Mar, 1997; The Daily Excelsior 17 Oct, 1997; Kalvandi et al. 2007; Hariri et al. 2021).

The morphology of genus *Eremostachys* has been characterized by a robust or erected pubescent stem, laciniate

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or crenate leaves, large calyces, large yellow, creamy or white corollas, beared nutlets and tuberous roots (Pignatti 1982). Phytochemical studies of genus *Eremostachys* have revealed the presence of many potent secondary metabolites viz. alkaloids, phenylethanoids, iridoid glycosides, acids, flavonoids, terpenoids, hydrocarbons and essential oils etc. Due to the variety of secondary metabolites present in the genus *Eremostachys,* this genus is well known for its medicinal properties viz. as strong antidepressant, free radical scavenging and cytotoxic activity (Delazar et al. 2004a; Delazar et al. 2004b; Delazar et al. 2005; Delazar et al. 2006). Some species like *E. azerbaijanica, E. glabra, E. labiosa, E. laciniata, E. laevigata, E. loasifolia, E. macrophylla* and *E. vicaryi* are excessively explored for their secondary metabolites and their medicinal importance (Delazar et al. 2004; Delazar et al. 2005; Erdemoglu et al. 2006; Navaei et al. 2006; Amiri et al. 2007; Calis et al. 2007; Nori-Shargh et al. 2007; Javidnia et al. 2008; Modaressi et al. 2009; Khan et al. 2010; Rustaiyan et al. 2011; Ali et al. 2012; Al-Jaber et al. 2012; Esmaeili 2012; Mughal et al. 2010 and 2012; Imran et al. 2012; Akhlaghi et al. 2015; Vaez et al. 2015; Asnaashari et al. 2016 a; Asnaashari et al. 2016 b; Faryabi et al. 2021; Hariri et al. 2021).

From India point of view, there is only one species *E. superba* Royale ex Benth., of genus *Eremostachys* that was identified as a critically endangered plant species due to lack of proper knowledge, grazing by herbivores, plucking of the flowers by travelers, and overexploitation by local people (Verma et al. 2003). It was described from Mohand and Khree Pass (Siwaliks of Saharanpur) by Royle in 1839, which was a very sophisticated and beautiful plant found in Uttarakhand, Himachal Pradesh, Jammu & Kashmir province of India (Sharma et al. 1981; Jain et al. 1984; Panwar et al. 2015; Hariri et al. 2021).

The genus *Eremostachys* is one of the important medicinal plants due to the presence of numerous potent secondary metabolites. The number of medicinal and pharmacological applications of the plant genus *Eremostachys* are also summarized in the paper. The chemical structure of the important reactive chemical ingredients of the secondary metabolites isolated and identified from the genus *Eremostachys* are given in the present paper. The important secondary metabolites of genus *Eremostachys* reported in the literature are compiled along with their pharmacological applications. It is well evident from the literature reports that substantive number of species of Genus *Eremostachys* got extinct or at the verge of extinction. The present review is aimed to recognize medicinal importance, traditional uses among society and also to document status report of ever becoming critically endangered species of medicinal flora (Hariri et al. 2021).

Taxonomic description of Genus Eremostachys (Ved et al. 2003).

Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division (Phylum): Tracheophyta
Class: Magnoliopsida (Dicotyledons)
Subclass: Magnoliidae Novak ex Takht.
Order: Lamiales
Family: Lamiaceae
Genus: Eremostachys

Species: *E. adenantha, E. azerbaijanica, E. baissunensis, E. glabra, E. labiosa, E. labiosiformis, E. laciniata, E. laevigata, E. lehmanniana, E. loasifolia, E. macrophylla, E. molucelloides, E. pulvinaris, E. speciosa, E. superba, E. thyrsiflora, E. vicaryi* etc.

Traditional Uses of Eremostchys: Conventionally, the genus *Eremostchys* is used by South Asian and West Asian countries for the treatment of various ailments. *Eremostachys* has been used as an anti-inflammatory and analgesic agent and applied topically for the treatment of bruises and localized pain and swelling (Said et al. 2002; Delzar et al. 2004b; Erdemoglu et al. 2006; Hariri et al. 2021).

Traditionally, *E. laciniata* is used in various illnesses viz, to treat allergies, headache and various liver diseases, asthma, cough & cold, alleviate inflammation and used as a herbal tea (from root and flower) (Said et al. 2002; Modaressi et al. 2009). The number of plants of this genus is also used for traditional and folk medicine for treating a number of ailments are described briefly in Table 1. In India genus *Eremostachys superba* Royle ex Benth is used to restore mulching by mixing it with cattle feed and fed to goats, cows, and buffaloes etc., which stop yielding milk (Khan et al. 2020; Hariri et al. 2021).

Pharmacological Importance: Genus *Eremostachys* is one of the important plants, which are known for their diversified medicinal and pharmacological applications (Table 2). Few plants of this species are widely studied viz. *E. laciniata, E. loasifolia, E. macrophylla, E. glabra, E. laevigata, E. azerbaijanica, E. labiosa, E. labiosiformis, E. pulvinaris* etc. However; most of the species are still need to be explored with respect to their pharmacological applications and secondary metabolites. From a medicinal point of view, genus *Eremostachys* is playing a key role in Ayurvedic and Unani medicine due to the presence of the number of chemical reactive secondary metabolites. The whole plant is important for medicinal purposes as all parts of the plant contain some vital secondary metabolites. Secondary metabolites reported in the literature along with their important pharmacological applications are summarized in Table 2 (i) (ii), (iii) and (iv) (Khan et al. 2020; Hariri et al. 2021).

Chemical structure of Secondary metabolites: Numerous secondary metabolites were identified from the genus Eremostachys. Sterols, essential oils, linear hydrocarbons,

iridoid glucosides, flavonoids, isoflavonoids, terpenoids, and their derivatives, acid derivatives and phenylethanoid glycosides etc. are found in a majority. Most of them are represented and specifies by their core structures as follows:

Table 1. Traditional uses of some species of genus Eremostachys

Species	Parts Used for Treatment	Traditional Uses
E. glabra	Rhizomes	Used as a native analgesic and anti-inflammatory agent in Iran (Delazar et al. 2004a).
E. laevigata	Whole plant	Used as therapeutics against many infectious diseases, as food preservatives and have shown insecticidal and antiparasitic properties (Burt et al. 2004). Also used in cosmetic and household products, (www.inchem.org).
E. laciniata	Roots, flower and rhizomes	Roots and flower decoction have been used orally for the treatment of allergy, headache and liver disease. It is known by the local name "Chelle-Daghi" in Iran and its rhizomes are used to relieve pain related to rheumatoid arthritis (Said et al. 2002 and Delazar et al. 2013), as an antioxidant (Erdemoglu et al. 2006), antibacterial (Modaressi et al. 2009), antidepressant (Nisar et al. 2011), antiinflammatory (Hariri et al. 2021) & analgesic in various places of middle south East & south Asia (Delazar et al. 2009).
E. macrophylla	Aerial and rhizome	Aerial & rhizome, used as a folk medicine in Iran, comprises therapeutic ingredients against joints pain, infectious wound healing, snakebite, rheumatism and antimalarial (Nori-Shargh et al. 2007, Mosaddegh et al. 2012, Asnaashari et al. 2015 and Asnaashari et al. 2016 (a and b)).
E. superba	Whole plant	Used as an antidepressant and antioxidant. This species is less reported towards medicinal importance except for the local report according to Gujjars, where they used root tubers as food to buffaloes to increase the milk production. It is used for curing mastitis and restoration of mulching in cattles (Verma et al. 2003 and Sharma et al. 2015) and against fish poisoning (Ajaib et al. 2014).
E. vicaryi	Whole plant and seed	Used for poisoning fish in the Eusufzai near Peshawar (Radcliffe-Smith et al. 1986) and seeds are utilized as cooling agents to lower fever in the Balochistan province (Pakistan) (Tareen et al. 2016).

Table 2(i). Secondary metabolites and Pharmacological Uses of some species of genus Eremostachys

Species	Secondary metabolites	Pharmacological application
E. adenantha Jaub. Et Spach	Dodecanal, tetradecanal, undecanal, tetradecanoic acid, hexadecanoic acid, 6,10,14-trimethyl-2-pentadecanone, caryophyllene oxide (from aerial part) (Javidnia et al. 2008).	Antioxidant (from leaves) (Firuzi et al. 2010).
E. azerbaijanica Rech. f	Tricosane, hexahydrofarnesyl acetone, 2-methyl-6-propyl-dodecane, flavonoid (luteolin-7-*O*-rutinoside), phenylethanoid (verbascoside) (Asnaashari et al. 2016a), sesquiterpenes, steroids, coumarins (Asnaashari et al. 2016b), Phlomisoside II, eremostachiin, alyssonoside, forsythoside B, lamalbide, pulchelloside I, sesamoside, 6-hydroxyloganin, shanzhiside methyl ester (from roots) (Modarresi et al. 2013, Fouladnia et al. 2012 and Asnaashari et al. 2018), dodecanal, hexadecanoic acid, 6,10,14-trimethyl-2-penta-decanone, tetradecanal, undecanal, tetradecanoic acid, caryophyllene oxide (Javidnia et al. 2008), carvone, β-caryophyllene, limonene, β-bourbonene, germacrene D, transcarveol, *cis*-calamenene (Manafi et al. 2010), hexahydrofarnesyl acetone, 2-methyl-6-propyl-dodecane (Asnaashari et al. 2016a).	Radical scavenging activity (Asnaashari et al. 2016a), antioxidant, antimicrobial, and cytotoxic activity (Asnaashari et al. 2017), antimalarial activity (aerial part showed IC_{50} values of 0.949 ± 0.061 mg mL^{-1} and rhizomes showed 0.382 ± 0.011 mg mL^{-1}) (Asnaashari et al. 2016b), antiproliferative (Delazar et al. 2017).

E. glabra Boiss. ex Benth.	furanolabdane diterpene glycoside (Eremostachiin) (Delazar et al. 2006), methyl ester, iridoid glycosides (6,9-epi-8-*O*-acetyl-shanziside 5,9-*epi*-penstemoside, 5,9-*epi*-7,8-didehydro-penstemoside (Delazar et al. 2004b), hexacosyl-(*E*)-ferulate, leucosceptoside A (Delazar et al. 2004a), iridoids (Barlerin, 8-*O*-acetyl-shanziside, penstemoside, 7,8-didehydro -penstemoside) (Jensen et al. 2007), *β*-sitosterol, verbascoside, stigmasterol, phlomisoside II, forsythoside B, 9-epi-phlomiol, lamalbide, 5,9-epiphlomiol, penstemoside, 9-*epi*-pulchelloside II, 6-hydroxy-7-*epi*-loganin, 6'-*O*-*β*-D-glucopyranosyl sesamoside, shanzhiside methyl ester, phloyoside II, hexacosyl-(*E*)-ferulate (from Rhizomes) (Delazar et al. 2013).	Free-radical scavenging activity, antioxidant (hexacosyl-(*E*)-ferulate showed RC_{50} = 0.0976 mg/mL and leucosceptoside-A showed 0.0148 mg mL^{-1}) ((Delazar et al. 2004a)) and antibacterial (Delazar et al. 2004b and 2005, Erdemoglu et al. 2006).
E. labiosa Bunge	*α*-Pinene, 1,8-cineole, 6,10,14- trimethyl 2-pentadecanone, sabinene, hexadecane, *α*-phellandrene, *β*-phellandrene, tetradecane, *p*-cymene (from aerial and stem part) (Rustaiyan et al. 2011).	Anticancer, anti-inflammatory, antileishmanicidal (Rabe et al. 2014).
E. labiosiformis (Popov)	Harpagide (from flowers), 9,12-octa-decadienoic acid, octadecanoic acid, hexadecanoic acid, 1,2-benzene-dicarboxylic acid diisooctyl ester, 9,12,15-octa -decatrien-1-ol (from aerial part) (Kooiman 1972).	Antioxidant, anti-Alzheimer (Samandari-Bahraseman et al. 2018), antibacterial (Vahedi et al. 2013).

Table2 (ii). Secondary metabolites and Pharmacological Uses of some species of genus Eremostachys

Species	Secondary metabolites	Pharmacological application
E. lacimiata a (L.) Bunge	Acidic iridoid glucoside (Calis et al. 2008), iridoid glucosides (phloyoside I, phlomiol pulchelloside I) (Modaressi et al. 2009), furanolabdane diterpene glycosides, monoterpenes, sesquiterpenes, iridoid glucosides and flavonoids (Navaei et al. 2006; Delazar et al. 2008; Eftekharsadat et al. 2011), luteolin, apigenin, 5,8-dihydroxy-6,7-dimethoxy-flavone, 5,7-dihydroxy-6,8-dimethoxy-flavone, luteolin 7-*O*-*β*-glucosides (Nisar et al. 2011), phlomisoside II, verbascoside, leucosceptoside A, martynoside, forsythoside B, apigenin 7-*O*-glucoside, luteolin 7-*O*-(6"-*O*-apiofuranosyl)-glucoside, apigenin 7-*O*-(6"-*O*-p-coumaroyl)-glucoside, sesamoside, 5-deoxysesamoside, 6-*β*-hydroxy-7-*epi*-loganin, 5-deoxy-pulchelloside-I, Chlorotuberoside, lamalbide, lamalbidic acid, phloyoside I (7-*epi*-phlomiol), phloyoside II, phlomiol, shanzhiside, shanzhiside methyl ester, 8-Oacetyl-shanzhiside methyl ester, dodecanol, widdrol, germacrene B and D, thujopsene, 3-octanone, (3Z)-hexen-1-ol, *n*-hexanol, benzacetaldehyde, 1-octen -3-ol, *α*-pinene, linalool, 6,10,14-trimethyl-2-pentadecanone, limonene, *p*-cymene, *δ*-cadinene, (2E)-dodecenal, dehydrolinalool, cyclo-pentadecanolide, (*E*)-*β*-ocimene, 1,8-cineole, terpinen-4-ol (Navaei et al. 2006, Al-Jaber et al. 2012 and Delazar et al. 2013) (aerial part).	Anti-inflammatory (Hariri et al. 2021 and Delazar et al. 2013), antibacterial (MIC = 0.05-0.50 mg mL^{-1}) (Modaressi et al. 2009 and Ur Rahman et al. 2015), free radical scavenging, antioxidant properties, anti-inflammatory, dietary supplement (Hariri et al. 2021, Mosaddegh et al. 2012 and Bajalan et al. 2017), effective in the treatment of mild and moderate Carpal Tunnel Syndrome (CTS) in combination with the wrist night splint, especially in alleviating the severity of the syndrome and increasing the palmer prehension power (Eftekharsadat et al. 2011), antipain (Gharabagy et al. 2013) anti-depressants (Nisar et al. 2011 and Hakimi et al. 2020).

E. laevigata Bunge	Benzaldehyde, 1,8-cineole, piperitenone oxide, *cis*-piperitoneoxide, 1-octen-3- ol, dodecanal, germacrene-D, *β*-caryophyllene, caryophyllene oxide (Amiri et al. 2007 and Esmaeili et al. 2012) (from whole plant).	Antibacterial, antioxidant activity (IC_{50} ($\mu g\ mL^{-1}$): 277.1 (flowers), 495 (stems), 212.6 (root) (Esmaeili et al. 2012), *β*-caryophyllene possesses anti-inflammatory, anti-carcinogenic activities and plant defense (Cai et al. 2002), germacrene-D is anti-insect (Altug et al. 2004), Dodecanal is non-toxic, food additive (GRAS in USA and inchem in UE) and used in perfumery as in soap, detergent, beauty care and household products (www.inchem.org).

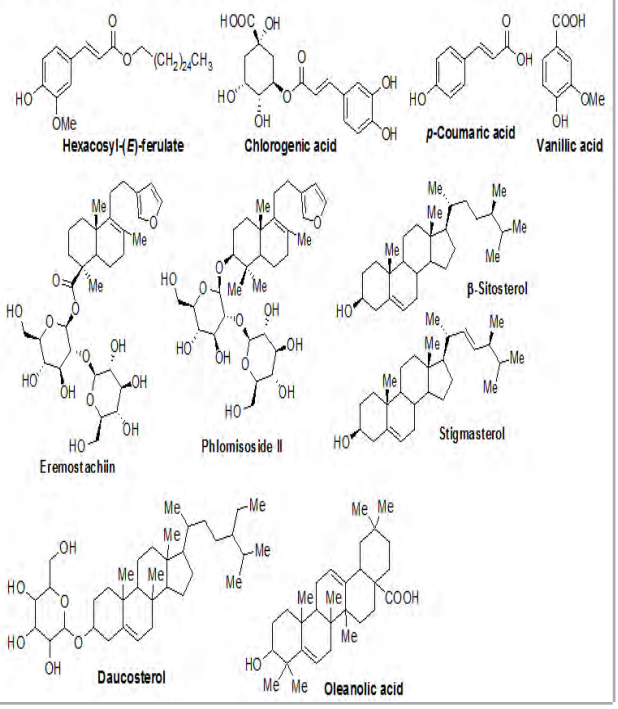
Table2 (ii).Secondary metabolites and Pharmacological Uses of some species of genus Eremostachys

Species	Secondary metabolites	Pharmacological application
E. laciniata a (L.) Bunge	Acidic iridoid glucoside (Calis et al. 2008), iridoid glucosides (phloyoside I, phlomiol pulchelloside I) (Modaressi et al. 2009), furanolabdane diterpene glycosides, monoterpenes, sesquiterpenes, iridoid glucosides and flavonoids (Navaei et al. 2006; Delazar et al. 2008; Eftekharsadat et al. 2011), luteolin, apigenin, 5,8-dihydroxy-6,7-dimethoxy-flavone, 5,7-dihydroxy-6,8-dimethoxy-flavone, luteolin 7-*O*-*β*-glucosides (Nisar et al. 2011), phlomisoside II, verbascoside, leucosceptoside A, martynoside, forsythoside B, apigenin 7-*O*-glucoside, luteolin 7-*O*-(6''-*O*-apiofuranosyl)-glucoside, apigenin 7-*O*-(6''-*O*-p-coumaroyl)-glucoside, sesamoside, 5-deoxysesamoside, 6-*β*-hydroxy-7-*epi*-loganin, 5-deoxy-pulchelloside-I, Chlorotuberoside, lamalbide, lamalbidic acid, phloyoside I (7-*epi*-phlomiol), phloyoside II, phlomiol, shanzhiside, shanzhiside methyl ester, 8-Oacetyl-shanzhiside methyl ester, dodecanol, widdrol, germacrene B and D, thujopsene, 3-octanone, (3Z)-hexen-1-ol, *n*-hexanol, benzacetaldehyde, 1-octen -3-ol, *α*-pinene, linalool, 6,10,14-trimethyl-2-pentadecanone, limonene, *p*-cymene, *δ*-cadinene, (2E)-dodecenal, dehydrolinalool, cyclo-pentadecanolide, (*E*)-*β*-ocimene, 1,8-cineole, terpinen-4-ol (Navaei et al. 2006, Al-Jaber et al. 2012 and Delazar et al. 2013) (aerial part).	Anti-inflammatory (Hariri et al. 2021 and Delazar et al. 2013), antibacterial (MIC = 0.05-0.50 mg mL^{-1}) (Modaressi et al. 2009 and Ur Rahman et al. 2015), free radical scavenging, antioxidant properties, anti-inflammatory, dietary supplement (Hariri et al. 2021, Mosaddegh et al. 2012 and Bajalan et al. 2017), effective in the treatment of mild and moderate Carpal Tunnel Syndrome (CTS) in combination with the wrist night splint, especially in alleviating the severity of the syndrome and increasing the palmer prehension power (Eftekharsadat et al. 2011), antipain (Gharabagy et al. 2013) anti-depressants (Nisar et al. 2011 and Hakimi et al. 2020).
E. laevigata Bunge	Benzaldehyde, 1,8-cineole, piperitenone oxide, *cis*-piperitoneoxide, 1-octen-3- ol, dodecanal, germacrene-D, *β*-caryophyllene, caryophyllene oxide (Amiri et al. 2007 and Esmaeili et al. 2012) (from whole plant).	Antibacterial, antioxidant activity (IC_{50} ($\mu g\ mL^{-1}$): 277.1 (flowers), 495 (stems), 212.6 (root) (Esmaeili et al. 2012), *β*-caryophyllene possesses anti-inflammatory, anti-carcinogenic activities and plant defense (Cai et al. 2002), germacrene-D is anti-insect (Altug et al. 2004), Dodecanal is non-toxic, food additive (GRAS in USA and inchem in UE) and used in perfumery as in soap, detergent, beauty care and household products (www.inchem.org).

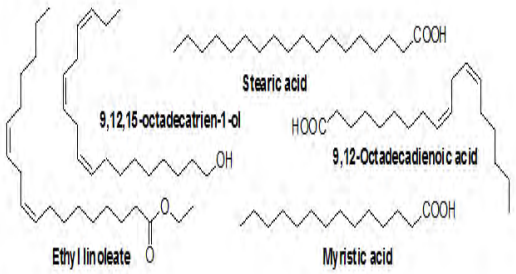
Table2 (iv). Secondary metabolites and Pharmacological Uses of some species of genus Eremostachys

Species	Secondary metabolites	Pharmacological application
E. pulvinaris Jaub. & Spach	Phenylethanoid glycosides (forsythoside B, leucosceptoside A, verbascoside) (Delazar et al. 2004) (from rhizomes).	Free radical scavenging activity and toxicity, antioxidant (RC_{50} = 0.0064, 0.0148 & 0.0079 mg mL^{-1} for forsythoside B, leuco-sceptoside A & verbascoside, respectively) (Delazar et al. 2004).
E. speciosa Rupr.	luteolin 7-*O*-*β*-D-glucoside Gella et al. 1972.	Antioxidant and anti-inflammatory (from epigeal parts) (Gella et al. 1972).
E. superba Bunge	less studied due to critically endangered species in India (Shrivastava et al. 2017 and Srivastava et al. 2018).	A very handsome plant used as an ornament (Duthie, 1903-29), tuberous roots are used for increasing lactation in cattle (Koul et al. 1997, Vaez et al. 2015 and Pant et al. 2011), treatment of liver, stomach and gout related diseases (Srivastava et al. 2018).
E. thyrsiflora Benth.	Alkaloids, steroids, flavonoids, phenols, tannins, saponins, terpenoids, fats, glycosides, coumarins, xanthoproteins, carbohydrates, carboxylic acids and volatile oils (Behlil et al. 2019).	Antioxidant activity (from the whole plant) (Behlil et al. 2019).
E. vicary Benth. ex Hook. f.	Vicarin, soforanarin B, luteolin 7-*O*-*β*-D-glucopyranoside, hamighriprasin (Calis et al. 2007).	Seeds are utilized as cooling agent to lower fever in the Balochistan of Pakistan (Ajaib et al. 2014).
E. baissunensis Popov	Barlerin, lamalbide, 5-deoxysesamoside (from aerial part) (Bobaev et al. 2015).	Not studied much.
E. lehmanniana	Fatty acids from seeds (Bagci et al. 2007)	*E. lehmanniana* Bunge is not studied much.

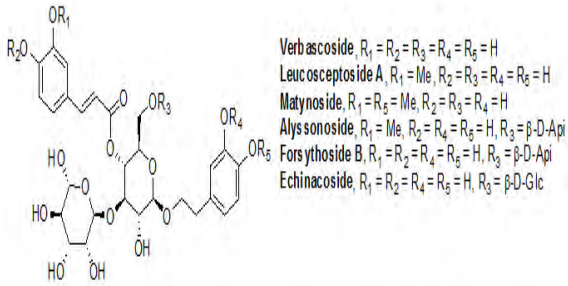
i) Di-terpenoids and Sterols:

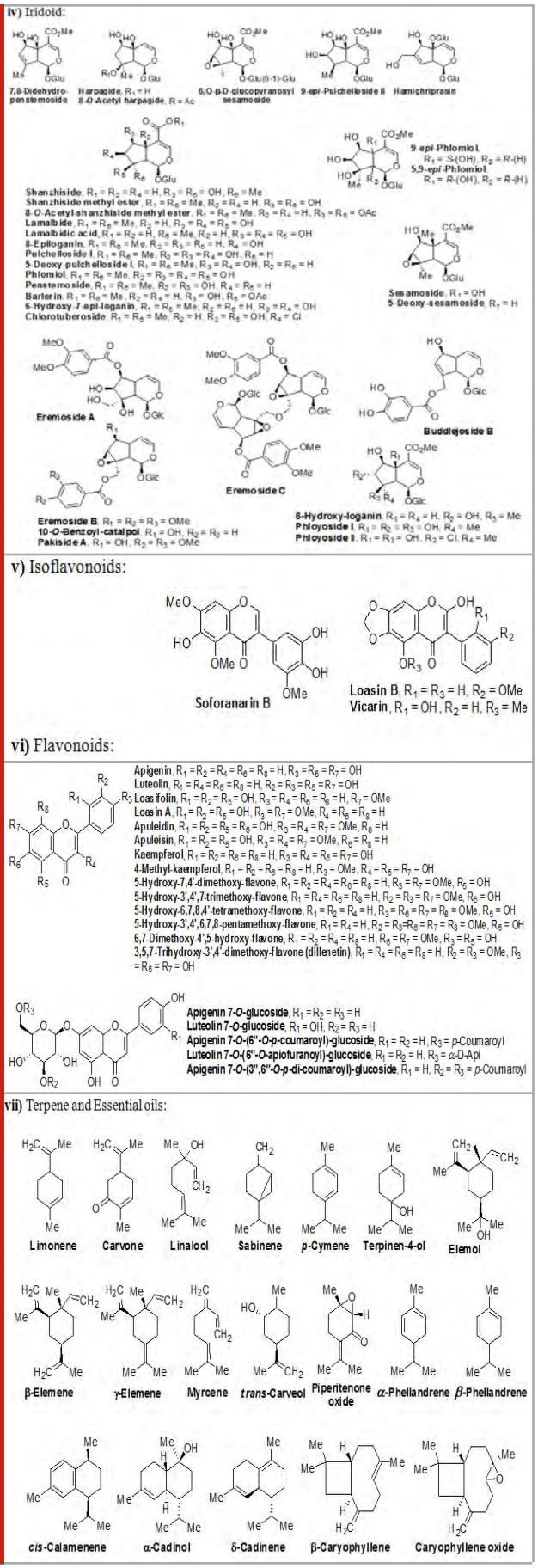


ii) Acids and fatty acids:



iii) Phenyl-ethanoid glycoside:





CONCLUSION

The findings of the present study has shown that the genus Eremostachys is very important with proven medicinal impacts due to the presence of numerous secondary metabolites and their known biological applications viz. antibacterial, anti-inflammatory, antioxidant, painkilling, antirheumatic, anti-poisonous. Further, it can be a potential agent towards antimalarial, anti-Parkinson's and anticancer etc. as few reports are based on such studies. Therefore, in this review, the important secondary metabolites extracted from the genus *Eremostachys* viz., flavonoids, isoflavonoids, iridoid glucosides (chemotaxonomic markers), phenyl-ethanoid glycoside, acids, hydrocarbons, essential oils, terpenes, diterpenoids and sterols etc. are summarized along with chemical structure. The traditional uses and pharmacological applications of this genus *Eremostachys* reported in the literature are compiled in tabular form. Unfortunately, only a few species (viz. *E. laciniata, E. azerbaijanica, E. glabra*, and *E. macrophylla*) have been majorly studied so far, however; most of the species of this genus are still need to be explored. The genus *Eremostachys superba* Royle ex Benth is an only endangered species in India, having an ornamental value as very few studies on their medicinal properties are reported in literature.

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Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Emergency Department Revisit Rate of Chronic Obstructive Pulmonary Disease Patients On Oral Corticosteroids: An Observational Study

Taha Ismaeil,*[1,2] Fatmah Othman,[2,3] Munyra Alhotye,[1] Haneen Almuzaini,[1] Rawabi Alharbi,[1] Nihal Aldulaimi and Alanoud Alsaif[1]

[1]Respiratory Therapy Department, College of Applied Medical Sciences, King Saud bin Abdulaziz university for health Sciences, Riyadh, Saudi Arabia.

[2]Research Unit, College of Applied Medical Sciences, King Saud bin Abdulaziz University for health Sciences, Riyadh, Saudi Arabia

[3]King Abdullah International Medical Research Center's, Riyadh, Saudi Arabia.

ABSTRACT

Studies report inconclusive results regarding the efficacy of oral corticosteroids to reduce the risk of re-visiting the Emergency Department (ED). The aim of this was to compare between COPD patients who received oral corticosteroids and re -admitted to the ED earlier than 60 days. An observational study was conducted at the ED from 2016 to 2018. A cohort of adult COPD patients, who received oral corticosteroids was assessed for any ED re-visit due to a COPD exacerbation. A total of 325 COPD patients included the study, 71% had no subsequent ED visit, and 94 (28%) patients had a repeat ED visit due to a COPD exacerbation. Of this ED re-visit group, 61% was within 60 days. The ED re-visit within 60 days group was more likely to have cardiovascular disease than the group with an ED re-visit after 60 days (51% vs. 45%, p-value 0.64). The use of oral corticosteroids could potentially reduce the severity of COPD exacerbation and ED re-visits.

KEY WORDS: CHRONIC OBSTRUCTIVE PULMONARY DISEASE, EXACERBATION, ORAL CORTICOSTEROIDS.

INTRODUCTION

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) (2022) reported that chronic obstructive pulmonary disease(COPD) could be the 3rd leading cause of mortality by 2021(Almagro et al. 2002; Criner and Han 2018; Kim and Aaron 2018; Global Initiative for Chronic Obstructive Lung Disease 2022). Exacerbations of COPD are important events that negatively influence the patient's health status, the quality of life, and the hospital admission rate (Alamoudi 2006; Mannino and Buist 2007; Simmering et al. 2016). As a COPD exacerbation is complex and challenging to manage, it is crucial to identify the risk factors as this has significant implications. Many studies identified risk factors associated with a COPD exacerbation; however, little evidence is available regarding the risk factors for emergency department (ED) readmission (Parekh et al. 2014; Erezaee et al. 2018; Bogart et al. 2020; Shin 2021). Based on the literature, 10% of the COPD patients discharged from hospital, were readmitted within 30-60 days, with more complications associated with mortality (Baker, Zou and Su 2013; Qahtani et al. 2017; Erezaee et al. 2018; Liao et al. 2018; Shin 2021).

There is a growing body of evidence regarding practical guidelines to reduce readmission and the factors that could contribute to reduce the risk of hospital readmission (Woods et al. 2014; Zafari 2021). Several studies examined the effect of prescribing oral steroids and subsequent hospital re-admission (Baker, Zou and Su 2013; Stefan et al. 2013; Woods et al. 2014; Simmering et al. 2016; Moran and Pavord 2020). The use of oral corticosteroids is recognized to relieve symptoms and exacerbations of the disease. In addition, randomized control trials provided evidence of short-term improvement in the symptoms of COPD exacerbation and pulmonary function (Lindenauer et al. 2010; Woods et al. 2014; Kim and Aaron 2018). However, evidence is still required regarding the effect of using oral steroids in reducing emergency re-visits. Thus, many studies have reported higher ED re-visit rates, ranging from 60% to 82% (Fidahussein et al. 2014; Erezaee et al. 2018; Zafari 2021). Some evidence did not focus on COPD patients who

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received oral corticosteroids. This study, therefore, aimed to identify a cohort of COPD patients who received oral corticosteroid and compare ED re-admission rate earlier than 60 days and ED re-admission rate after 60 days.

MATERIAL AND METHODS

A retrospective cohort study was conducted with all patients who presented with a COPD exacerbation at the ED of King Abdulaziz Medical City in Riyadh from 2016 to 2018. The inclusion criteria were middle-aged (>40 years old) patients with at least one month of active records in KAMC, and discharged from the hospital with an index diagnosis of COPD exacerbation or presented at the ED due to a COPD exacerbation. The International Classification of Diseases (ICD-10) were used to identify patients with COPD with acute exacerbation (ICD-10 code J44.1). The date of admission or ED visit was considered the index date. Of this group of patients, we identified a cohort who received a prescription for an oral corticosteroid, through the prescription data linked to the patient's electronic medical file. This cohort was assessed for any ED re-visit due to an exacerbation of COPD.

We excluded COPD patients who received oral corticosteroids for other diseases, such as rheumatoid arthritis, anaphylaxis, or angioedema. The main outcome of this study was an ED re-visit within 60 days of discharge from the index date. The re-visit was identified as an admission in the ED due to shortness of breath or shallow, labored breathing, and coughing. Due to the possibility of multiple COPD-related ED re-visits during the study period, we focused only on the first ED re-visit after the index date. Each patient's clinical and demographic variables were retrieved from their electronic medical file. This included age (categorized into ≤65, 66-75, ≥75), gender, body mass index (BMI)(categorized into normal, overweight, and obese), comorbidities (diabetes mellitus, cardiovascular diseases, stroke, or hypertension), type of oral corticosteroid prescribed, and admission/discharge date. Descriptive statistics were used to analyze the data.

Continuous variables, if normally distributed, are presented as the mean and standard deviation or by median and interquartile range if not, and the categorical variables as the proportion. We calculated the frequency and proportions of the groups with an ED re-visit within or after 60 days, and compared the two groups in relation to the demographic and clinical variables using a chi-square test or Fischer exact test as appropriate. A p-value of less than 0.05 was considered significant. All analyses were performed using STATA 15. Patients with missing covariates were excluded from conducting a complete case analysis. This research was approved by the Institutional Research Board of King Abdullah International Medical Research Center and registered with protocol number SP19/173/R.

Table 1. Demographic characteristics of COPD patients treated with oral corticosteroids with an ER visit within and after 60 days

Characteristics	All patients N=94 (%)	Patients with an ED re-visit > 60 days N= 58(%)	Patients with an ED re-visit < 60 days N=36(%)	P-value*
Age category				0.45
≤65 year	14(15)	8(13)	6(17)	
66-75 year	34(37)	19(33)	15(42)	
≥75 year	45(48)	31(53)	14(40)	
Gender				0.33
Male	36(38)	20(34)	16(44)	
Female	58(62)	38(66)	20(56)	
Body Mass Index(BMI)				0.12
Underweight	22(23)	18(31)	4(11)	
Normal	21(22)	10(17)	11(30)	
Overweight	22(23)	13(23)	9(25)	
Obese	29(31)	17(29)	12(33)	

* p-values were obtained using chi-square tests or Fisher's exact tests (for numbers less than five in each group). P<0.05 was considered as significant.

RESULTS AND DISCUSSION

A total of 325 COPD patients presented at the ED with a COPD exacerbation and received a prescription for an oral corticosteroid on the index date. The mean age was 72 ± 15 years, and 64% were female. Of this cohort, 28% (n=94) re-visited the ED due to a COPD exacerbation during the study period. The median time between the first ED visit and the ED re-visit was 3 months (IQR 1–6 months). The mean age of this group was 75 ± 10 years, and 62% were female. Shortness of breath was the most frequent symptom (82%). Prednisolone was the most frequently prescribed oral

corticosteroid (93%), followed by hydrocortisone (3%). Of the 94 patients, 38% (n=36) had an ED re-visit within 60 days and 61% (n=58) after 60 days.

Table 1 demonstrates the demographic characteristics of the cohort with COPD with an ED re-visit. Compared with the after 60-day ED re-visit group, the group who re-visited within 60 days was younger (13% < 65 years vs. 17% < 65 years respectively, which was not significant). In terms of BMI, 29% of the after 60 days group were in the obese category compared to 33% in the within 60 days group. The difference was not significant.

Table 2 demonstrates the clinical characteristics of this cohort. The within 60 days group were more likely to have cardiovascular diseases (51% vs. 46%, p-value=0.64) compared to after 60 days group. The proportion of ED re-visits in the current study is consistent with similar studies, reporting rates of 16% to 24%. However, other studies reported a higher ED re-visit rate, ranging from 60% to 82% (Lindenauer et al. 2010; Fidahussein et al. 2014; Parekh et al. 2014; Shirakawa 2021).

Another study reported that 1 in 11 COPD patients is hospitalized within 30 days after discharge, highlighting the presence of important risk factors for early hospital readmission. The difference may be due to the studies investigating the ED re-visit rate for all COPD patients and within 30 days from the index date. The current study focused on COPD patients with an exacerbation who received an oral corticosteroid, as the aim was to determine the effect of the oral steroid on the re-visit rate, hospital admission, and healthcare cost (Erezaee et al. 2018; Shirakawa 2021). For COPD patients, ED re-visits occur frequently and many studies focused on the identification and mitigation of the associated risk factors (Decramer and Janssens 2013). The severity and progression of the disease, with the presence of comorbidities, have a direct impact on the ED re-visit rate and length of hospital stay. In the current study, cardiovascular comorbidity was higher in the group re-visiting within 60 days compared to the counter group. This finding was consistent with previous studies (Shirakawa 2021).

The prescription of oral corticosteroid for the treatment of an acute exacerbation of COPD is recommended in many guidelines (Global Initiative for Chronic Obstructive Lung Disease 2022); however, its efficacy in reducing the ED re-visit rate is still debated (Parekh, et al. 2014; Woods et al. 2014; Erezaee et al. 2018; Colak 2021). Many studies recommended using the ED as a potential setting to implement re-admission reduction strategies for COPD patients. The studies support targeting subgroups of patients with severe and unstable COPD to reduce the overall ED re-visit rate and hospital re-admission. The finding of the current study is in agreement with this point as we restricted the sample to patients with COPD who presented at the ED with an exacerbation, and a relatively small proportion (28%) had an ED re-visit within the study period (Colak 2021).

Table 2. Clinical comorbidity of COPD patients treated with oral corticosteroids with an ED visit within or after 60 days

Characteristics	All patients N=94 (%)	Patients with an ED re-visit > 60 days N= 58(%)	Patients with an ED re-visit < 60 days N=36(%)	P-value*
Diabetes mellitus				0.41
No	35(38)	20(34)	15(43)	
Yes	58(62)	38(65)	20(57)	
Cardiovascular disease				0.64
No	48(52)	31(53)	17(49)	
Yes	45(48)	27(46)	18(51)	
Hypertension				0.94
No	13(14)	8(14)	5(14)	
Yes	80(86)	50(86)	30(85)	
Stroke				0.40
No	88(95)	54(93)	34(97)	
Yes	5(5)	4(7)	1(3)	

* p-values were obtained using chi-square tests or Fisher's exact tests (for numbers less than five in each group). P<0.05 was considered as significant.

The main strength of this study is that we were able to identify the second visit of the COPD patients through the electronic medical files, using a valid diagnosis, which reduces misclassification bias. However, there are some limitations that may affect the generalizability of the results. Though we collected information regarding the comorbidities of the cohort, many other factors, such as underlying disease activity, the burden of other comorbidities, the number of rehospitalizations in the stated period, and current medication were not collected, which

may influence the result of the study. Patients may also have had a previous visit to the ED before the start date of the study, which may affect the re-admission risk of the cohort. Further studies could be directed to identify the effect of such variables in exploring the association between the prescription of oral corticosteroids and ED re-admission (Colak 2021).

CONCLUSION

The findings of the present study aimed to estimate the 60 days ED re-visit rate of COPD patients with an exacerbation who received oral corticosteroids in their initial visit. The re-visit rate was 28% of the patients, and of this group, 38% re-visited the ED within 60 days, providing evidence of a low ED re-visit rate. Reviewing the management plan for COPD patients presenting at the ED, an improved treatment strategy is required to reduce the risk of readmission, reducing the healthcare cost as well as improving the patient's quality of live.

Conflicts of Interests: Author declare no conflict of interests to disclose.

Ethical Clearance Statement: This study was approved by the IRB office of King Abdullah International medical centre, study number SP19-173-R.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Removal of Heavy Metal Ions using Activated Carbon by Mixed Plants

S. Vasanthan[1]*, A. Murugesan,[2] A. Kistan[1] and A. Selvam[1]

[1]Department of Chemistry, Panimalar Institute of Technology, Chennai, Tamil Nadu, India.

[2]Post Graduate & Research Department of Chemistry, Government Arts College, Ariyalur, Tamil Nadu, India.

ABSTRACT

Environmental and industrial problems arising from polluted water workably influence the relevance of separation of metal ions. Most of the industries in to-days world dump their wastewater into river, pond or sea which pollutes the water and increases the pollution level. Hence, it is increasingly important to purify the polluted surface water and also industrial effluents, especially for the exclusion of metal ions, by employing several physico–chemical processes. The different sources of heavy metal pollution are geological weathering, mining and industrial processing of ores leads to leaching of metals ions from waste, metal excretions from animals and run-off from agricultural fields using metallic biocides. The CPACs were chosen because of its cheapness and easy carbonization from the abundant carbonaceous agricultural wastes/ by-products. BDST model used to predict the presentation of a column for adsorption of metal ions. The performance of the column charged with granular CAC and CR [Tulsion CXO – 9(H)] was also studied and compared.

KEY WORDS: ACTIVATED CARBON, BIOCIDES, CARBONIZATION, GRANULAR, INDUSTRIAL EFFLUENTS.

INTRODUCTION

Industries such as electroplating, pickling, galvanizing, leather, metal finishing and processing, chemical etc, are also some other sources. The metal ions are highly toxic due to their bioaccumulation tendency and required affinity for the sulphydryl (-SH) groups of enzymes / proteins, thus preventing the enzymatic activity and / or disrupting the cellular structures (Bayader et al. 2018). Generally, heavy metals reason for irritation, nerve tissue damages, cardiac strain, heart diseases, disturbed metabolism, kidney malfunction, hyper-tension, ruin of central nervous and renal systems, brain damage and cancer. Therefore, an economical way to achieve this without losing creativity and maintaining strict reset limits has become a challenge to human ingenuity and duty (Fergusson 1990; Söderholm et al. 2019). This has led to the continued refinement of existing treatment techniques and the recognition and development of promising emergent technologies like adsorption (Benjamin and Victoria 2020; Mofijur et al. 2021).

The removal of metallic ions up to date a economic approach remainders a great trouble even as some of successive structures have industrialized with adsorption strategies. Activated carbon (AC) adsorption has advantages over traditional water treatment and reuse methods in terms of initial investment, simplicity of design, ease of operation and freedom of toxic substances. (Van 1983; Rahim et al. 2021). Commercial AC(CAC) in powder and granular forms have the most common adsorbent and broadly was used but are expensive. This has contributed to the search for inexpensive adsorbents other than CAC. References are made to the cultivation and use of beneficial adsorbents such as chitin and chitosan, silica, wood, peat, natural clay, bagasse heartwood, dyed cellulosic material, apple waste, waste AC, GAC models, fibers and polymers/resins. it's possible. absorbent. However, most effective a completely constrained quantity of statistics is to be had on using chemically prepared activated carbons (CPACs) from agricultural wastes, by product of organic material (Poots et al. 1976; Filippi and Krukonis 1980; McKay et al. 1980; McKay et al. 1982; Poots et al. 1986; McKay and Bino1987; Pollard 1992; Maranon and Sastree 1992; Shukla et al. 1992; El-Geundi 1993; McKay 1998; Annesini and Monticelli 2000; Sivakumara et al. 2020).

Fixed bed/column processes are commonly used for pollution control methods such as ion adsorption through ion exchange beds or carbon adsorption beds. Several models have been introduced into the industry to study data and predict outcomes for different adsorption schemes. (Tien

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1994; Subin Park and Junghyun 2016). Although these models based on important mass transfer mechanisms with external films, pores and bottom diffusion have been proposed, solutions of some partial differential equations involving solids and dynamic parameters are required (Löhner et al. 2021).

Shortcut models based on pilot plant testing method remain used mostly for confirmation relatively than information collection, money and saving time. The bed depth facility time (BDST) model and mass transfer zone [MTZ] model (Alan 1952; WalkerL and Weatherley 1997). Adsorber performance provides simple tactics and quick predictions. The BDST model has been successfully used to describe the dye adsorption of the column. The purpose of this effort is to study the capacity of CPACs such as SC and SDC to eliminate metal (Cu2+, Pb2+ Cr3+ and Zn2+) ions from aqueous solution, by means of the column technique. CPAC was chosen because of its low cost due to the high volume of carbonaceous agricultural waste/by-products and its ease of carbonation. BDST model used to predict the performance of metal ion adsorption columns (Mamdouh 2006). The performance of column charged with granular CAC and CR [Tulsion CXO – 9(H)] was also studied and related (Vithanage et al. 2015; Afroza et al. 2020; Löhner et al. 2021).

In the fixed bed depth service time model, the basic principle of the strategy is to predict the effectiveness of the adsorbent material with which it can withstand the removal of a certain number of contaminants from the solution before regeneration is required (Mohamed et al. 2020). Required period of time is called the service time(t) of the bed. Hutchins projected a simple approach to fixed bed absorbers to relate the service time with the process variable quantity like, initial concentration, flow rate and adsorption capacity, by equation (1) (Arunachalam et al 2021).

$$t = [(N_o Z) / C_o V] - \{(1/k_a C_o) \ln [(C_o/C_b)-1]\} \quad (1)$$

where, C_o = initial concentration of metal (mg dm^{-3}) (Zümriye Aksu, Jülide Yener 2001)

C_b = break through adsorbate concentration (mg dm^{-3})
k_a = BDST adsorption rate constant (dm3 mg^{-1} min^{-1})
V = velocity (cm min^{-1})
Z = bed height (cm)

The theoretic deepness of adsorbent (AC) adequate to avoid the adsorbate concentration from beyond C_b at t = 0, termed the bed depth (Z_o, in cm) can be attained, when the service time is zero (t = 0) and given by equation (2):

$$Z_o = (V/ k_a N_o) \ln [(C_o/C_b)-1] \quad (2)$$

By determining the service time t for the formation depth Z from the experimental data, we can estimate No and ka from the slope of the graph and the values of the intersection (at t = 0), respectively. Graph of the critical formation depth equation. Reciprocal value of slope remains the rate at which the adsorbent bed is consumed, and increasing this particular value by the adsorbent's outward bulk thickness gives adsorbent utilized rate to continuous discharge waste water of acceptable quality (Elwakeel et al. 2020). BDST is written as simplified method as follows:

$$t = AZ + M \quad (3)$$

where, slope,

$$A = (N_o / C_o V) \quad (4)$$

$$M = (1/k_a C_o) \ln [(C_o/C_b)-1] \quad (5)$$

The value of straight line presented is used to explain the working of the bed, if there is initial concentration $C_{o,1}$, to a new value $C_{o,2}$, Hutchins projected that new slope A_2 and new intercept M_2 can give by eqns.(6) and (7), similarly.

$$A_2 = A_1 (C_{o,1}/ C_{o,2}) \quad (6)$$

$$M_2 = M_1 (C_{o,1}/ C_{o,2}) \ln \{[C_{o,2}/ C_{o,b})-1] / [C_{o,1}/C_{o,b})-1]\} \quad (7)$$

(McKay et al. 1998) detailed that, When the calculated data is important for changing the permeate volume flow rate in a similar adsorption system, the new slope (A2) through the unaltered segment (M2 ; M2 = M1) can be written as:

$$A_2 = A_1 (Q_1/ Q_2) = A_1 (V_1/ V_2) \quad (8)$$

Apart from BDST model, MTZ model also predicts the design parameters similar to the BDST model.

MATERIAL AND METHODS

The activated carbon prepared by Sol gel method (Nurul et al. 2021). Chromium (Cr), Lead (Pb), Zinc (Zn), Copper (Cu) and were determined by spectrometric method using Atomic Absorption Spectrometer - Model: PerkinElmer-Analyst -400. The obtained results were formulated, estimated and mentioned according to the standards prescribed below 'Indian standard drinking water specification IS 10500: 1992' of Bureau of Indian Standards [BIS].

For the fixed bed experiments, the groundwater from the column is collected at regular intervals of time (30 – 45 min.) and the metal ions were estimated spectrometric method using Atomic Absorption Spectrometer - Model: PerkinElmer-Analyst -400 (Allen and Minear 1982; Rao and Ramakrishna 1982; Jeffery et al. 1991; Lahrich et al 2019).

RESULTS AND DISCUSSION

Effect of initial concentration: The specific concentration of metal ions in the raw water is an important parameter and major determinant, but a given adsorbent capacity only absorbs a certain amount of metal ions. So, for the more concentrated solution of an incoming, the small amount of adsorbent can purify. Many experiments have done to study the effect of changing the initial concentration on the rate of metal ion removal from solution. An increase in initial metal ion concentration increases the slope of intercept curve, decreasing the volume of influent treated

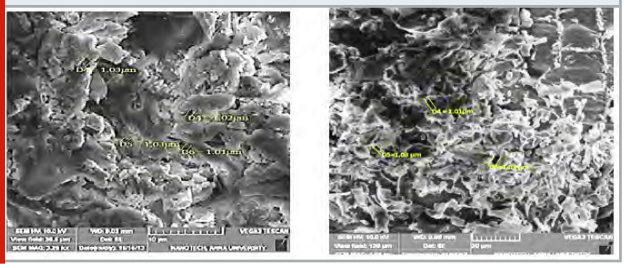
earlier the adsorbent renewal and also results in an early breakthrough and exhaustion of the bed/column (Talat et al. 2018; Abdullah et al. 2019; Bounaas et al. 2021).

Activated Carbon Characterization: The Activated carbon prepared from three different plants of stems used (Neem, Mango and palm tree) (bulk density, porosity, pore volume, ash content, Average particle size, iodine number etc,) and characterized by SEM (Bounaas et al. 2021). The data of the prepared samples are shown in Fig1.

Table 1. Characteristics of Activated carbon prepared from Mixed Plants

Parameter	Observed Value	Standard Value
Bulk Density	0.521 gm/cm^3	1.285 gm/cm^3
Porosity	1.253 cm^{3}g^{-1}	1.365 cm^{3}g^{-1}
Iodine Number	1022 mgs/gm	576.86 mgs/gm
Average particle size	0.76 mm	0.81 mm
Ash content	8mg/g	10.65 mg/g

Figure 1: SEM images of Activated carbon prepared from three different stems of plants



Consequence of contact time on removal of heavy metals: The result of contact time on the exclusion of Activated carbon was evaluated for various concentrations (3 to 9 g/L), at regular time interval 15 to 80 minutes shows a increase in metal concentration removal with increase in time and concentration of Activated carbon (Marrakchi et al. 2020). The increase in thickness of activated carbon increases the number of active sites on the surface get increases, which involved in removal of heavy metal ion in groundwater samples. It is explained that the part of degradation this metal concentration (Pb, Cr, Zn and Cu) progressively increased 3 to 9 g/L, afterward there is no removal of metal concentration. (Fig 2 & 5). This indicates that the fluid to adsorbent mass transfer rate of the metal ion increases with the increase in initial concentration (Co). This is expected, since the concentration gradient across the film surrounding the adsorbent particle will be higher at the higher concentrations of metal ions (Felebuegu et al. 2006; Dev et al. 2020). Increasing the concentration of metal ions entering the continuous stream decreases the output. This is due to high initial concentration (Co) soaking the adsorbent rapidly, thus reducing the break through time (Awan et al. 2021).

Consequence of catalyst dosage on removal of Pb, Cr, Zn and Cu: The experiments done with changing the amount of dosage from 3 to 9 g/L for groundwater samples. The solutions kept under sunlight illumination for 15 to 80 minutes. As a result, shown remarkably greater in removal of heavy metal concentration from 40 mg/L dosage. It is well understood that the removal heavy metal concentration from groundwater activity increase with the increase in the dosage of Activated carbon (Fig 2 to 5) (Sujatha et al. 2021).

Table 2. Concentration of heavy metal in ground water samples before adsorption.

S.No	Heavy metals	BIS (IS 10500: 1991)	R-1	R-2	R-3	R-4	R-5	R-6	R-7	R-8	R-9	R-10
1.	Chromium	0.05	0.528	0.834	0.538	0.24	0.451	1.06	0.31	0.201	0.507	0.211
2.	Lead	0.01	0.324	0.156	0.232	0.258	0.125	0.26	0.526	0.291	0.123	0.199
3.	Zinc	5 – 15	7.32	9.24	6.45	12.16	11.84	8.16	8.28	4.95	6.9	4.06
4.	Copper	0.05 - 1.5	4.45	7.94	5.58	3.42	6.84	5.88	4.56	3.28	6.24	4.48

Table 3. Concentration of heavy metal in ground water samples after adsorption.

S.No	Heavy metals	BIS (IS 10500: 1991)	R-1	R-2	R-3	R-4	R-5	R-6	R-7	R-8	R-9	R-10
1.	Chromium	0.05	0.201	0.507	0.211	0.092	0.124	0.384	0.086	0.174	0.18	0.082
2.	Lead	0.01	0.291	0.123	0.199	0.225	0.092	0.235	0.493	0.258	0.09	0.166
3.	Zinc	5 – 15	4.95	6.9	4.06	9.79	8.84	5.78	5.89	2.56	4.51	1.67
4.	Copper	0.05 - 1.5	3.28	6.77	4.41	2.22	5.63	4.71	3.4	2.11	5.6	3.24

Table 4. The percentage removal of Chromium, Lead, Zinc and Copper with respect to time using CPAC Dosage = 3 g/100 mL, pH = 4, Temp = 300 K

Time in mins	% Removal of heavy metal with respect to dosage			
	Cr	Pb	Zn	Cu
15	55.85	51.53	59.56	56.45
30	59.42	52.53	62.53	57.86
90	67.54	61.23	69.85	63.85
120	71.23	64.78	71.53	65.42

Figure 2: The percentage removal of Heavy metal with respect to contact time

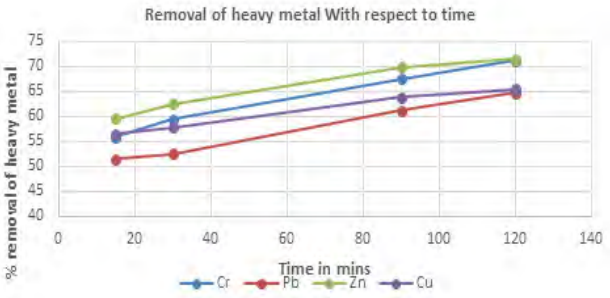
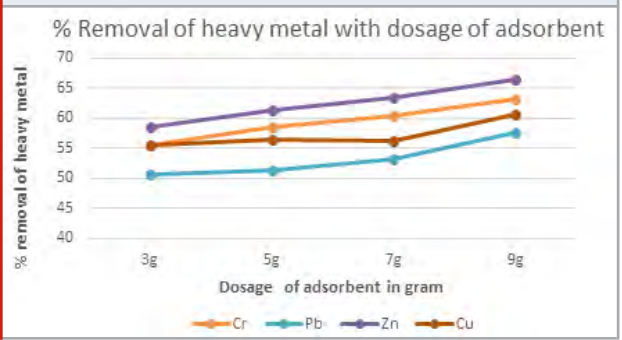


Table 5. The percentage removal of Chromium, Lead, Zinc and Copper with respect to dosage of Activated carbon Contact time 30 minutes, pH = 4, Temp = 300 K

Dosage amount	% Removal of heavy metal			
	Cr	Pb	Zn	Cu
3g	55.62	50.53	58.56	55.53
5g	58.63	51.23	61.42	56.53
7g	60.53	53.23	63.54	56.12
9g	63.17	57.53	66.51	60.54

Figure 3: The percentage removal of Heavy metal with respect to dosage of adsorbent



% of removal heavy metal concentration = (Absorbance at initial –Absorbance at final)/Absorbance at initial) X 100

Table 6. The percentage removal of Chromium, Lead, Zinc and Copper with respect to pH using CPAC Dosage = 3 g/100 mL, Contact time 30 minutes, Temp = 300 K

pH	% Removal of heavy metal with respect to pH			
	Cr	Pb	Zn	Cu
1	51.53	49.53	48.53	43.53
2	54.56	53.23	51.53	48.46
3	60.53	55.45	53.59	56.53
4	65.53	57.86	59.53	62.53
5	60.27	42.53	50.42	52.42
6	40.23	37.89	35.53	42.53
7	35.23	32.21	29.53	36.35
8	30.26	27.53	24.53	27.53
9	25.63	22.53	22.53	21.53

Figure 4: The percentage removal of Heavy metal with respect to pH

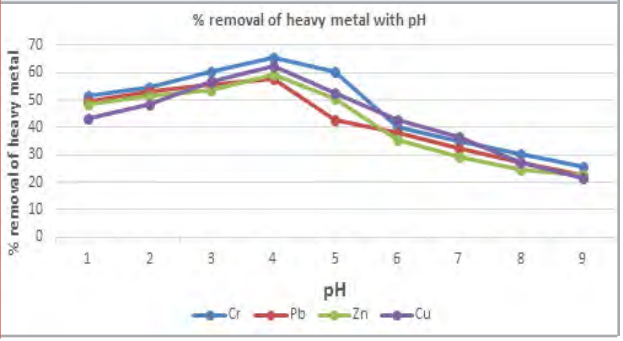


Table 7. The removal percentage of Chromium, Lead, Zinc and Copper with respect to Temperature using CPAC dosage = 3 g/100 mL, Contact time 30 minutes, pH 4

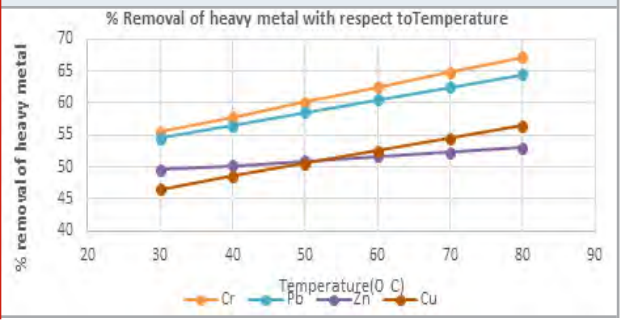
Temp. (0 C)	% Removal of heavy metal with with Temperature			
	Cr	Pb	Zn	Cu
30	55.53	54.53	49.53	46.53
40	57.86	56.53	50.23	48.53
50	60.19	58.53	50.93	50.53
60	62.52	60.53	51.63	52.53
70	64.85	62.53	52.33	54.53
80	67.18	64.53	53.03	56.53

The percentage removal of removal heavy metals was calculated using above formula and experimental data obtained from different experiment.

Effect of pH and Temperature on removal of heavy metals: The removal of heavy metal is high at pH 4, after pH 4 the percentage removal of heavy metal decrease, this is due to increase repulsion between adsorbent and heavy metal ions (Almomani et al. 2020). The removal percentage

of heavy metal increase with increase time up to 80 minutes and further rising temperature decrease the percentage removal of heavy metal, this is due to desorption takes place above 800C (Jayanthi et al. 2021).

Figure 5: The percentage removal of Heavy metal with respect to temperature Heavy metal



CONCLUSION

The findings of the present study has observed that the amount of wastewater treated before the initial breakthrough was directly proportional to the capacity of the adsorption tower and increased as the contact time increased. The effect of contact time on the amount of metal ions adsorbed by other metals also increased. Activated carbon was effectively produced by sol-gel method and it was characterized by SEM. The produced Activated carbon was utilized for removal Pb, Cr, Zn and Cu metals from groundwater samples. It is clear that the part of exclusion of heavy metals of groundwater gradually improved from 50 to 80 percent. The percentage removal of heavy metals gradually improved with contact time up to 120 minutes. This environmentally good Activated carbon material used for removal of heavy metal such as Pb, Cr, Zn, Cu. It is concluded that activated carbon prepared from mixed plants more efficient and very cheap than other types of adsorbents. Also, this study is very useful for researcher and public to get an idea about the removal of heavy metals ion in groundwater.

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Conflict of Interests: Authors declare no conflict of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Relationship of Strength with Pain and Function of Hand in Female Patients with Chronic Rheumatoid Arthritis

Choudhry Dimple,[1] Yadav Joginder,[2] Singh Harpreet,[3] Savarna[1] and Dhankher Poonam[1]

[1]College of Physiotherapy, Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Sciences, Rohtak, Haryana, India

[2]Faculty of Physiotherapy, Shree Guru Gobind Singh Tricentenary University, Budhera, Gurugram, Haryana, India

[3]Department of Medicine, Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Sciences, Rohtak, Haryana, India

ABSTRACT

Rheumatoid arthritis is a chronic systematic inflammatory disease which is characterised by pain and functional loss in an individual with bilateral involvement of hands, resulting in loss of joint integrity and bony deformities. Grip strength decreases the functional ability and hamper the Activity of daily livings (ADLs). The objective of present study was to assess the relationship between grip strength with key strength, pain and function of rheumatoid hand. A Total of 103 female Rheumatoid arthritis patients of age group 20 to 60 years as per inclusion and exclusion criteria were enrolled for this study. Grip strength was measured by Hand dynamometer and key strength was measured by digital pinch meter, pain score of severity and interference were quantified by Brief Pain Inventory (BPI) scale, hand function score was calculated by short form score for assessment and quantification of chronic rheumatoid affection of hand (SF-SACRAH). There was a significant positive correlation between right hand Grip strength with key strength of right hand ($r= .559$, $p<0.01$) and hand function score ($r= -.230$, $p<0.05$). The positive correlation of left-hand grip strength with left hand key strength ($r= .616$, $p<0.01$) and pain severity score ($r= -.198=$, $p<0.05$) was also found. The study concludes a positive correlation between grip strength of right hand with key strength and function of right hand in chronic rheumatoid arthritis patients whereas severity of pain shows positive correlation with left hand grip strength. Rheumatoid patients need a better rehab management or their functional status.

KEY WORDS: BPI, GRIP STRENGTH, KEY STRENGTH, RA, SF-SACRAH.

INTRODUCTION

Rheumatoid arthritis is chronic inflammatory autoimmune disease, which is characterised by symmetrical involvement of joint pain and functional loss (Ellegard 2019; Tanaka 2021). According to WHO (2019), rheumatoid arthritis is the second most common cause of disability which affects the productive years of adult life and approximately 50 percent of rheumatoid arthritis patients were not able to perform their full-time job in developed countries. Rheumatoid arthritis is a progressive disease which results in destruction of synovial layer of joint. The mechanism of destruction of joint in rheumatoid arthritis is well known and the osteoclasts have the potential role in erosion of focal bone. Several types of osteoclastic activity are seen by electron microscopy in area of subchondral bone of metacarpal heads of rheumatoid joint (Smolen et al. in 2019). In early rheumatoid arthritis the inflammation affects the activities of daily livings (ADLs) because of pain and swelling. As disease progresses joint become worsen and hamper the functional activity of patients (Tanak 2019). The grip force and pinch force get decreased due to involvement of MCP joint and functional activity and ADLs ability of an individuals is also hampered due to muscular weakness in these patients (Juan et al. 2019; Ellegard 2019; Tanaka 2021).

A compensatory intervention programme such as assistive devices and joint protection splints are needed to improve ADLs in rheumatoid arthritis disease (Kopruluoglu et al. 2020). Despite the pharmaceutical intervention physical therapy was also play remarkable role in management of chronic RA patients. Large joints respond well but small joints need more attention of physical therapist for functional status. The objective of this study was to measure the grip

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and key pinch strength of both the hand and correlate them with severity and interference score of pain and hand function in chronic rheumatoid arthritis female patients. This study will help to provide guidance for management of functional disabilities of hand in female patients of chronic rheumatoid arthritis. These patients may need a better protocol for better functional status (Tanaka 2021).

MATERIAL AND METHODS

The study was conducted in Pt.B.D. S PGIMS Rohtak from July (2016) to June (2017). Total 103 female subjects were enrolled in study as per inclusion criteria who signed the Helen ski declaration form. Ethical clearance was obtained from the institutional ethical committee of Pt. B.D. Sharma PGIMS Rohtak wide letter No: IEC/17/387 dated 25.03.17 for data collection, before commencement of study. Female subjects between age group of 20-50 years, who clinically diagnosed RA as per ACR criteria 1987 at least 2 years and subjects on DMARDs more than 2 years with no hand deformities were included in study. Severe anaemic, evidence of hypothyroid, renal, cardiac and pulmonary disease, who has undergone any recent fracture and hand surgery and pregnant females were excluded from study (Brorsson et al. 2009). Grip strength is a muscle strength of flexor group of muscle and it was quantified by hand dynamometer. It was measured in sitting position with shoulder slightly in abduction and zero degree in medial and lateral rotation, elbow in flexed to 90-degree forearm was in zero degree of supination and pronation, and wrist in 0 to 30 degree of dorsiflexion and 0 to 10 degrees in ulnar deviation (Robert et al. 2011; Salaffi et al. 2021).

Key strength was measured by digital pinch gauge. Key strength was measured because it was usually used to hold key and other utensils used in daily activities. Position of subjects were sitting with shoulder slightly in abduction and in lateral rotation, elbow was flexed to 90 degree, forearm was in zero degree of supination and pronation and wrist in 0 to 30 dorsiflexion and 0 to 10 degree in ulnar deviation. Examiner held the distal end of gauge to prevent dropping (Mathiowetz et al. 1985; Silva et al. 2018). Brief pain inventory scale was used to measure pain severity and interference score in this study. İn Chronic Rheumatoid Arthritis, various physical functions of patients were measured in terms of qualitative and quantitative data. Pain was also meassured in quantitative form so as to check after treatment whether condition of patients is improved or not. Brief pain inventory was a self report questionnaire which include severity of pain and interference of pain.

Severity of pain was assesssed by mean value of four questions of various level of pain during past few weeks which were measured on 0 to 10 Visual Analogue Scale. Pain interfere score was calculated by mean value of five questions of various activity of daily living like walking, mood, relationship with others and sleep etc (Somers et al. 2009). SF-SACRAH was a score for assessment and quantification of chronic rheumatoid affection of hand, which was used to measure the functional activity of rheumatoid hand. It is a self report questionare which is concerned mainly with hand function, pain and stiffness. İt consists of five questions out of which three were based on various functional hand activities and one each for pain and stiffness. Each question rated 0 to 100 score, whereas 0 meant patients hand activities was possible without any diffiiculties, 50 meant hand activities were possible with some difficulties and 100 meant unable to do (Kumar et al. 2012; Stummer et al. 2017).

RESULTS AND DISCUSSION

A total of 103 female patients of chronic rheumatoid arthritis were participated in the study and all of them completed the study. The results were analysed with the software SPSS-16.0 for window version. Mean and SD were calculated for Grip strength, key strength, pain scores and hand function. Correlation was checked between the various variables (grip strength, key pinch strength, pain severity score, pain interference score, hand function score) by bivariate two tailed test of correlation using Pearson coefficient. In all statistical analysis, $p<0.05$ was considered as significant.

Figure 1.1: grip strength measuremed by hand dynanometer.

The demographic data of all patients was such as age, duration of disease, Duration of DMARDs, blood reports, grip and key strength of both the hand, hand function score and pain severity and interference score were shown in table 1.1.

Table 1.2. Represent the correlation of the right-hand grip strength with other variables. Right hand grip strength shows positive correlation between with left hand grip strength ($r= .738$, $p<0.01$), key strength of right hand ($r= .559$, $p<0.01$) and hand function score, SF-SACRAH ($r= -.230$, $p<0.05$).

Table. 1.3. Represent the correlation of the left-hand grip strength with other variables. Left hand grip strength show positive correlation between with left hand key strength ($r= .616$, $p<0.01$) and pain severity score ($r= -.198$, $p<0.01$) and hand function score, SF-SACRAH ($r= -.230$, $p<0.05$).

This study was conducted to evaluate the correlation between grip strength with key pinch strength of both the hands, pain severity and interference score and hand function score in chronic rheumatoid arthritis patients. In this study grip strength of right hand showed positive correlation with key strength of right hand ($r=.559$, significant $p<0.01$) and with

hand function score SF-SACCRAH (r=-.230, significant p<0.05). Whereas grip strength of left hand showed positive correlation with key pinch strength of left hand (r=.616, significant p<0.01) and with pain severity score (r=-.198, significant p<0.05).

Table 1.1 the demographic data of the subjects (n=103).

	min	Max	Mean ± SD
Age (years)	20	50	40.45±7.58
Residential status	Urban 58	Rural 45	
Duration of Disease (years)	2	15	5.19±3.05
Duration of DMARDs (years)	2	10	3.43±1.86
Family history	Yes 43	No 60	
Occupation	Home maker 38	Working 65	
Rheumatoid factor	Positive 81	Negative 22	
Blood report			
Hb	6	14.7	10.74±1.75
ESR	12	100	50.94±15.86
Dexa score	-2.8	1.7	-.06±1.42
Grip strength (Kg)			
Right hand	2.8	14.1	8.80±2.18
Left hand	3	15.3	8.73±2.51
Key strength (Kg)			
Right hand	1	9.3	4.54±1.73
Left hand	1.2	7.2	4.26±1.69
Pain			
Severity score	3	9.25	6.24±2.16
Interference score	2	8.75	6.71±1.88
Hand function score	16	94.3	59.27±37.00

SD: standard deviation min: minimum max: maximum

Table 1.2. Correlation of right grip strength with other variables.

Variables	Pearson's correlation coefficient (r)	p-value (by 2 tailed test)	Significant
Left grip grip strength	.738*	.000	Significant at the 0.01 level (2-tailed).
Right hand key strength	.559*	.000	Significant at the 0.01 level (2-tailed).
Pain severity score	-.181	.067	NS
Pain interference score	-.043	.664	NS
SF-SACRAH	-.230	.019	Significant at the 0.05 level (2-tailed).

*: significant p<0.01 NS: non-significant

Table 1.3. Correlation of left-hand grip strength with other variables.

Variables	Pearson's correlation coefficient (r)	p-value (by 2 tailed test)	Significant
Left hand key strength	.616*	.000	Significant at the 0.01 level (2-tailed).
Pain severity score	-.198*	.045	Significant at the 0.05 level (2-tailed).
Pain interference score	-.161	.104	NS
SF-SACRAH	-.181	.067	NS

*: significant p<0.01 NS: non-significant

Dellhag and Bjellee et al. (1995) reported that hand grip force was significantly correlated with hand function. Terslev et al. (2003) identified the pathological processes which are responsible for this are thought to be 'rheumatoid cachexia' (loss of muscle cell mass and destruction of muscle architecture because of the autoimmune, catabolic nature of the condition) as well as disuse atrophy of muscle, which hamper the functional activity of hands. Adams et al. (2004) and Ellegaard et al. (2019) described that Pain is also affect the functional activity of hands and accordingly cause the ADLs limitations (Ellegaard et al. 2019; Salaffi et al. 2021).

Uutela et al. (2018) also reported that muscle strength of hands were lowered in rheumatoid arthritis patients and it was significantly correlated with hand function. Bjork et al. (2011) reported that women feel more pain as compared to men and pain severity affect the hand function as disease progressed. Hallert et al. (2012) also reported that despite of all intervention at regular follow up in rheumatoid patients the disability is progresses as disease progressed because of joint damage (<2years). Dedeoglu et al. (2013) found the positive correlation between hand strength with pain and functional activity in rheumatoid arthritis patients. Approximately 30% reduction in hand function were found as compared to healthy individuals. Bohannon RW. et al.in (2013) found that grip and key pinch strength were associated to disability and impairment, level of disease activity, joint surface damage, discomfort, and disease duration in the rheumatic population. Salaffi et al. (2021) described that joint pain and swelling were common symptoms resulting in diminished grip strength and function of hand (Salaffi et al. 2021). The limitation of study was wide age group 20-50 years and dominant hand was not considered. Future research is needed to find the relationship between dominant hands with functional activity and quality of life in chronic rheumatoid arthritis patients. This study was design to evaluate the correlation between grip strength of hands with pain severity and function in chronic rheumatoid hand.

CONCLUSION

The findings of the present study concluded that grip strength of right hand shows the positive correlation between key pinch strength of right hand and function of hand in Chronic Rheumatoid arthritis patients. Whereas grip strength of left hand showed positive correlation with key pinch strength of left hands and pain severity score. The study states that research hypothesis is accepted.

Conflict of Interests: Authors declare no conflict of interests to disclose.

Ethical clearance Statment: Ethical clearance was obtained from the institutional ethical committee of Pt. B.D. Sharma PGIMS Rohtak wide letter No: IEC/17/387 dated 25.03.17 for data collection, before commencement of study.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Synthesis and Application of Latex-Based Ethylene-Propylene Copolymer on Methacrylic Acid

Shixaliyev Kerem Seyfi
Azerbaijan Oil and Industrial University, Baku, Azerbaijan

ABSTRACT

The latex synthesis in the presence of ethylene-propylene copolymer and methacrylic acid (MAA) was considered for this experiment. The kinetic regularities of the synthesis process were determined and the optimal quantities and ratios of the components were determined both experimentally and by mathematical modeling. It was determined that a modified latex with optimal values is obtained when the amount of methacrylic acid in the latex is 5 parts by weight. The absorbent composition was prepared latex-based based on synthesized EPC - + methacrylic acid. The prepared absorbent was impregnated with viscose (22B) and 222K kapron cord and a rubber-cord bond were determined.

KEY WORDS: ETHYLENE-PROPYLENE COPOLYMER (EPC), MODIFICATION, METHACRYLIC ACID (MAA), SYNTHESIS.

INTRODUCTION

Synthetic latexes are widely used in various industries. Latex is an important product used in the tire industry, in the production of rubber-technical products, in the medical industry, and in the construction industry (Shikhaliyev 1980; Shikhaliyev 2020). Latex is the main absorbent component in the production of carcass parts of tires in the tire industry, which provides a woven material-rubber adhesion bond (Bilalov 1973; Bilalov 2005; Bilalov 2008; Bilalov 2010). Recently, the production of latexes by synthetic methods is mainly obtained by the emulsion method (Ososhnik 2007; Nurmukhametova 2012; Amirov 2017; Shikhaliyev 2020).

New latexes are being purchased on an industrial scale and the equipment used for their synthesis is being updated. New emulsifiers and coagulation agents are used in technological processes. Synthetic latexes are widely used in various industries. Latex is an important product used in the tire industry, in the manufacture of rubber products, in the medical industry, and the n construction industry. Latex is the main adhesive in the tire industry, which provides a cord-rubber connection in the manufacture of the carcass part of the tire. Recently, the production of latexes by synthetic methods is mainly by emulsion. New latexes are currently being used on an industrial scale, and the equipment used to synthesize them has also been updated (Nabil et al. 2013; Hayeemasaeetshal 2013). New emulsifiers, surfactants are used in technological processes (Koblov et al. 2012; Mikheeva et al. 2013; Mikheeva et al. 2014). However, none of these adhesives can fully ensure the rubber-cord connection. Taking all this into account, we have synthesized a latex based on EPC (Shikhaliyev 2020).

Recently, the ethylene-propylene copolymer has been used in the production of military tires. The adhesive that provides the cord-rubber connection of the EPC-based carcass part has not yet been synthesized. In the production of tires used in military aviation based on EPC, ensuring the cord-rubber connection of their carcass part, the synthesis of latex-based on EPC is the most pressing problem (Shikhaliyev 1980; Shikhaliyev 2020). The contact of non-woven goods (a cord, cloth, apron, etc.) with rubber is almost zero without adhesion. Therefore, it is necessary to use adhesives to ensure a rubber-woven bond (Bazenov 2010). Our literature review has shown that the production of synthetic latexes is the most pressing problem and material in demand on an industrial scale (Bilalov et al. 1973; Bilalov et al. 2004; Shikhaliyev 2020).

Korkut (2012) and Riyajan (2012) considered the sample to be the most popular butadiene-styrene latexes and therefore recommend the use of absorbent formulations with particularly active accelerators for vulcanization of latex products. The search for vulcanization systems will continue for a long time. n the tire industry, a latex-based absorbent is used to absorb cords and technical parts. For this purpose, an aqueous solution of various rubbers is used. However, the latex-based ethylene-propylene copolymer

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has not been used so far. As it is known, some rubbers are mainly made of EPC. Taking all this into account, the object of research is the production of EPC + PMMA flat latex-based ethylene-propylene copolymer based on methacrylic acid (Shikhaliyev 2020).

MATERIAL AND METHODS

Ethylene propylene copolymer and methacrylic acid were used as raw materials for preparing an aqueous dispersion of EPC and modified EPS-PMAK latex. A residual pressure 4MPa was considered for this study. To obtain an aqueous dispersion of modified latex EPDM, a laboratory setup and a special experimental technique have been developed. Ethylene propylene copolymer and methacrylic acid were used as raw materials for preparing an aqueous dispersion of EPC and modified EPS-PMAK latex. residual pressure 4MPa (Shikhaliyev 1980; Shikhalizade et al. 2010; Shikhaliyev 2020). To obtain an aqueous dispersion of modified latex EPDM, a laboratory setup and a special experimental technique have been developed. The composition of the copolymer was determined by IR spectroscopy. The spectra were recorded on a UR-20 spectrometer in a special range from 400 to 3500 cm-1. The crystallinity of the copolymers was determined by X-ray diffraction on a URS-50nm instrument. The intrinsic viscosity of copolymers was determined in decline at a temperature of 30 ° C in Ebberson viscometers.

The adhesive is a key factor used in the formation of the rubber-cord system. Thus, after impregnation of the cord with an absorbent composition based on EPS, the fibers of the woven product provide a high adhesion of the rubber-cord system, forming a strong adhesion bond with the rubber. Spills in the cord-rubber-adhesive system should be considered separately for each system. This is because spillage can occur between either cord-rubber or rubber-adhesive. In all cases, the strength of the cord-rubber-adhesive system has a biphasic property and Silva have high adhesion to both the textile product (Shikhaliyev 1980; Shikhalizade et al. 2010; Shikhaliyev 2020).

For the preparation of absorbent composition, the absorbent content should be 8.0% dry residue and pH 8.8-9.0. Technical carbon to a predetermined EPC latex to determine the dry residue of the absorbent composition. Phenol formaldehyde resin modified by Naibova (Naibova 2015) and. An absorbent drop was prepared by adding 10% NaOH. The liquid residue of the absorbing composition was calculated to the nearest 0.0012. The absorbent composition was placed in a drying cabinet at a temperature of 165-175 ºC and dried until constant weight. The dry residue (p) is calculated as a percentage:

$$P_{\text{dry residue}} = \frac{m \cdot 100}{m_n}$$

where - m is the mass of dry residue, g; mn – a mass of the absorbent composition. It was determined using a pH meter to determine the pH of the absorbent composition. The absorbent composition was prepared according to the recipe given in Table 1.

Table 1. Recipe for the optimal absorbent composition studied

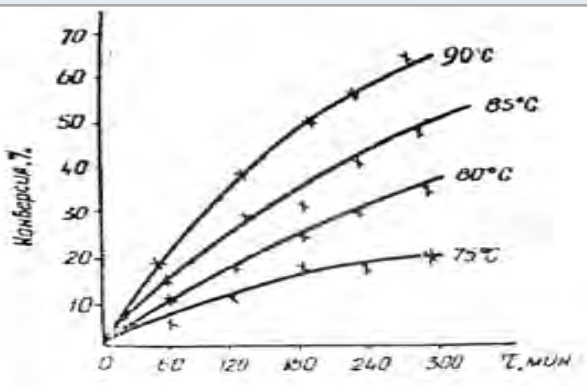
Ingredient name	100 parts by weight parts by mass	Components	Dry residue of the component due to the absorbent composition
LateEPC+PMMA Modified resorcinol	100	240	63,2
formaldehyde resin	53,2	268	34,2
Technical carbon	37,2	509	13,1
10% of NaOH	25,9	-	“,6

SF-282 resorcinol-formaldehyde resin is used in standard absorbent formulations. However, due to the economic inefficiency of this resin, Assoc. We used a synthesized and modified FFQ by Naibova (Naibova 2015). For this purpose, we used technical formaldehyde. In this case, we increased the dry residue of the absorbent to 5%. In this case, the absorbent composition easily penetrates between all the threads of the cord, soaks it thoroughly, and gives the threads a special softness, which led to a noticeable increase in the rubber-cord connection. The rubber-cord bond was determined by the standard H-method. After the cord was impregnated with an absorbent compound, it was rubberized on a calendar with a thickness of 0.1 mm on both sides. A double-sided rubberized pattern was vulcanized by adding it to a specially prepared performance.

The vulcanization process was carried out at a temperature of 155°C -160°C for 20 minutes and a pressure of 3.5 Mpa was considered. The "whiskers" on the edges of the sample are cut with a knife. The sample should be 25 ± 1 mm. The sample was fixed in the tanks on the fighter and the machine was started. The breaking strength was determined by the moment on the scale when the cord pulls on the fighter.

The test was performed several times and the result was an average score. It was determined that the viscosity of the absorbent composition should be 5-8%. However, the concentration of EPC + PMMA copolymer in the used latex should not be less than 10%. Only in this case can the cord strands be thoroughly soaked in the absorbent composition (Naibova 2015).

Figure 1: Construction scheme of methacrylic acid to raise the temperature from 75C to 90C.



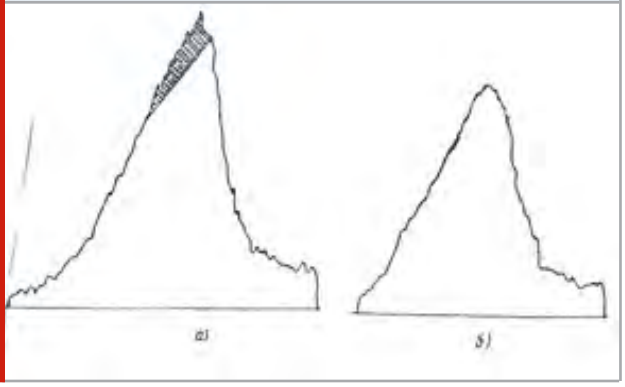
RESULTS AND DISCUSSION

Investigation of the kinetics of graft copolymerization: In the laboratory, a special device was installed to carry out the synthesis process and to obtain latex in this device, we first cut EPS into small pieces, put them in a three-necked flask, and stirred until completely dissolved in toluene. Once completely dissolved, we raised the temperature to 80C and added methacrylic acid drop by drop to the mixture for 10 minutes. After transferring the methacrylic acid to the complete reaction flask, we performed a new synthesis by adding benzoyl peroxide. As a result of the synthesis, a new modified latex was obtained. Absorbent composition is prepared based on the obtained latex. EPS stable aqueous dispersion was modified with methacrylic acid in the presence of benzoyl peroxide and nitrogen at different temperatures (75°C to 90°C). General kinetic regularities of copolymerization reaction with EPC and methacrylic acid, copolymerization rate constants, reaction sequence and speed, monomer half-life and activation energy, monomer conversion, etc. was appointed.

Table 2. Monomer configuration, reaction temperature, and reaction time

Temperature, °C	Reaction time, hours	Monome\ polymer	Monome conversion, %	The viscosity of latex, seconds	Surface tension, din / sm
80	8	0,02	89	3,12	42,0
80	88	0,04	86	3,16	42,1
80	6,5	0,05	82	3,15	42,4
85	6,5	0,04	92	3,16	43,3
85	6,5	0,08	90	18	42,0
85	5	0,10	88	3,19	43,2
90	5	0,02	94	3,19	42,4
90	5	0,02	97,5	3,16	41,0
90	5	0,05	99,4	3,14	41,8

Figure 2. X-ray of the copolymer containing EPS (a) and EPS + PMMA (b)The proportions of components for latex production are given in Table 3



The rate of solidification of the monomer at different temperatures was determined from the dry residue sample of the reaction mixture. The results showed that the duration of the construction reaction of methacrylic acid depends on the temperature. EPC methacrylic acid builds very slowly at 70 ° C. Raising the temperature from 75 ° C to 90 ° C increased the reaction rate.

Use of modified EPC latex to increase the adhesion of rubber-cord bond: The results of IR spectroscopy confirm the presence of -COOH groups in the copolymer chain. At 675, 1100, 1300, 1750, and 1800 cm –1, peaks characteristic of carboxyl-containing polymers appear in the IR spectrum. These streaks were observed after 10 hours. X-ray studies showed that the EPS crystallization rate used in the study

was about 8%, but this was not the case with the original polymer. MAA helps to destroy the crystal grades present in vaccinated samples and causes a sharp increase in the rate of conversion of methacrylic acid during the process (Figure 1).

Table 3. Ratios of components

System In ph.	Amount of sulfonyl,%	Amount of azeotropes,%	Surface tension Din/sm	Viscosity, Seconds
11	4	24	58	2,18
11	6	18	49	2,04
11	8	10	43	1,09
11	10	8	42	1,04
8	10	9,0	43	1,04
9,0	10	8,5	42	1,04
10	10	8	42	1,04
12	10	8	42	1,04

Table 4. Physical and mechanical properties of films.

Polymer	at the break, MPa	Relative extension, %	Residual elongation, %	Note
Original EPC	8,0	225	83	After holding for 3 days at 20 ° C, the samples of the films were thermostatic at 130 ° C for 30 min.
EPC precipitated from an aqueous dispersion	9,0	200	74	
Graft copolymer EPC + PMAA (5 wt.%)	24,0	285	38	

The missing properties of EPC were obtained by copolymerizing it with MAA to obtain a modified latex, and the kinetic regularities of the copolymerization reaction were determined. It is shown that the optimal mode: temperature - 90 ° C, time - 5 hours. In this section, the monomer conversion reaches 99%. To clarify the effect of the composition of the modified latex EPC on the foaming and strength properties of films and rubbers based on it, the physical and mechanical properties of films and rubbers were determined (Shixaliyev 2020; Bazhenov et al. 2021; Alizade 2022). The data obtained are shown in Table 4 The grafted EPC is distinguished by its high strength and elasticity, which gives the right to judge the structural ability of -UNC. The study of the physicomechanical properties of rubbers made it possible to assume that the -COOH groups introduced into the copolymer as a result of grafting are involved in the formation of the vulcanization structure. Modified EPDM containing 5 wt% acids has higher strength (18.0 MPa) compared to unmodified EPDM (15.4 MPa). The physical and mechanical properties of the films are shown in table 4 (Alizade 2022).

After vulcanization of the samples, the analysis of their physical and mechanical properties showed that EPC latex provides the strength of the cord-rubber bond in the range of 10.5 kg / 2.5 cm, which was applied to the elastic polymer, cover meets the requirements.

CONCLUSION

The findings of the present study shows that the aqueous dispersions of EPC latex were synthesized using various emulsifiers with high technological stability. The presence of the main factors affecting the stability and adhesive properties of the EPC aqueous dispersion has been established. It has been shown that the best stability for 6 months is achieved at a pH value of 12. EPC water emulsion was modified in the presence of MAA by graft copolymerization. Gross kinetic regularities of EPC with MAA in the emulsion were determined. It is shown that the optimal conditions are: temperature - 90 °C, time - 5 hours. Under these conditions, the monomer conversion reaches 99%. The physicochemical and adhesive properties

of modified latexes containing from 2 to 20 wt% of grafted PMAA have been studied. It has been established that the high bond strength of the cord with rubber is achieved by using EPDM latex containing 5 wt. % grafted PMAK. It is shown that the physical and mechanical properties of films and rubber compounds based on a graft copolymer significantly exceed those of unmodified copolymers.

Conflict of Interests: Author declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Perception and Preference of Under Graduate Students on Different Parameters of Online Education during COVID -19 in South Bengal, India

Tapas Kumar Ghosh
Department of Computer Science, Bankura Sammilani College, West Bengal, India

ABSTRACT

The Pandemic from the Corona Virus (SARS-CoV-2) has impacted the worldwide Education Sector tremendously. Due to it educational institutes across the state of West Bengal have been closed since March-2020. The Pandemic forced the educational community in West Bengal to shift their teaching-learning activities to online platforms. Teachers and students quickly adapted to synchronous i.e. interactive online classes along with asynchronous i.e. video-and other materials-based modes of teaching learning. Various online education platforms and facilitating tools are used heavily during this crisis period. This survey paper aims to get a detail report on the perception and preferences for different online teaching-learning parameters used by UG level learners of different general degree colleges along with colleges of professional courses like BBA/BCA and engineering colleges from the districts of Bankura and Purulia of West Bengal. The students preference for different attributes of online learning may be useful for designing a suitable online class environment. A survey work was done in online mode using google form. The form was circulated among several WhatsApp group of students of different colleges through their teachers. The result showed that almost 96% of the students prefer to use Google Meet or Google classroom with their smart phone for online classes. It was also observed that frequent loss of Internet connection in rural areas is more prominent than in the Urban and Semi Urban areas of South Bengal. Students preference has utmost importance to attract them for attending online classes. This article will be helpful to attract students in online classes in this crisis period..

KEY WORDS: ASYNCHRONOUS LEARNING, INFORMATION AND COMMUNICATION TECHNOLOGY, INTERNET, ONLINE LEARNING, SYNCHRONOUS LEARNING.

INTRODUCTION

First reported case of the Covid-19 disease was in December 2019 in China's city Wuhan. Due to its highly contagious nature this disease spreads all over the world. Governments of different countries have advised their people for frequent hand washing, wearing masks, maintaining safe distance, and to avoid gathering in a small area. To control the transmission of the Covid-19 virus lockdown and stay at home have become the only solution to flatten the curve (Sintema 2020). The lockdown in response to COVID-19 have interrupted conventional education system. Many countries across the globe have to close their educational institutes to protect their students from viral exposures. The educational community of different countries were forced to make collected efforts to maintain learning continuity during this period (Khan et al. 2021).

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In this context online learning has become inevitable in our country also. Students have to depend more on their own devices to continue learning through the Internet from their home. Teachers were also learning new online teaching concepts for which they may not have prior experience. E-learning has many advantages like easily accessible, affordable, flexible, life-long learning, learning anytime from anywhere etc. Online learning is assumed to be easily accessible and it can even reach to rural as well as remote areas. It is also a relatively low cost mode of education in terms of the cost of transportation, accommodation, and the total cost of institutional learning. Technology enabled online lectures are necessary for blended mode of learning and flipped classrooms (Khan et al. 2021).

The flipped classroom is a simple concept of e-learning where students are provided learning resources such as articles, pre-recorded videos and YouTube links at home and practice working through it. Then the students can use their online classroom time for clearing doubts through discussion with faculty and friends (Doucet et al. 2020). Students can

enhance their skills like problem solving, critical thinking and self learning through it. They can learn anytime from anywhere, and thereby become able to develop new skills. The outbreak of Covid-19 Pandemic again adds one more logic favouring online learning. Several researchers had investigated for different class of students' perception and preferences for online learning during the Covid-19 outbreak, and many of them had discussed about the challenges faced. For example, found that online classes are more difficult to manage than traditional face to face interactive classroom for the agricultural graduates from different universities of National Agricultural Research System (NARS) in India. This is due to the technical constraints, delay in feedback and incompetency of the instructor to handle effectively the Information and Communication Technologies (Muthuprasad et al. 2021).

Similarly, previous research has shown that Indian undergraduate medical students have lowest preference for both synchronous and asynchronous learning methods due to poor internet connection speed (Mondal et al. 2021). Also, Dhawan (2020) has reported that factors like security features, speed of Internet, availability of bandwidth, digital literacy levels of the learners etc affect the choice of a particular technology. Findings of another study Mandal and Mondal (2020) reported that 21% students from districts of Bankura and Purulia of West Bengal missed more than 40% of online classes due to poor network connectivity in their locality of residence. On the positive side, researchers from different parts of the globe have reported that students also acknowledge the advantages of e-learning during this pandemic (Razami and Ibrahim 2021).

They can continue their education remotely from their residence, recorded audio-video lectures have facilitated their study and revision process and their self-directed learning skills have developed also during Covid-19 Pandemic. Hasan and Hassan (2020) suggested that online learning environment should be student-centric, so that they can learn easily, and can get opportunities for interaction through use of different media. All these findings motivate our study to understand perception and preferences of under graduate level learners from the districts of Bankura and Purulia of South Bengal, so that a suitable online learning environment can be provided.

MATERIAL AND METHODS

UG level students from different general degree colleges along with colleges of professional courses from the districts of Bankura and Purulia of West Bengal were taken as respondents for this survey. The survey was conducted from the 25th of October, 2021, to the 3rd of November, 2021. A preliminary questionnaire was set using the information collected from above mentioned different research papers and informal discussions with few students who were attending the online classes since March 2020. First the model of questionnaire was tested with 30 randomly chosen respondents from different colleges and their feedbacks were considered for preparing the final questionnaire. The questionnaire was fed into a Google form, link of which are sent to students through several Facebook groups as well as WhatsApp with the help of teachers from different colleges of the districts of Bankura and Purulia in West Bengal.

RESULTS AND DISCUSSION

For limited time constraint the study was restricted only in the districts of Bankura and Purulia in South Bengal. The data collected from respondents were analyzed and presented in the form of Tables and Figures.

Figure 1: Students' Experience of online learning before and after March (2020)

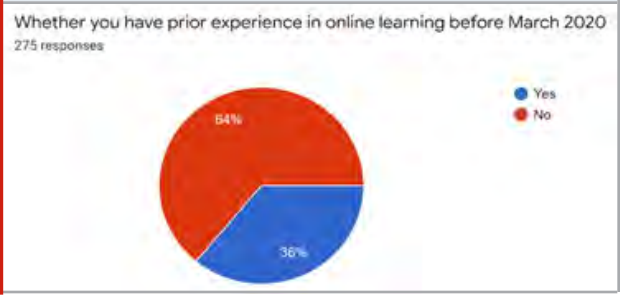


Table 1. Demographic Details of Students

Field Attribute	Categories	Percentage
Gender	Male	66.20%
	Female	33.80%
Residence	Rural	53.50%
	Semi Urban	13.40%
	Urban	33.10%
Discipline	Science	32.60%
	Arts	34.10%
	Commerce	0%
	Professional Courses (BBA/BCA)	32.60%
	Engineering	0.70%

Total 283 students participated in the survey, among which 95 (33.8%) are female and 186 (66.2%) are male. Majority of them were from rural areas of South Bengal and have no prior experience of online learning [Figure-1]. They are from disciplines of Science Arts and professional courses with few from engineering also [Table 1]. Almost 92% of them use Smartphone and only 8% have Laptop or Personal Computer for online learning [Figure-2].

For successful running of online classes students must have some skill in information and communication technology. From Table 2 we may conclude that only few students (10%) may have to face problem for attending online classes due to their low level skill in ICT. It also shows that students have equally likely preferences for synchronous as well as asynchronous learning.

In most cases, colleges were flexible to offer online teaching through their own portal or different platforms. But figure3

shows that majority of students (96%) were using Google Meet/Google Classroom. Only 4% from total respondents were using other platforms like Webex, Zoom, College portal or other software. Google Meet might be considered as the most flexible ICT tool to use for synchronous classroom experience that can be easily accessible and also students and teachers can use it easily.

Table 2. Students' skill level in ICT with preference on different modes of online learning.

Your Skill level in Information and Communication Technology.	High (17.4%)	Medium (72.2%)	Low (10.3%)
Which mode of learning (Synchronous or asynchronous) you prefer most?	Asynchronous i.e. Video and other material based learning (27.8%)	Synchronous i.e. online classes (24.8%)	Both (47.4%)

Figure 2: Devices used for online education.

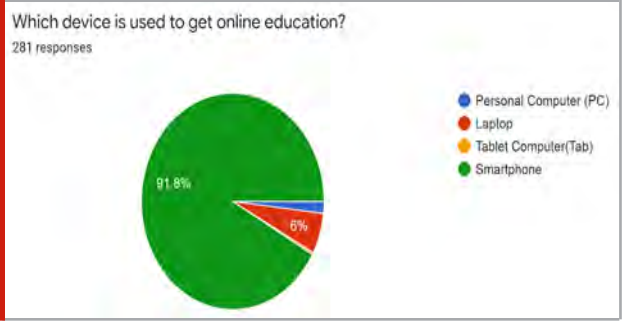


Figure 3: Preferred online learning platform

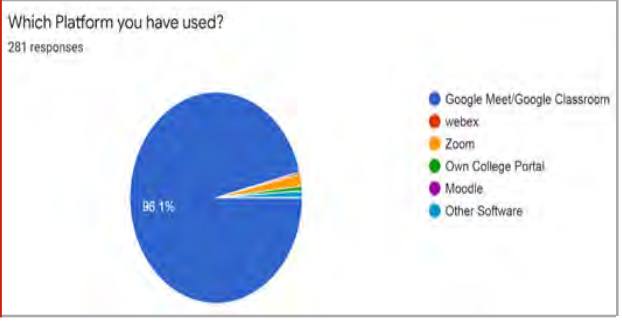


Figure 4: Loss of network connection

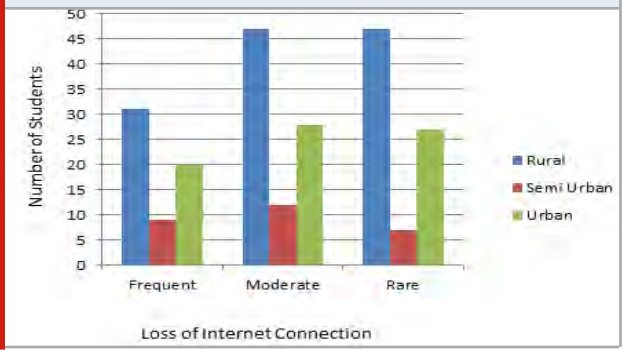
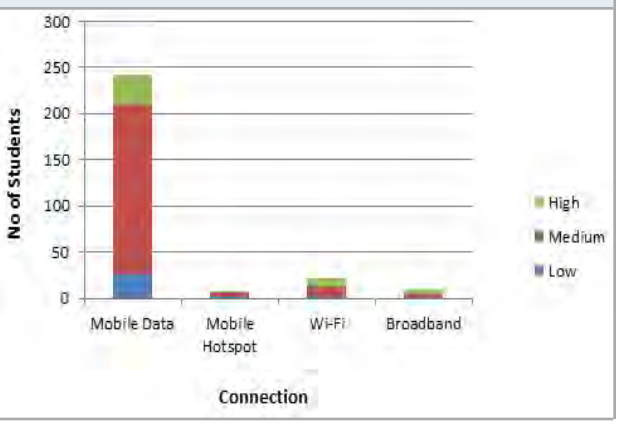


Figure 5: Data Rate in different type of Connection



In the introduction we have discussed that uninterrupted internet connection is a challenge for smooth conductance of online education. Figure 3 indicates that among 115 students from rural area of Bankura and Purulia district 21% have been suffering from frequent loss of internet connection 69% reported that loss of internet connection is moderate or rare in their locality. A similar result was found by (Mandal and Mondal 2020). Only 10% students have no loss of Internet connection during their academic activities. Picture is almost similar in urban and semi urban area also. 25% of them reported frequent connection loss where as 62% have moderate or rare connection loss and 13% have no connection loss. Figure 4 also indicates that majority of students (87%) were continuing their digital learning through their Smartphone mobile data (Mandal and Mondal 2020).

Below in Table 2 we summarize the findings about different parameters of digital learning from home environment. The data also shows that many students are ready to opt for blended mode of learning in future, however traditional face-to-face lecture class is the most preferred method. Table 4 reveals that majority of students like to ask their professor for clearing doubts during or after online classes. It also shows that students' want class updates through WhatsApp and they also like descriptive type questions for their evaluation through examination however more than 40% of them like MCQ for their examination.

Table 3. Respondents perception on learning from home

Your experience with learning digitally from home.	Comfortable (67%)	Not Comfortable (33 %)	
During learning activities are you distracted with other activities at home?	Yes (26.3%)	No (41.6%)	May be (32.1%)
Which mode of learning would you like for coming years?	Face to face interactive offline classes (41.4 %)	Online Classes (28.9 %)	Blended Mode of both offline And online (29.7 %)

Table 4. Preferred parameter of online classes and examination.

What is your most preferred method for clearing doubts in online learning?	Ask the professor during/after an online lecture. (73.3%)	Post query in a group of your class and get help from your friends. (16.3%)	Go through Online material With explanation. (10.4%)
Which mode of communication would you like for class updates?	Through college portal or E-Mail (16.3%)	Through WhatsApp (81.5%)	Through text messaging (1.5%)
According to you in which format online exam should be conducted?	MCQ only (42.9%)	Blend of short and long answer questions (46.2%)	MCQ, long and short answer based questions (10.9%)

CONCLUSION

The findings of present study indicate that to prevent the spread of the novel corona virus, online education has become the primary means of instruction. It is the most effective solution in front of educational community in West Bengal to catch up with the curriculum. The perception and preferences of students about different parameters of online education system is an important criterion which we have tried to summarize in this paper. This article may help to develop a useful online learning system in this crisis period.

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Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Biological and Computational Approach to Modify Bacterial Size and Reduce its Antibiotic Consumption Targeting MREB Bacterial Cytoskeletal Protein

Prarthana J[1,*] Nayana B,[1] Jayanthi MK,[2] Chagalamari Akhila,[2] Shashank M Patil[3] and Ramith Ramu[3]

[1]Department of Biotechnology, Sri Dharmasthala Manjunatheshwara College, Ujire, Karnataka, India

[2]Department of Pharmacology, JSS Medical College, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India

[3]Department of Biotechnology and Bioinformatics, School of Life Sciences, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India.

ABSTRACT

Amongst the cytoskeletal proteins of bacteria, MreB is known to have very crucial role in modulating shape of the bacteria. Present study involves the use of biocide (A-22) which minimizes the bacterial size augmenting with minimal antibiotic consumption. Intended experiment is designed to be carried out on selected pure strains of gram-positive and gram-negative bacteria namely *Lactobacillus rhamnosous* ATCC 7469 and Pseudomonas aeruginosa ATCC 27853 respectively. The pure strains are exposed to biocide and changes in the shape is recorded by means of Foldscope (Origami based paper microscope, Prakash Labs) and in-vivo assessment done using antibiotic sensitivity assays with different antibiotics. The novel biocide specifically targeting bacterial cytoskeletal protein, that determines rod shape among bacterial population. The said compound is also experimented as combinational drug along with conventional antibiotics to reduce antibiotic dose needed to kill and to overcome antibiotic resistance. The A-22 has reduced nearly 60-70% antibiotic usage. In *Pseudomonas aeruginosa* ATCC 27853 when tested for MIC using A-22 and different antibiotics, it was found that 0.5 µg/ml of ampicillin, 1 µg/ml of streptomycin and 5 µg/ml erythromycin were effective in curtailing bacteria against conventional antibiotic concentrations ampicillin 128 µg/ml streptomycin 32 µg/ml, erythromycin 64 µg/ml. Compared to doses of antibiotics required to kill bacteria, the combinational drug of biocide and antibiotic have shown promising effects in killing bacteria at very less concentration, this can useful for treating most diseases caused by antimicrobial resistance bacterial populations.

KEY WORDS: ANTIBIOTIC SENSITIVITY, BIOCIDE, CYTOSKELETAL PROTEINS, FOLDSCOPE, MREB.

INTRODUCTION

Eukaryotes and prokaryotes contain cytoskeletal protein structures that are essential to stabilize cell membrane and also provide rigidity to the cell (Soufo and Graumann 2007; Vats et al. 2009). In prokaryotes it helps in maintaining cell morphology, cell growth, cell division and chromosome segregation. The cytoskeletal proteins of eukaryotes namely actin, tubulin, and intermediate filaments are homologous to cytoskeletal proteins of bacteria MreB, FtsZ, and crescentin respectively (Daisuke et al. 2008). Among these, MreB protein determines rod-shape in bacteria: MreB which is an actin homolog is recognized as significant protein in maintaining rod shape in the bacteria such as *Pseudomonas aeruginosa, Escherichia coli, Caulobacter* and *Bacillus subtilis* (Busiek et al. 2015; Awunii 2020).

It was first identified in *Escherichia coli* as a protein necessary in maintaining cell shape (Doi et al. 1988). MreB belongs to the superfamily of HSP70–actin–sugar kinase and it was known in forming spirals which can traverse the longitudinal axis of cells of *B. subtilis*, this suggests that bacteria are having an internal actin-like cytoskeleton to maintain cell shape which is analogous to eukaryotes (Jones et al. 2001; Awuni and Mu 2019). It is considered to be conserved actin homolog in prokaryotes and is mainly encoded in the chromosomes of the bacterial species and also helps in variety of cellular activities. A22 - biocide changing rods into cocci: S-(3,4-dichlorobenzyl) isothiourea

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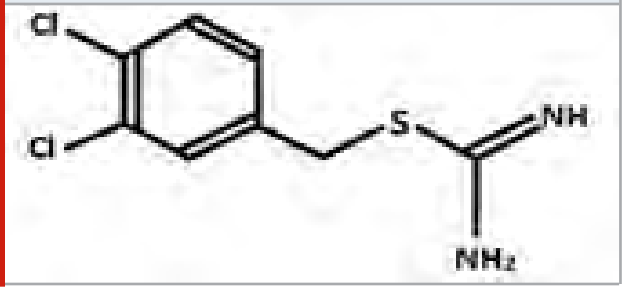
(A22) biocide is a derivative of S-benzylisothiourea which is chemically designated as [(3,4-dichlorophenyl)methyl] thiocarboxamidine (Figure 1) (Iwai et al. 2002; Carballido-Lopez et al. 2006; Awunii 2020).

Figure 1: Structure of A-22



The A22 is known to be acting like a reversible inhibitor of a bacterial cell wall protein MreB which leads to change in shape of bacteria from rods to the coccoid form and also it prevents assembly of MreB into long rigid polymers. As a result of change in shape various properties of the bacteria can be affected, such as the cell division, the acquisition of nutrients, motility, the clamping surfaces, and pathogenesis (Bonez et al. 2016). To check the activity of A22 in changing rods to cocci the pure strains were used in this study which includes *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Lactobacillus rhamnosus* ATCC 7469 and Lactobacillus casei ATCC 393 (Valik et al. 2008; Percival and Williams 2014; Moradali et al. 2016; Awuni 2020).

P. aeruginosa and *E. coli* were used in the study as they are pathogenic microorganisms and they show resistance to most of the antibiotics. As a case combination of biocide and antibiotics are used to inhibit the bacterial growth. In the current scenario, several phytochemical formulations have been used to treat microbial diseases (Patil et al. 2021a; Patil et al. 2021b; Patil et al. 2021c). Small molecules like bioactive peptides have also been used, indicating the significance of small chemical compounds (Patil et al. 2020). In the present study MreB polymerization is targeted using A22, MreB is very essential for cell wall biosynthesis and is also conserved in all rod shaped bacteria. This study mainly involves the use of combination of biocide and antibiotics against strains such as *P. aeruginosa* and *E. coli* because of their resistance to almost all the antibiotics. Their size reduction is established with minimum amount of conventional antibiotic to kill them. By this treatment of many severe diseases caused by these bacteria can be made possible.

MATERIAL AND METHODS

The pure strains of the bacteria from ATCC were taken and streaked on the agar plates. *Pseudomonas aeruginosa* and *Escherichia coli* was grown on Nutrient agar (NA) and incubated at 37°C for 24 hrs whereas, *Lactobacillus rhamnosus* was grown on De Man, Rogosa and Sharpe (MRS) agar and incubated at 37°C for 24 hrs (Bonez et al. 2016). Different broth was used for inoculating different bacteria. For *P. aeruginosa* Mueller Hinton borth (MHB) and agar was used for inoculating and plating respectively, whereas for *E. coli* Luria bertani broth and agar (LBA) was used for inoculation and plating respectively. *L. rhamnosus* was inoculated and plated on MRS media. Initially the cultures were exposed to different concentration of biocide (A22) given in the Table 1 and incubated at 37°C for 24 hrs.

After 24 hrs, the optical density of the culture was measured at 660nm using UV-VIS Double Beam Spectrophotometer 2205 (Systronics) and the size of the bacteria was measured using micrometry shown in the Table 2 (Awuni and Mu 2019). Then the lawn culture was prepared using sterile swab and different concentration of antibiotics was added and plates were incubated at 37°C for 24 hrs and the zone of inhibition was measured which is given in the Table 3. The bacteria were inoculated with combination of biocide and antibiotics and after 24 hrs of exposure the optical density of the culture was measured using UV-VIS Double Beam Spectrophotometer 2205 (Systronics), and the graph obtained is given in the Figures below (Al-Khayyat et al. 2019). Molecular docking simulation is performed to understand the interaction of ligands with the target proteins at the molecular level. In this study, the crystallographic structure of MerB protein was retrieved from the RCSB PDB database (PDB ID: 1JCE).

On the other hand, chemical structure of the compound A-22 was drawn and 3D optimized using ChemSketch. Protein and ligand preparation, as well as binding site prediction steps were completed according to the previous studies conducted by Patil et al. (2021d) and Patil et al. (2021e). AutoDock Vina 1.2, an open-source command line software designed for the docking of the molecular entities was used for the docking simulation (Trot and Olson 2010). The visualization of docking simulation was done using BIOVIA Discovery Studios Visualizer (2021), an open source visualizing GUI software. Druglikeliness and pharmacokinetic analysis, also known as ADMET (adsorption, distribution, metabolism, excretion, and toxicity) predictions, were performed to assess its oral bioavailability of potential drug candidates in silico. In this study, chemical structure of the compound A-22 in SMILES format was submitted to the ADMETlab server (https://admetmesh.scbdd.com/).

For the druglikeliness evaluation, Lipinski's rule of five was considered. For pharmacokinetic evaluation, CACO-2 permeability, human intestinal absorption (HIA), volume distribution (VD), cytochrome P (CYP) inhibition, hERG blocking, and AMES toxicity parameters were considered (Patil et al. 2021f). As the druglikeliness and pharmacokinetic studies were performed in silico, there were no ethical clearance, patient consent, or any kind of approvals required.

RESULTS AND DISCUSSION

The different bacterial cultures viz., *P. aeruginosa, E. coli* and *L. rhamnosus* were exposed with 1, 3 and 0.5 µg/ml concentration of the biocide. The optical density of the culture after exposure with biocide and control (without biocide) was recorded and also the size of the bacteria

measured is given in the Table 1. After the cultures were exposed to biocide, they were subjected to antibiotic assay against different antibiotics and the zone of inhibition formed is given in the Table 2. The optical density of the culture was recorded and the graph obtained is shown below (Figure 2, 3, and 4). The MIC of the experimental molecules has been shown in Figure 5.

Table 1. Optical density and the size of the bacteria after exposure to A-22 biocide

Bacteria	Biocide concentration	Optical density (660nm)	Size (µm)
Pseudomonas aeruginosa	Control	0.612	7.5
	1 µg/ml	0.410	5.0
Escherichia coli	Control	1.453	2.5
	3 µg/ml	0.751	1.875
Lactobacillus rhamnosus	Control	1.684	8.0
	0.5 µg/ml	1.571	7.5

Table 2. Zone of inhibition formed using different concentration of different antibiotics

***P. eruginosa* - 1 µg/ ml biocide concentraton**			***L. rhamnosus* – 0.5 µg/ ml biocide concentration**			***E. coli* – 3 µg/ ml biocide concentration**		
Ampicillin	0.5	8	Erythromycin	5	13	Ampicillin	1	N.Z
	1.0	9		10	16		2	N.Z
	1.5	10		15	18		3	N.Z
	2.0	12		20	20		4	14
Streptomycin	5	8	Chloramphenicol	2	8	Erythromycin	10	12
	10	11		4	13		20	14
	15	12		6	15		30	14
	20	13		8	18		40	14
Erythromycin	5	10	Streptomycin	2	7	Ciprofloxacin	0.012	N.Z
	10	13		5	11		0.013	N.Z
	15	15		7	14		0.014	10
	20	15		10	18		0.015	14
Chloramphenicol	5	11	Note: All the concentrations are given in µg/ ml, whereas the zone inhibition is given in mm.			Streptomycin	1	N.Z
	10	13					2	9
	15	15					3	10
	20	16					4	10
Ciprofloxacin	0.2	7				N.Z.: No zone observed during the experiment		
	0.3	9						
	0.4	10						
	0.5	11						

Figure 2: Graphs showing growth percentage of *Pseudomonas aeruginosa* against different experimental compounds A) ampicillin, B) streptomycin, C) erythromycin, D) chloramphenicol, and E) ciprofloxacin

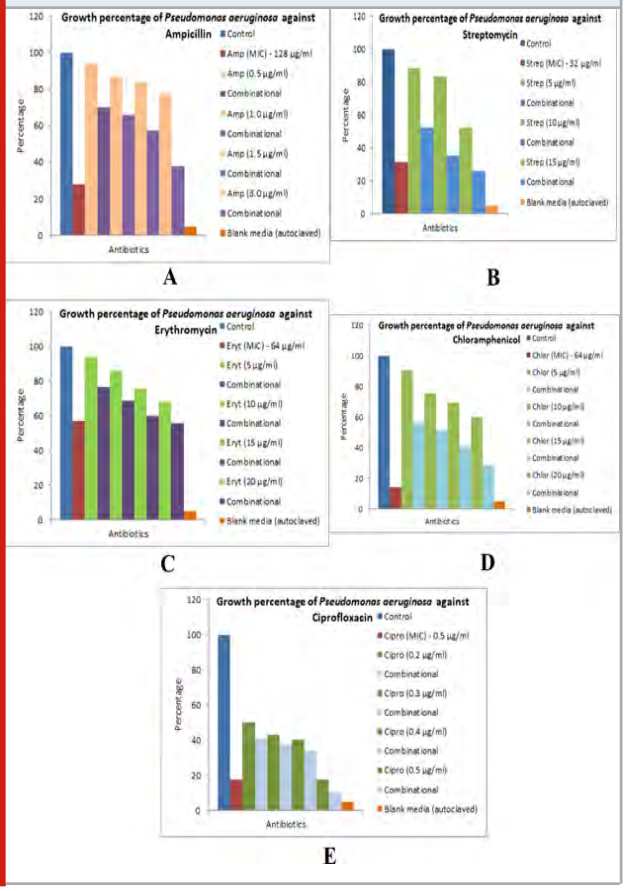
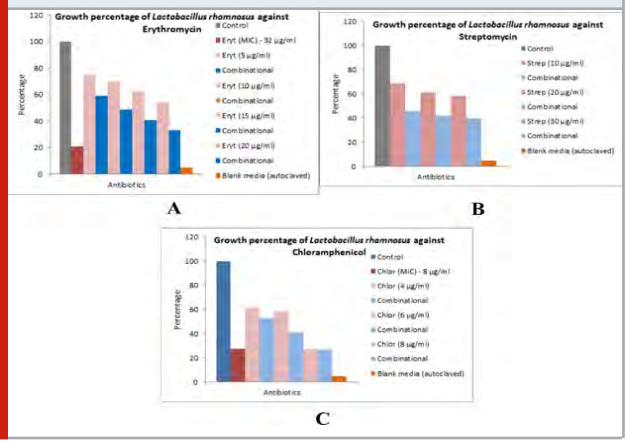


Figure 3: Graphs showing growth percentage of *Lactobacillus rhamnosus* against different experimental compounds A) erythromycin, B) streptomycin, and C) chloramphenicol



During the molecular docking process compound A-22 formed 3 hydrogens with GLY 68, PRO 103, and THR 158 residues of the protein. It also formed 2 hydrophobic alkyl bonds with LEU 312 and VAL 315. In addition, an electrostatic pi-anion bond with ASP 9 of the protein. The compound was predicted with a binding affinity of -8.7

Figure 4: Graphs showing growth percentage of *Escherichia coli* against different experimental compounds A) ampicillin, B) streptomycin, C) erythromycin, D) ciprofloxacin

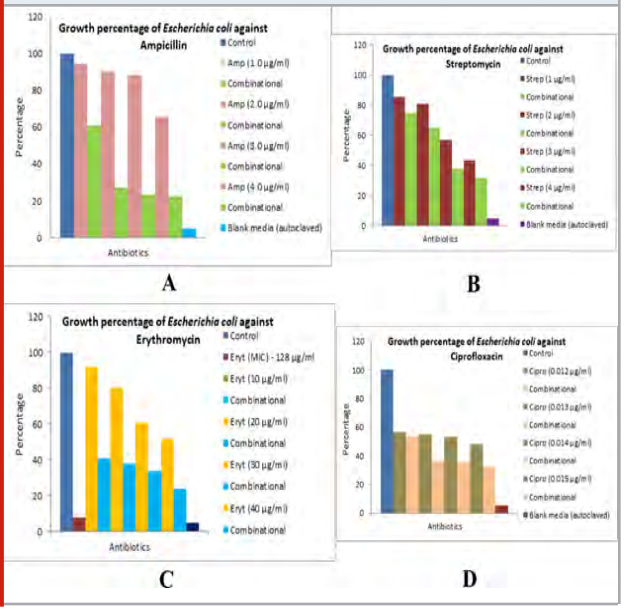
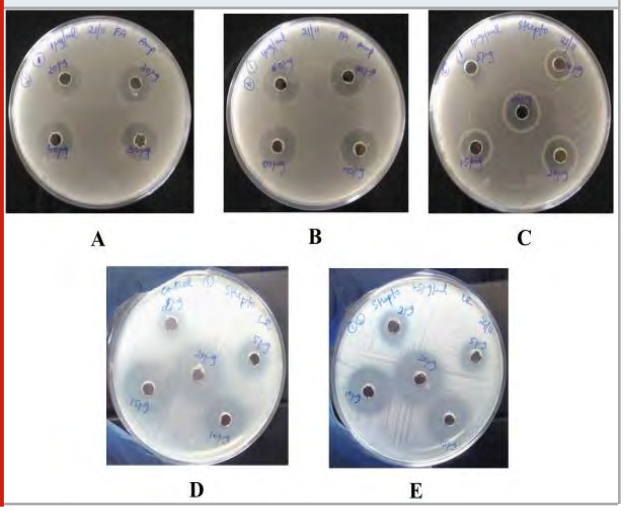


Figure 5: Image of *Pseudomonas aeruginosa* culture showing zone of inhibition for different concentration of A and B) ampicillin, C) streptomycin, D) Image of *Lactobacillus rhamnosus* culture showing zone of inhibition (diffuse growth present) for different concentration of streptomycin without exposing to biocide, E) after biocide exposure

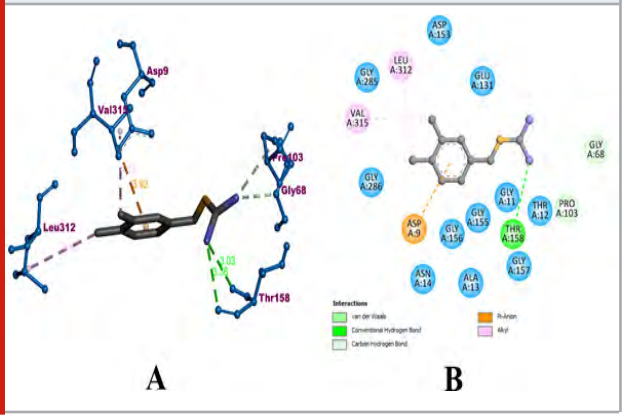


kcal/mol. The binding interaction of A-22 with MerB has been visualized in Figure 6. The bound amino acids have been colorized according to their binding type. Whereas, the surrounding amino acids have been colored in teal.

During the evaluation of druglikeliness according to the Lipinski rule, the molecular weight (MW) of A-22 was found to be 227.07 g/mol, which has not exceeded the limit of 500 g/mol. clogP value represents the partition coefficient between n-octanol and water to measure the hydrophilicity. Low hydrophilicities and therefore high clogP values cause

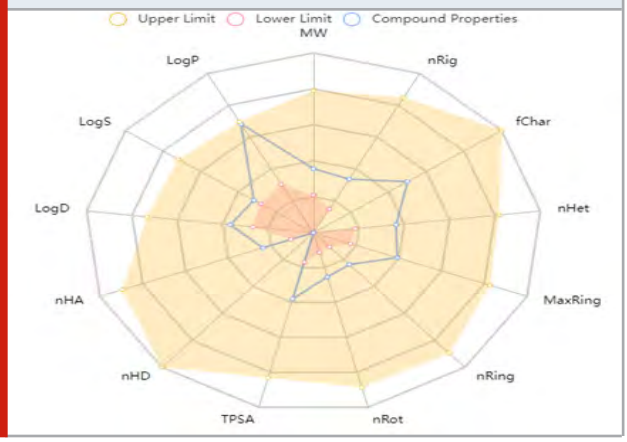
poor absorption or permeation. In this study, the clogP was found to be 1.65. Hydrogen bond acceptors were predicted to be 2, whereas the limit has been set for 10. In case of hydrogen bond donors, A-22 was predicted to have 2, with the limit set for 5. Therefore, compound A-22 never violated the rule of Lipinski rule. In case of pharmacokinetic analysis, Caco-2 cell line permeability was predicted to be -4.97 for A-22.

Figure 6: Visualization of binding interaction of the compound A-22 with MerB protein in A) 3D and B) 2D



The limit of Caco-2 value is -5.15 log units. Human intestinal absorption value was predicted to be positive (0.008). Volume distribution was predicted to be 4.628, which should be in the range of 0.04-20L/kg. In case of cytochrome P inhibition, A-22 was predicted with no inhibition of cytochrome P enzymes. The human Ether-à-go--Related Gene (hERG) is blocking was predicted with positive value (0.032). Furthermore, AMES toxicity value was predicted to be positive (0.236), which indicated absence of risk of toxicity. Table 3 represents the druglikeliness and pharmacokinetic analysis of the compound A-22. Figure 7 visualizes the druglikeliness and pharmacokinetic analysis of A-22.

Figure 7: Visualization of druglikeliness and pharmacokinetic analysis of the A-22. Presence of blue line represents the compound A-22 within the boundaries of all the parameters used.



The alarming increase in antibiotic resistance is due poor public health, inexpensive antibiotics which is causing threats in neonatal sepsis, causing therapeutic failures in bacterial infections (Laxminarayan et al. 2015). It is alarming that although bacterial resistance continues to emerge, the rate at which antibiotics are being developed is decreasing (Pulcini et al. 2012). MreB is a promising drug target because it is conserved and essential in most rod-shaped bacteria, MreB has been associated with essential subcellular processes including cell wall biosynthesis and maintenance of cell shape (Doi et al. 1988; Bean and Amann 2008; Awuni 2020). Determinations of the DNA-sequence of the MreB-gene and of the gene-products of the Mre-region that function in formation of the rod shape of *Escherichia coli* cells, cell division, cell wall morphogenesis MreB have been identified as potential targets for antibiotics.

The nucleotide binding site is an important target for antibiotics development because nucleotide binding plays a crucial role in the structure and dynamics of MreB (Wachi and Matsuhashi 1989; Jones et al. 2001; Soufo and Graumann 2005; Bean and Amann 2008; Awuni and Mu 2019). ATP induces the polymerization of MreB into filaments required for cell wall biosynthesis (. Interestingly, the polymerization of MreB induces ATP hydrolysis, which serves as a timing process to coordinate depolymerization (Bean and Amann 2008; Gunning et al. 2015). Thus, ATP is required by MreB to function properly and any molecule that could compete with ATP for binding to the nucleotide binding pocket could be a bactericidal agent (Awuni 2020).

The results obtained from this study are the first to evaluate the effectiveness of biocide A22 by inhibiting cytoskeletal protein Mreb on the strains *Pseudomonas aeruginosa* ATCC 27853 and *Lactobacillus rhamnosus* ATCC 7469 and to reduce consumption of antibiotics due to decreased size. The MIC value of A22 on *Pseudomonas aeruginosa* was found be lower in this study, compared to the MIC reported in previous studies (Bonez et al. 2016). The drastic reduction in the antibiotic consumption, below MIC after the exposure of the strains to biocide was observed in this study. The result reported in the study provides an alternative method to inhibit multi-drug resistant (MDR) microorganisms. The results obtained helps indicates that the biocide A22 used in the study brought about change in bacterial conformation by targeting its cytoskeletal protein MreB, and also reduced antibiotic consumption of bacteria (Awuni 2020).

Apart from the *in vitro* evaluations, *in silico* studies conducted on MreB inhibition also indicates that compound A-22 has the higher inhibitory potential. A recent study showed that MreB protein can be inhibited by few of the 100 natural compounds tested. Apart from amentoflavone and rutin, the other compounds failed to achieve significant inhibition. The compounds were also reported with insignificant druglikeliness and pharmacokinetics results (Al-Khayyat et al. 2019). In another study, phytochemicals from Leucas aspera were screened for the inhibition of MerB in silico. Among them, leucasperone B and penicillin were found to be the potent inhibitors of the protein. However, compound A-22 has been proved with the better

outcomes during in silico analysis in comparison with these studies, with respect to binding interaction, druglikeliness and pharmacokinetics analysis. From these we can say that A22 can be used as a novel drug for treating diseases caused by MDR *Pseudomonas aeruginosa* and also usage of high dose antibiotics can be stopped which can prevent many side effects caused by antibiotics to humans (Sharavanan et al. 2019; Awuni 2020).

Table 3. Druglikeliness and pharmacokinetic analysis of compound A-22.

Categories	Types of parameters	A-22
Druglikeliness based on Lipinski's rule of five	Molecular weight	227.07 g/mol
	No. of hydrogen bond donors	2
	No. of hydrogen bond acceptors	2
	cLog P	1.65
Adsorption	Caco-2 permeability	-4.97
	Human intestinal absorption (HIA)	0.008
Metabolism	CYP1A2 inhibition	No
	CYP2C19 inhibition	No
	CYP2C9 inhibition	No
	CYP2D6 inhibition	No
	CYP3A4 inhibition	No
hERG blocking	Clearance (CL)	0.032
Toxicity	AMES toxicity	0.236
Distribution	Volume distribution (VD)	0.071

CONCLUSION

The findings of this study are the first to assess the efficacy of biocide A22 in suppressing the cytoskeletal protein MreB of *Pseudomonas aeruginosa* ATCC 27853 and Lactobacillus rhamnosus ATCC 7469, as well as the reduction in antibiotic consumption due to reduced size. This study discovered a significant decrease in antibiotic consumption below the MIC level and there is nearly 60-70 percent antibiotic usage after the strains were exposed to biocide. In addition, our computational investigation also suggests that biocide A-22 inactivates MreB. During druglikeliness and pharmacokinetics analysis reveals A-22 shows no toxic effects. Therefore, we conclude biocide A-22 as a potent anti-bacterial agent against MDR bacterial species.

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Conflict of Interests: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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On the Woody Species Diversity and Population Structure of the Gola Natural Vegetation, Eastern Hararghe, Oromia, Ethiopia

Abdulbasit Hussein* and Tasisa Temesgen

Department of Natural Resource Management,Haramaya University College of Agriculture and Environmental Science, Dire Dawa, Ethiopia

ABSTRACT

The present study has analyzed the diversity and population structure of woody species in Gola natural vegetation. So far, the expected form of vegetation diversity relationships with different land use in study sites has not been known. A total of 73 quadrats were established systematically within three land-use types. Vegetation parameters and species type were recorded. There were 52 woody species found in total. The Fabaceae family had the most species, accounting for 15.3% of the total plant species. The PA site had Shannon's diversity index value that was significantly higher than the other two land-use types (P= 0.042). The dynamics of woody plants diversity about land use is of major need for sustainable management of forests, and the present study provides valuable information for forest management, and it may help to develop testable hypotheses on other tropical forests.

KEY WORDS: DIVERSITY, FARMLAND, GRAZING LAND, GOLA, PROTECTED AREA.

INTRODUCTION

Biological diversity refers to the diversity and evenness of species among and among living things and ecological complexes. The species is one of the most important analytical elements of the plant community (Laigle et al. 2021). Species richness is a simple way to measure biological diversity (Hillebrand et al. 2018). As anthropogenic activities destroy plant cover and biological variety, anxiety is rising in many parts of the world. Africa is predicted to have 650,000,000 hectares of forest cover, accounting for 17% of global forest cover and multiple biodiversity hotspots (Hegde and Enters 2000; Carney et al. 2014). Ethiopia is considered one of Africa's most significant countries in terms of biological resources. One of the most important concerns confronting humanity today is the degradation of the cover of the forest (Reynolds et al. 2007; Atsri et al. 2020; Hasan et al. 2021).

The lack of local communities' participation living near conservation zones in conservation initiatives is a key impediment to overall forest protection in Ethiopia (Woldemariam and Teketay 2001; Lemenih et al. 2014). To reduce the threat to natural forests, the country devised a wide range of conservation initiatives, such as water management, tree plantations, and replanting, rehabilitation, and restoration projects. These strategies were created to enhance vegetation protection and community members' lifestyles (Birhanu et al. 2021). The eastern part of Ethiopia has been putting in place well-known conservation measures on degraded landscapes for some years, with area protection being the most popular conservation method in this region (Bardgett et al. 2021; Gebo et al. 2021; Yami and Mekuria 2022).

Gola is home to a diverse range of natural plants and wildlife. Perhaps, as befitting a recently formed area of protection, it is lacking in basic vegetation and information on the environment. To fill the current knowledge gap in the research area's woody species, a detailed species diversity analysis of plants and vegetation population structure was carried out in three land-use categories: protected area, farmland, and grazing land. The main aim was to identify woody species and their diversity in connection to population structure to evaluate the effectiveness of conservation methods in the research region in eastern Ethiopia.

MATERIAL AND METHODS

Gola natural vegetation is located in Goro Gutu District, Eastern Ethiopia. The district has a total area of 531 km2. The protected part of Gola natural vegetation harbours

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different wildlife types and is reflected as an important support for upcoming indigenous community development. There is shared grazing space and farms used in the community near to the Gola protected area, with no limit to access resources. Three land cover types were studied as follows: protected area; farmland and grazing land used by the community.

Transect lines and sampling quadrats were created for each land use type based on area proportion. 6 transect lines in total were laid (two in each land cover type). 73 research quadrats were systematically chosen along the transect lines (20 from PA, 28 FL land, and 25 from GL). Quadrats of 20mx20m (400m2) were erected systematically at every 100 m interval for tree data gathering. There were five 5m x 5m sub quadrats used for shrubs and climbers in the major quadrats' four at corners and one center and averaged. A clipper and a hypsometer were used to measure the diameter and height of woody vegetation respectively (Kent and Coker 1992). The DSH/ DBH of branching shrubs and trees at breast height were recorded and averaged. Vegetation with multiple stems or forks that were less than 1.3 m tall was also counted as a single tree (Kent and Coker 1992). On the field, plant species were identified. Samples were delivered to the herbarium for species identification that had not been identified in the field. Shannon diversity index (H') was vital to choose an index that's more sensitive to richness when comparing diversity among samples and environments. Therefore, the species diversity across land-use types was estimated using the Shannon diversity index as follows:

$$H' = \sum_{1}^{s} pi \ln pi \quad (1)$$

where H' = Shannon index of diversity
Pi = the fraction of species.
Evenness (E), is a frequently used and well-known method for measuring community evenness (Pielou, 1966).

$$E = \frac{H'}{H'max} \quad (2)$$

where E = Shannon index of evenness;
H' = Shannon index of diversity;
H'max = lnS, and
S = total species number.
Importance value index was used to measure the relevance of all species to the study area, the IVI, relative frequency, frequency, and abundance were estimated using the method suggested in previous studies (Kent and Coker 1992a; Jha 1997).

IVI=Relative Frequency+ Relative Dominance+ Relative Density (3)

$$\text{Relative Frequency } (\%) = \frac{\text{Frequency of any species}}{\text{Total frequency of all species}} \times 100 \quad (4)$$

$$\text{Relative Density } (\%) = \frac{\text{Number of individual of each species per ha}}{\text{Total number of individuals of all species per ha}} \times 100 \quad (5)$$

$$\text{Relative Dominance}(\%) = \frac{\text{Basal area of each species}}{\text{Total basal area of all species}} \times 100 \quad (6)$$

$$\text{Basal area(BA) } (\%) = \frac{\pi d^2}{4}, d = \text{Diameter at breast height} \quad (7)$$

The BA is a measurement of domination and is used to elucidate the cross-section of the tree stand. It is derived using the method below (Kent and Coker 1992):

$$BA = \frac{\pi d^2}{4}. \quad (8)$$

Where; BA = Basal area, d = DBH/DSH, and π= 3.14
The Jaccard similarity index was used to calculate the coefficient of similarity between land-use types (Chidumayo 1997).

$$J = \frac{a}{a+b+c}. \quad (9)$$

J = Jaccard coefficient of similarity, a= Species Number shared to both samples, b= species number present in the first site only and, c= species number found only in the second area.

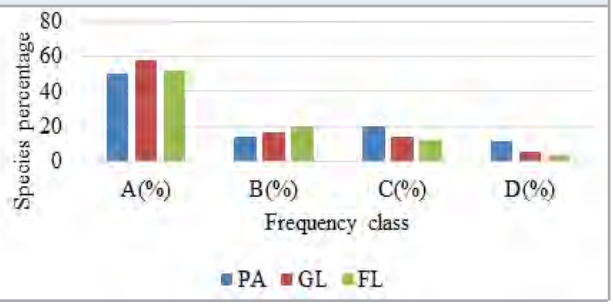
Following the requisite data collection, both qualitative and quantitative statistics were carried out. Percentages, figures, and means were used to present descriptive analyses of population structure. In addition, to examine diversity between land-use types, one-way analysis of variance is an example of inferential statistics were used for diversity indices (evenness and Shannon Weiner diversity index) and species richness. Every statistical analysis was carried out using R software (version 4.1.2) using lattice, vegan, permute, and biodiversity R package at the 5% level of significance.

RESULTS AND DISCUSSION

Woody species composition: A maximum of 52 species of woody plant 33 genera and 24 families were found in the area of the study. The growth forms of these species were distributed as follows: 6 tree/shrubs 7 climbers, 16 trees, and 23 shrubs. There were 44 plant species collected in PA, 19 of which were shrub species, 14 of which were tree species, and 7 and 4 respectively for climbers and tree/shrubs. In GL, as in PA, shrubs dominated with 17 species, followed by trees (12), tree/shrubs (4), and climbers (3). Similarly, shrubs have 13, trees have 9, and tree/shrubs and climbers have 2 and 1species respectively at the FL site. The species number (52) found in the research region was larger than the species number found in Ethiopian forests, such as the Jabi Tehnan forest in the northwestern part of the country

(Asmare and Gure 2019). On the other hand, the overall number of plants found at the study site was found to be lower than that reported from communal grazing grounds in South Tigray (Wayu et al. 2019). Various environmental variables in various parts of the country could cause such discrepancies in the study. Forest management and ownership may reveal their status and divergence, with highly protected natural vegetation having a higher number of individuals than community forest and open access regions (Sinasson et al. 2021; Tadesse et al. 2021).

Figure 1: Frequency class of woody species [(D ≥75, C = 51 – 75, B = 26– 50, A ≤25) (GL, grazing land FL, farmland and PA, protected area)]



The variation in woody diversity and abundance among each land-use type exposed the increasing influence of vegetation preservation through effective conservation measures such as area enclosures and restoration practices. According to Gebre et al. (2019), the loss in woody species diversity in grazing land could be an indication of vegetation species' increased vulnerability to livestock and/or human intervention at maturation or early stages of rejuvenation. Heavy trampling may lead to a decline in plant species density and diversity over time (Mussa and Yunus 2022; Zegeye 2022).

The Fabaceae family was found to have the greatest species, accounting for 18.18%, 25%, and 32 % of all plant species found in a protected area, grazing, and farmland, respectively, followed by Euphorbiaceae in FL (16.00 %) and GL (11.11%). However, Euphorbiaceae and Oleaceae, with 11.36% of species, were the second-highest lifeforms family in PA. Other previous studies also suggested that Fabaceae were the dominant family in their study area (Abunie and Dalle 2018; Eshetu and Hailu 2020). This situation might arise due to a wide variety of adaptations to different environmental management (Alimi et al. 2021; Bora et al. 2021).

Table 1. Mean ± SD of Gola natural vegetation diversity, evenness, and richness

sites	Shannon diversity index (H')	Evenness (H′/Hmax)	Species richness
Protected area	3.54 a±0.81	0.90^{a} ±0.02	19.70^{a}±7.51
Grazing land	2.99 ab ±0.76	0.87^{b}±0.04	14.30^{a} ±7.62
Farmland	2.20 b±0.62	0.62^{b}±0.07	11.20^{a} ±7.84
P-value	0.041*	0.048 *	0.81

Table 2. The similarity of woody species across the study sites

Sites	Protected area	Grazing land	Farmland
Protected area	0		
Grazing land	0.79	0	
Farmland	0.51	0.66	0

The density of Woody Species: Gola natural vegetation has a mean density of 8844.16 individuals per hectare. *Acacia senegal* had the largest density of single woody species, with 2240 and 960 individuals/ ha in protected areas and grazing land, respectively, while *Grewia schweinfurthii* had 420 individuals/ha/ ha in FL. The highest concentrations of some species, such as *L. camara*, maybe due to their unpleasant features for both wild and domestic animals, in addition to a diverse variety of seed distribution methods and highly reproductive capabilities (Hishe et al. 2021; Koricho et al. 2021). According to the same study, open-grazed lands showed lower vegetation density when compared to a protected area (Wu et al. 2021; Wachiye et al. 2022).

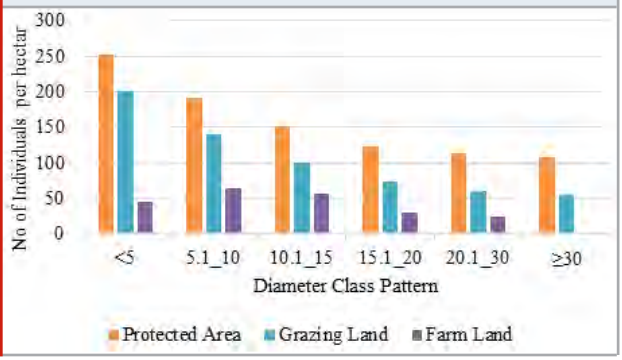
Frequency: Woody plant species were grouped into four different frequency groups in this study. In this study, a considerable proportion of woody plant species were found in lower frequency classes, while a small percentage of many species were found in the upper-frequency groups. This suggests that the species composition of the study sites was generally heterogeneous. PA had 11.33 percent a greater number of species in a higher frequency class than grazing and farmland, which had just 5.6% and 45 of plants, respectively. While in the lowest class of frequency, grazing land had a higher percentage of species (58%) than farmland (52.33%), and protected area (50.25%) (Fig 2). Thus, the outcome verifies the existence of a floristic heterogeneity to a high degree in each land-use type (Balemlay and Siraj 2021; Akodéwou and Godron 2022).

In both PA and GL, *Acacia bussei* was the greatest commonly identified species of plants, while Grewia schweinfurthii has been the maximum often identified in farmland. The least often documented species of plant in a protected area, grazing land, and farmland was *Olea europaea, Grewia velutina,* and *Sansevieria ehrenbergii* respectively, each of which was only found in one plot. The absence of often frequent for some species vegetation is related to a higher level of human involvement and livestock grazing. To improve the frequency distribution of such species, conservation must be prioritized (Mewded et al. 2021).

Woody Species Diversity, Evenness, and Richness: The average woody species diversity and evenness were 3.18 and 0.86, respectively. The species diversity and evenness were significantly different among the three land-use categories. PA had a significantly higher Shannon diversity index value than the FL. Even though GL was somewhat greater than farmland, the GL did not significantly differ from each other (Table 1). This could be due to regular habitat disturbances in grazing and farming areas, as a result of human and livestock grazing and extra agricultural use during the day (Beche et al. 2022).

The mean evenness of the three land-use categories fell significantly (p=0.041) from the protected area through the grazing land to the farmland. In farmland, a low average evenness rating indicates that only just a few species occupy the area. While in a protected location, great evenness suggests that species distribution is continuous (Cruz-Alonso et al. 2021).

Figure 2: Species distribution by diameter



Jaccard Species Similarity Coefficient: Jaccard's coefficient of similarity was computed to compare the similarity of family, species, and genus of the three land-use. For PA and GL, the Jaccard's similarity of woody species was 79% of species. Similarity values of 66% were found between the GL and FL. However, there were 51% of species similarities between PA and FL. The highest species level similarity value was found between PA and GL, followed by GL and FL, whereas the lowest was found between PA and FL.

Basal area: The mean BA of Gola Natural vegetation was 33.11m2 ha-1 of plant species that have DBH > 2.5 cm. PA, GL, and FD each had a total basal area of 43.73 m2/ha, 31.59 m2/ha, and 22.79 m2/ha, respectively. PA had higher coverage of the basal area than another two sites (P 0.04) and was followed by GL. This could be attributed to the presence of a more proportion of mature and larger trees in PA, resulting in larger diameters as a result of minor human interference activities such as tree logging, farming, and animal grazing. FL, on the other hand, has much less coverage of the basal area. This directly related tendency revealed that anthropological involvement had an impact on vegetation's basal areas (Calbi et al. 2021). *Acacia seyal* (4.53 m2/ha), followed by *Euphorbia adjurana* (3.8 m2/ha), covered the maximum amount of mean basal area at the protected area. The greatest percentage of the average BA was accounted for by Acacia bussei (3.62 m2/ha) and Acacia senegal (3.14 m2/ha), followed by *Acacia tortilis* (3.51 m2/ha) and *Acacia bussei* (2.54 m2/ha) at grazing and farmland, respectively. In this research, basal area evaluation crosswise each species exposed that the studied region was dominated heavily by a small number of woody species. This also shows that species with the largest basal area do not always have the highest density, implying that species are different in size (Kaushal et al. 2021; Rasquinha and Mishra 2021).

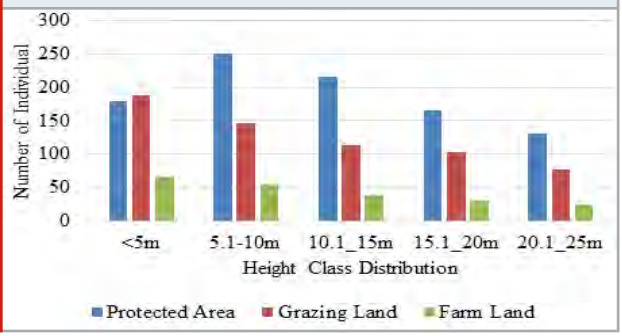
Importance value index: *Acacia senegal* (27.99), *Grewia schweinfurthii* (25.78), and *L. camara* (16.36) were the highest three species with the IVI in PA. These species could be critical in a protected area. Woody species such as *Jasminum abyssinicum* (0.68), *Jasminum grandiflorum* (0.69), and *Jasminum schimperi* (0.71), were found to have lower IVI. This means that these species have the smallest ecological impact on the place. Their decreased IVI could indicate that these woody species are in danger and, among other things, require immediate conservation measures (Ayalew and Alemu 2021; Mewded et al. 2021). Woody species with the highest IVI at the GL include *Acacia senegal* (19.02), *Acacia tortilis* (17.76), and *Acacia brevispica* (17.76). This shows that in grazing areas, these are the most ecologically significant species. Woody plants with lower IVI at the GL, such as *Barbeya oleoides* (0.76), *Withania somnifera* (0.79), and *Jasminum abyssinicum* (0.81), attract conservation action based on their ecological significance (Ayalew and Alemu 2021; Mewded et al. 2021).

The three most ecologically significant species with the greatest IVI values at the farmland site are *Grewia schweinfurthii* (24.50), *Acacia senegal* (22.48), and *Acacia negrii* (21.50). In all three study sites, *Acacia senegal* exhibited the highest IVI. Because most of the factors are next to each other, this shows that there is some similarity in the sites. Dracaena ombet (0.90), *Sansevieria ehrenbergii* (1.50), and *Grewia villosa* (1.57) were the least ecologically relevant species at this site based on their IVI values. Each

of the three sites has an entirely distinct least significant species. This also suggests that the state of anthropogenic disturbances such as farming and grazing varies greatly among the three land-use types (Ghanbari et al. 2021; Muluneh et al. 2021; Pandey 2021).

Woody Species Distribution by Diameter Class: In the protected area and grazing land sites, the Species in the DBH class was divided into six categories. In FL, however, a maximum of five diameters classes are allowed. The overall DBH class distribution of woody species in a protected area and grazing land vegetation showed an inverted J-shape distribution. This illustrates where the DBH class distribution of the species was most common in smaller diameters and vice versa. The first two diameter classes in protected areas and grazing land were accounted for 48.32% and 53.41% of DBH frequency in total, respectively. This showed that mature and large diameter class trees were being harvested by locals for various uses (fencing, farm implementing, house construction, and fuelwood). Locals harvested trees with DBH more than 30cm for construction and charcoal manufacturing, according to similar reports (Gurmessa et al. 2021). However, in farmland diameter patterns of woody species, the majority of DBH frequency (55.01 percent) was restricted between the second and third diameter classes, indicating that there were more individuals in the middle diameter classes but fewer in the lower and higher diameter classes (Fig 3). This shows that the species in the community has a low reproductive capability (Calbi et al. 2021; Laigle et al. 2021).

Figure 3: Distribution of height class at the study site)



Height Distribution: The natural plants of Gola were divided into five classes based on height. In contrast to the DBH class, the height class distribution in the GL and FL sites has an inverted J-shape design, with a steady decrease towards the largest highest class. In both grazing and farmland, this finding revealed the development and strong regeneration state of the species population. However, in PA, it exhibits an uneven pattern dominated by the largest trees and plants. In PA, the majority of species achieved the medium canopy layer classification, indicating that the species number was highest in the middle classes and reduced as height increased. The increased number of large-sized individuals in the upper height class in natural vegetation suggests the presence of a significant number of mature vegetation species for reproduction (Balemlay and Siraj 2021). For protected areas, this argument holds are true. This is due in part to the residents' absence of large-scale woody exploitation (Gemechu and Jiru 2021; Tamiru et al. 2021).

CONCLUSION

The findings of the present study have shown that the plant species of the research site were conquered by shrubs that were dominated by the Fabaceae family. Woody cutting, agriculture, and overgrazing are examples of human pressures on the majority of species, all of which are considered environmental matters that have contributed to the degradation of the vegetation. The diversity and structure of woody species showed variation across the three land-use types, which increased in the PA whereas decreasing through the adjacent GL to FL. These types of comparative analysis contribute to understanding the role of conservation on the natural vegetation in Ethiopia, practicing wide-scale conservation activities of degraded landscapes.

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Conflict of Interest: Authors do not have a conflict of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Dimensions of Quality in Healthcare: Perceptions of Patients from Saudi Public Hospitals

Muhammad Attar*
Applied College, Natural Sciences and Engineering Department,
University of Jeddah, Jeddah, Kingdom of Saudi Arabia

ABSTRACT

Healthcare quality is driven by multiple dimensions. The present study aimed to examine the main dimensions of healthcare quality from the perspective of patients in the Kingdom of Saudi Arabia public (KSA) hospitals. A patient satisfaction survey was designed to discover the perceived dimensions of quality in the KSA healthcare sector. The steps involved in identifying the quality dimensions relating to healthcare are presented in the paper. The principal component analysis (PCA) lead to identifying the components with total variance explained and result in identifying three meta dimensions. This included wellness support, compliance with Standards, and exceptional service and immediate care. The research findings have provided a platform for emerging and discovering patient needs, direct improvement efforts in such a critical service sector and can be used as a basis for developing new measures to discover patients' needs.

KEY WORDS: HEALTHCARE QUALITY DIMENSIONS, PRINCIPAL COMPONENT ANALYSIS (PCA), PATIENT NEEDS.

INTRODUCTION

The healthcare industry has a significant importance in the global economy. This is because of its critical role in maintaining the health of people and providing high-quality healthcare services. Most countries provide a large expenditure on the health sector to maintain the health of people. For instance, Fuchs (1998) pointed out that in 1997, the US spent around 8% of its GDP on healthcare. Moreover, Estes et al. (2013) expected that by 2020, the expenditures on healthcare will reach 20% of the US national GDP. The UK increased the expenditure on the health system as a percentage of GDP from 5.9% in 1981 to 9.6% in 2017 (OECD 2020). Indeed, the COVID-19 pandemic resulted in enormous changes in health care delivery systems and has a major impact on the global economy. Various governments went through major health investments for maintaining the health of people (Faruk et al. 2021).

The Ministry of Health (MoH) in the Kingdom of Saudi Arabia (KSA) is responsible for providing healthcare services and managing the healthcare sector through health directories across the kingdom (AlYami and Watson 2014). Additionally, military hospitals are controlled and supported by the Ministry of Defense and Aviation controls (Mufti 2000; MoH 2002; Faruk et al. 2021). The healthcare sector has been given a top priority in Saudi Arabia's Vision (2030). The vision aims to improve the quality of life across the Kingdom in several fields including the health of people. To respond to the patient needs, MoH has made an extensive effort to improve the healthcare system, develop the infrastructure of hospitals' facilities, and provide affordable medical services to patients. It also has contributed positively to improving healthcare services and satisfying patient needs by adopting the most advanced technology applications based on a world-class standard. The efforts have been made to achieve a high level of healthcare quality. However, this did not result in improving the medical services provided to the patients (Ishfaq et al. 2016). The healthcare sector in KSA developed greatly over the past years with increasing demand for healthcare services. In (1970), the total number of populations in the KSA was 5.8 million and increased to 34.2 million by 2019. The total number of beds were increased from around 9000 to 77000 while the total number of hospitals was shifted from 74 to 498 (MoH 2019; Faruk et al. 2021).

To improve performance in the delivery of healthcare services in the KSA, there is a need for assessing, and improving productivity in hospitals (Al-Hanawi and Makuta 2022). The healthcare system includes several dimensions that are rapidly changing over years. Thus, identifying the main dimensions of quality in healthcare is necessary to improve the medical services provided to the patients.

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Patients as the main stakeholder in the healthcare system have significant importance when assessing healthcare quality (Potts et al. 1984; Bensing 1991). Patient-Centered Outcomes Research Institute (PCORI) (2014) identified the healthcare stakeholders which are patients, clinicians. researcher, purchasers, hospitals/health systems, policymakers, payers, industry, and training institutions (Faruk et al. 2021). Many studies showed that patient perceptions of healthcare service quality are important and should be prioritized when evaluating the healthcare system (Gabel et al. 2003; Iversen et al. 2012).

Table 1. Healthcare organizations and quality constructs

Health Organization	Abbreviation	Location	Year	Quality Constructs
The Institute of Medicine	IOM	USA	2001	effectiveness, safety, responsiveness, timeliness, efficiency, and equity.
Organization for Economic Co-operation and Development	OECD	France	2006	effectiveness, safety, and responsiveness.
The Institute for Healthcare Improvement	IHI	USA	2007	individual experience, populations health, and per capita costs of care
European Commission	EC	Belgium	2014	effectiveness, safety, responsiveness, efficiency, and equity.
The World Health Organization	WHO	Switzerland	2018	effectiveness, safety, responsiveness, timeliness, integration, efficiency, and equity
The Australian Health Performance Framework	AHPF	Australia	2019	effective, appropriate, efficient, responsiveness, accessible, safe, continuous, capable, and sustainability.

Specifically, studies in the field of patient satisfaction related to healthcare services in KSA have only focused on measuring efficiency levels among public hospitals (Alatawi, Niessen and Khan 2020; Faruk et al. 2021). Therefore, the aim of this study was to identify the main healthcare quality dimensions in KSA public hospitals based on patients' perceptions. In order to achieve this aim, the current study utilized the principal component analysis (PCA) to explore the relative contribution of each quality dimension in healthcare as perceived by patients. These perceptions can be expected to be considered for improving patient perception and satisfaction in KSA public hospitals.

MATERIAL AND METHODS

The current study examined the main dimensions of healthcare quality from the perspective of patients in KSA public hospitals. The survey questionnaire instrument was constructed and included closed-ended questions rated on a five-point Likert-type scale. It contained demographic characteristics about the patients such as nationality, marital status, sex, age, and education level. The Software Statistical Package for Social Sciences (SPSS) 20.0 was used to analyze the data. The principal component analysis (PCA) was utilized to explore the main dimensions of healthcare service quality in KSA public hospitals. PCA is a multivariate statistical method used for reducing the dimensions or components of the dataset into a small number to find out the internal uncorrelated variables (Hair et al. 2010). Four faculty members and two medical staff from different public hospitals evaluated the survey questions. They participated in a focus group meeting to clarify the survey questions and their relation to the main research objective. The discussion outcomes resulted in the selection of 36 out of 62 questions.

A pilot study was conducted with 29 undergraduate students who visited the medical center of the university to ensure the clarity and readability of the questioner's items. The 36 statements included in the survey questionnaire revealed a high level of clarity and the average time required for completing the questionnaire was less than five minutes. The sampling plan criteria included five public hospitals located in Jeddah city. These hospitals were convenient and mostly visited by patients from different cities located in the western region of KSA. The results presented in this study were based on descriptive statistics and multivariate statistics methods.

RESULTS AND DISCUSSION

Healthcare quality has been receiving much concern from researchers and several studies have been conducted. This is because of difficulties in defining the quality of healthcare service from only the patients' point of view

who use the service. According to Panchapakesan et al. (2009) improving quality in healthcare can be achieved by recognizing the perceptions of stakeholders. The Institute of Medicine (IOM) (1990) in the US described quality in healthcare as "the degree to which health services for individuals and populations increase the likelihood of desired health outcomes and are consistent with current professional knowledge" (Institute of Medicine 1990; Panchapakesan et al. 2009; Faruk et al. 2021).

Table 2. General information of the respondents

Description	Frequency	(%)
Nationality		
Saudi	471	79
Non-Saudi	99	21
Marital Status		
Single	393	68.9
Married	177	31.1
Sex		
Male	354	62.1
Female	216	37.9
Age		
18-25	313	54.9
26-35	139	24.4
36-50	85	14.9
>50	33	5.8
Education Level		
High School	124	21.8
Bachelor	272	47.7
Master	98	17.2
PhD	76	13.3
Visiting Frequency		
Weekly	86	15.1
Monthly	118	20.7
Every 6 month	207	36.3
Yearly	159	27.9

Previous studies showed that there is no generic model or instrument developed to measure patients' perceptions of quality dimensions in healthcare. Several organizations have identified quality dimensions in healthcare with different quality constructs (Faruk et al. 2021). In (2001), the IOM identified six healthcare quality dimensions, which are effectiveness, safety, responsiveness, timeliness, efficiency, and equity. These dimensions have been used as a tool for quality assessment and measurement of the healthcare system. According to Leatherman and Sutherland (2003) the most common health quality dimensions used in the USA, Canada, and the Organization for Economic Co-operation and Development (OECD) countries are access, effectiveness, communication, and safety. The OECD (2006) proposed three main dimensions of healthcare quality ,which are effectiveness, safety, and responsiveness. The WHO (2006) proposed the following quality dimensions in healthcare effectiveness, safety, acceptability, responsiveness, efficiency, access, and equity.

In (2018), the World Health Organization used the same IOM dimensions and added the integration dimensions, which formed the overall health quality dimensions. The Institute for Healthcare Improvement (IHI) (2007) in the USA proposed the framework for safe, reliable, and effective care that included three main dimensions: the individual experience, the populations' health, and the per capita costs of care. The European Commission (EC) (2014) identified five dimensions of healthcare namely, effectiveness, safety, responsiveness, efficiency, and equity (Faruk et al. 2021). The Australian Health Performance Framework (AHPF) (2019) is a tool used to measure the performance of health care in Australia based on nine dimensions effective, appropriate, efficient, responsiveness, accessible, safe, continuous, capable, and sustainability. Table 1 shows the healthcare organizations and quality constructs.

A total of 672 respondents participated in this study. Out of collected questionnaires, 102 were excluded because of missing data. This results in (84.8 percent) response rate. The first part of the questionnaire included general information about the patients. This information is shown in table 2. As shown in the above table (79 percent of patients) were Saudi and (21 percent) were non-Saudi. Moreover, (68.9 percent) of the respondents were single and (31.1 percent) were married. Most of the participated patients were male (62.1 percent) whereases (37.9 percent) were female. More than half of the respondents were younger than 35 years and most of them belonged to the college degree category. The demographic information indicated that patients used to visits public hospitals every 6 months. The quality dimensions that considered in this study were derived from Gronroos (1988), Garvin (1984), and Parasuraman et al. (1985), and IOM (2001). The healthcare quality dimensions with its definitions are listed in Table 3.

The second part of the survey questionnaire instrument contained 36 statements and constructed based on 13 dimensions. The reliability of the questionnaire scales was assessed using Cronbach alpha to ensure the trustworthiness of the answers gathered from the patients. According to Hair et al. (2010), the acceptable Cronbach's alpha is greater than 0.7 and reveals a high level of internal consistency. The Cronbach's alpha, mean, and standard deviation for 36 items were calculated and presented in Table 4. All identified dimensions have coefficients higher than 0.7 and the total reliability was 0.81, indicating high overall reliability (Hair et al. 2010: Faruk et al. 2021).

In order to know the numbers of components to consider, a scree plot was used where stress values are plotted versus the number of dimensions. As illustrated in Figure 1 the scree plot represented three components. Therefore, all components with an eigenvalue greater than one were considered. The overall significance of the correlation matrix is zero, the approximate chi-square value was 7285.981, and the Kaiser-Meyer-Olkin (KMO) was 0.920. According to Kaiser (1974) the KMO value greater than

0.6 indicates the sampling adequacy and the factor analysis performed is appropriate. The Components with total variance are shown in Table 5.

The items that represent the quality dimensions with high loading values were considered and a total of three components were obtained. According to Hair et al (2010) items can be included when the loading values exceed 0.5 onto a factor, and items less than 0.5 should be excluded. As shown in Table 6, three components have eigenvalues greater than 1.0 and account for 57.02 percent of the total variation in the data. The component weights and dimensions are represented in table 6. The results of PCA showed that out of 13 healthcare quality dimensions, only seven dimensions reflected the perception of patients in KSA hospitals. These dimensions were access, recovery, conformance, facility, reliability, feature, and responsiveness. Thus, 18 items were excluded as they were not meeting this criterion (communication, competence, security, courtesy, understanding, credibility). This is because of the low factor loading to the proposed factors (Faruk et al. 2021).

Table 3. Healthcare quality dimensions

Construct	Definition
Reliability	The service provided is consistent and serves the patient right every time
Responsiveness	Readiness and response to patients need immediately
Competence	Obtain the skills needed and knowledge to perform the service.
Access	The healthcare services are easy to reach and access
Courtesy	The healthcare staff are polite, respectful, and friendly to patients
Communication	Patients can communicate with all medical staff members
Credibility	The trustworthiness, and honesty of all medical staff members
Security	The healthcare services are free from error, danger, and risk
Understanding	Recognize and understand patients' needs.
Tangibles	The physical components of healthcare services
Features	The supplementary characteristics of healthcare services
Conformance	The healthcare provider follows the healthcare standards, and procedures.
Recovery	The healthcare provider takes immediate action and solves any issues that could be occurred

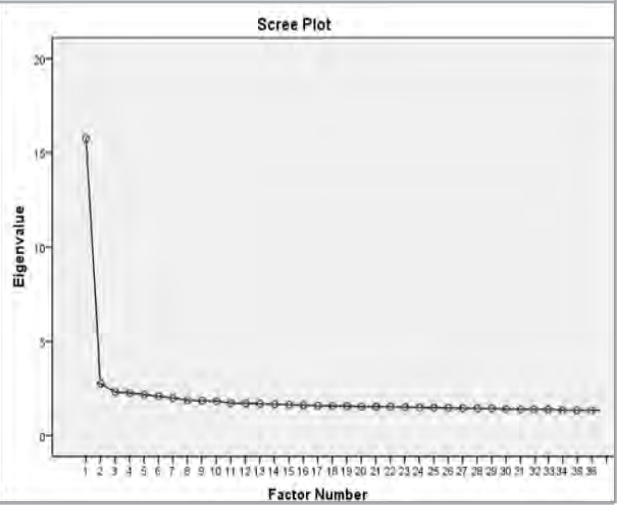
Table 4. The healthcare quality dimensions and Cronbach alpha values

Health Care Quality Dimensions	Code	Item	Cronbach Alpha	Mean	SD
Access	Access1	The hospital location is easy to reach	0.899	2.78	1.27
	Access2	The hospital operational systems are working properly			
	Access3	Easily accessible by telephone and website			
	Access4	I can make an appointment and receive healthcare services			
Reliability	Reliabi1	Provides medical services as promised	0.852	2.77	1.24
	Reliabi2	Committed to providing medical services on time			
	Reliabi3	The medical services provided are trusted			
Communication	Commu1	The staff spoke using clear language	0.867	2.21	1.65
	Commu2	The doctor provides a clear explanation about the medications			
	Commu3	The hospital sent me an appointment reminder by text message			
Competence	Compet1	The staff are experienced and efficient	0.830	2.75	1.26
	Compet2	The doctors have high skills and knowledge			

Table 4 Continue

	Compet3	The nurses cooperate effectively with patients			
Security	Secur1	The hospital has several security men	0.710	2.69	1.26
	Secur2	Patient personal information is secured			
	Secur3	The hospital is equipped with surveillance cameras			
Facility	Facility1	Modern tools and equipment's are used to provide the service	0.950	2.73	1.22
	Facility2	Good atmosphere and decoration			
	Facility3	The hospital placed directions signs			
Responsiveness	Resp1	The medical services provided over 24 hours	0.932	2.77	1.25
	Resp2	Staff respond immediately to patients' complaints			
	Resp3	Staff inform patients of scheduled appointments			
Courtesy	Court1	Staff are friendly, polite, and respectful	0.627	2.73	1.26
	Court2	The clean and neat appearance of staff			
	Court3	Good understanding of the patient's needs			
Feature	Feature1	The hospital provides free water bottles	0.910	2.84	1.31
	Feature2	The hospital provides free WIFI internet			
	Feature3	The hospital has a mobile application or website for scheduling an appointment			
Conformance	Conform1	The time required to see the doctor is acceptable	0.770	2.80	1.26
	Conform2	The doctor prescribed the medication. according to the patient's age			
	Conform3	The patient medical report does not include any mistakes			
Understanding	Understand1	The hospital medical staff made an effort to understand my needs.	0.611	2.61	1.54
	Understand2	The doctor described the right medicine			
	Understand3	The medical staff assign the right doctor to me			
Credibility	Cred1	The hospital name and its reputation are trusted	0.701	2.70	1.25
	Cred2	The medical results are accurate			
	Cred3	The patient has confidence in doctor qualifications.			
Recovery	Recov1	The hospital staff take immediate action in case of emergency	0.880	2.73	1.23
	Recov2	The doctor informs the patient about the required medicine dosages.			
	Recov3	The doctor provides alternative recovery plans.			

Figure 1: Scree plot for the survey items



As a result, access and recovery dimensions pertained to the first component. The second component included two dimensions namely, conformance and facility. The third component resulted in obtaining three dimensions reliability, feature, and responsiveness. The PCA showed that seven dimensions explain 57.02 percent of the total variability in the count. Access and recovery belonged to the first component. This indicated that public hospitals should facilitate access to patient information, treatment details, and tests needed to be consistent with a patient's recovery plans. The meta dimension can be labeled as "wellness support". This result is consistent with Musa et al. (2021) study who emphasized on creating wellness programs within primary health (Musa et al. 2021).

According to the institute of medicine (IOM) (2001), the availability of information is important for patients and their families to have the right for choosing the healthcare providers that offer medical and treatment services. Conformance and facility pertained to the second principal component. This means that patient view regarding the appropriateness of medical facilities, and relates to the fulfillment of healthcare standards. This is suggested the "healthcare compliance with standards" meta dimension. The dimension can be assessed through accreditation and audit activities. This is supported by the fact that compliance

with accreditation standards provide many benefits to the hospitals in term of improving performance and patient safety (Hussein et al. 2021).

Indeed, most hospitals in Saudi Arabia work effectively to achieve Joint Commission International (JCI) accreditation. The JCI accreditation requirement is to execute patient satisfaction surveys and share these results with them (JCI 2008). Hospitals that have sought JCI accreditation have made the necessary steps to conduct patient satisfaction surveys. This required standard, prepared by the JCI to obtain patients' opinions on the medical care received and has been considered as a part of the quality indicators for improvement. However, some hospitals in Saudi Arabia have not used patient satisfaction data to improve the quality of their care, only collecting the survey data to comply with JCI requirements (Hussein et al. 2021).

Table 5. Components with total variance explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative	Total	% of Variance	Cumulative %
1	1.144	7.153	30.247	1.144	7.153	30.247	1.133	7.081	29.092
2	1.122	7.010	37.257	1.122	7.010	37.257	1.125	7.034	36.126
3	1.019	6.370	57.022	1.019	6.370	57.022	1.098	6.861	57.022
..	....	...	...						
36	.741	4.630	100.000						

Table 6. Component weights and dimensions

Health care quality Dimensions	Factor		
	1	2	3
Access 1	0.96		
Access 2	0.947		
Access 3	0.919		
Access 4	0.762		
Recovery 1	0.657		
Recovery 2	0.615		
Recovery 3	0.521		
Conformance 1		0.944	
Conformance 2		0.874	
Conformance 3		0.777	
Facility 1		0.654	
Facility 2		0.566	
Reliability 1			0.852
Reliability 3			0.801
Feature 1			0.786
Feature 2			0.698
Responsiveness 1			0.599
Responsiveness 3			0.573

The third principal component included reliability, feature, and responsiveness. This suggested supplemental services to patient needs in a short time. For instance, speed up the medical reports' turnaround time by improving the medical transcription process using an advanced information system. Also, reducing the patients waiting time, which includes the total time required to see a doctor and time needed to obtain prescribed medicines. The proposed meta dimension is "exceptional service and immediate care". Lee and Yoon (2021) highlighted the usefulness of artificial intelligence (AI) based technology applications in hospitals, which help in improving the accuracy of medical diagnosis, creating new value for patients, and increasing the efficiency of operational processes. The health care meta quality dimensions in KSA public hospitals are represented in Table 7.

Table 7. Health care meta quality dimensions in KSA public hospitals

Wellness Support	Compliance with Standards	Exceptional Service and Immediate Care
Access Recovery	Conformance Facility	Reliability Feature Responsiveness

CONCLUSION

The findings of the present study showed that wellness support, compliance with standards, and exceptional service and immediate care are the most critical quality dimensions that influence patients' perceptions in KSA public hospitals, and can be used as the best predictors of overall patients' perceptions. A longitudinal study can be conducted to validate the identified dimensions and to find out changes in patients' perceptions. Other directions of study include comparing patient perceptions of healthcare quality dimensions in different regions of KSA. The main purpose of this study was to identify the main healthcare quality

dimensions in KSA public hospitals based on patients' perceptions. This application provides a platform for the hospitals to discover opportunities for improvement and satisfy patients' needs. To ensure satisfactory performance, public hospitals should provide unique medical services to patients, which are in alignment with the Kingdom's 2030 vision.

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Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Prophylactic Effect of *Cucumis melo* on Chromium Vi-Induced Male Albino Rats

G Malathi and J Vadivelu*

Post Graduate and Research Department of Biochemistry, Sri Akilandeswari Women's College, Wandiwash, Tamil Nadu, India

ABSTRACT

The production of reactive oxygen species (ROS), which induce oxidative stress in humans, is linked to the negative effects of Chromium 6. The protective action of *Cucumis melo* L. fruit extracts was evaluated in this study in an animal model of hematological and biochemical parameters, which was induced by chromium VI ($K_2Cr_2O_7$). The purpose of this study was to analyse the efficacy of *Cucumis melo* L. on chromium VI ($K_2Cr_2O_7$)-induced rats. For 42 days, male albino rats (160–20 g) were given the stated oral LD50 dosage of chromium VI ($K_2Cr_2O_7$) (10 mg/kg body weight). After 42 days, chromium-induced rats were administered with two different concentrations of *Cucumis melo* L. and ascorbic acid (250 mg/kg, 500 mg/kg body weight) for 7 days. Following therapy, blood was drawn and analysed for a variety of biochemical markers. The results revealed that ingestion of either plant extract, ascorbic acid, or their combination on chromium 6 induced rats significantly increase the activity of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and decreased the activity of Gamma Glutamyl transferase (γ-GT) was recorded. This study has proven that fruit extract particularly its combination with ascorbic acid has a potential prophylactic effect. *Cucumis melo* is vital for modifying the Chromium (VI) induced toxicity on male albino rats. Indeed, the recommended fruits should be consumed to the Chromium (VI) deposited harmful region since they may protect cells from environmental stress.

KEY WORDS: BIOCHEMICAL CHANGES, CUCUMIS MELO. L, KIDNEY, LIVER AND POTASSIUM DICHROMATE.

INTRODUCTION

Environmental toxins such as chromium, which is made up of chromium (0), chromium (III), and chromium (IV), hurt human health (VI). According to the Environmental Protection Agency's (EPA) list of the eight most frequent pollutant heavy metals, chromium is one of them (EPA) (Eman and Farag 2020). Chromium, a transition metal element, is found in abundance in the earth's crust, including rocks, volcanic dust, gases, and soils, in addition to plants and animals. The toxicity of chromium 6 is greater than that of chromium 3. The human body requires trivalent chromium to increase the action of insulin in bodily tissues for sugar, protein, and fat consumption. Furthermore, chromium may enhance cascade ranges in pig and broiler chicks. Hexavalent chromium is typically produced by industrial operations (Genchi et al. 2021).

Hexavalent chromium compound potassium dichromate is used in tanning, dyeing, pharmaceuticals, photography, alloys, dry battery production, stainless steel manufacturing, electroplating, and wood preservation. Several papers on pharmacotherapeutics, genotoxicity, and its deleterious implications are available (Czarnek et al. 2021). Few studies have attempted to relate potassium dichromate at various dose levels to hematological and biochemical alterations in rats using an adequate animal model (Ezekaibeya et al. 2020). According to clinical evidence, Cr (VI) exposure can cause serious kidney damage (Wu et al. 2020).

The major cause of cellular malfunction and death is the formation of free radicals as a result of a decline in Cr 6 levels within the cell. (Wu et al. 2020). Plants have an important part in the treatment of illnesses, as seen by their widespread use in all major medical systems. Several medicinal plants may have health-promoting pharmacological effects (Luo et al. 2020). Long-term use of natural remedies without evidence of harm to health might indicate that a chemical is safe (Silva et al. 2020). In the field of medicine, many plants are yet to be investigated. One of these plants is *Cucumis melo* L. It is a Cucurbitaceae family annual climber whose fruit is mostly used as a vegetable. This plant can be found in rural and coastal areas. Researchers found that both *Cucumis melo* and *P. dactylifera* plant extracts, as well as

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their combination, exhibit potential hepatoprotective effects in streptozotocin-induced rats (Manchali et al. 2021).

Because of its excellent nutritional content, the fruit is typically consumed as a vegetable (Ganji et al. 2020). This fruit was high in carbohydrates, protein, fats, and vitamins. The aqueous extract of ripe fruit contained water-soluble vitamins such as ascorbic acid, phenylalanine, glutamine, and asparagine (Leon et al. 2021). The goal of the study is to see how prolonged low-dose chromium exposure affects several hematological and biochemical markers in male albino rats. *Cucumis melo* L. fruit was also analyzed, and all essential components were confirmed to be present in adequate amounts for active prophylactic efficacy.

MATERIAL AND METHODS

Cucumis melo L. fruits were harvested in Vandavasi, Thiruvannamalai, Tamil Nadu, India, for extract extraction. From December to January of 2019-2020, all plant materials were gathered.

Siddha Central Research Institute, Chennai (Central Council for Research in Ayurveda and Siddha, New Delhi, Ministry of Health & Family Welfare, Government of India), Reference No: C14022001M, verified the fruits. Extraction took place at room temperature under normal circumstances. Aqueous was used to extract around 5g of shade-dried powder from *Cucumis melo* L. fruits, which was then maintained in a boiling water bath for 30 minutes. The extract was filtered before being concentrated in a water bath at 100°C. For drugs and chemicals, Chromium 6 was purchased from scientific Lab Chemicals, Chennai, and was used to make the suspension in a dose of 10 mg/kg body weight for the respective groups. All other chemicals were purchased from Sisco Research Laboratory, Chennai.

Male albino rats weighing 160 to 180 g were procured from the Biomedical Research Unit and Laboratory Animal Centre, BRILAC/SDCH/SIMATS/IAEC/3-2020/049 Chennai, and were cared for accordance to CPCSEA norms under the supervision of the Animal Ethical Committee. All animals were kept in regular circumstances (25 1 °C, 12 h light/12 h dark cycle) with free access to food and water for 7 days prior to the experiment. According to Giang et al. 2021, the rats are evenly allocated into four treatment groups, each with six rats.

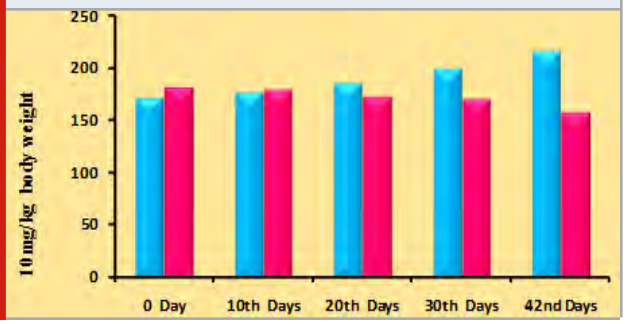
For 50 days, the animals in various groups were given the following treatments orally by gavages: GROUP I: The untreated rat (Normal Control). GROUP II: K_2 Cr_2 O_7 induction (10 mg/kg body weight) was given for 42 days. GROUP III: Treatment with Ascorbic acid (500 mg/kg body weight) for 7 days after induction of K_2 Cr_2 O_7 (10 mg/kg body weight) for 42 days. GROUP IV: K_2 Cr_2 O_7 was induced (10 mg/kg body weight) for 42 days and then treated for 7 days with Cucumis melo L. Aqueous Extract (500 mg/kg body weight). GROUP V: K_2 Cr_2 O_7 was induced (10 mg/kg body weight) for 42 days and then treated for 7 days with Ascorbic acid (500 mg/kg body weight) and *Cucumis melo* L. aqueous extract (500 mg/kg body weight). Animals were slaughtered by cervical dislocation after anaesthesia at the end of the treatment period. The Indian Council of Medical Research rules were followed to the letter during the sacrifice. Blood and tissue were taken and utilised for biochemical assessments after the liver and kidney were removed and thoroughly cleansed in normal saline.

Freshly removed Liver and Kidney were rinsed in cold saline (0.9 percent NaCl), blotted dry, put into a cold beaker, and chopped thoroughly with scissors for tissue homogenates. Then a 0.25M sucrose solution (8 ml per gramme wet weight) was added. The homogenate was then filtered and centrifuged for 10 minutes at 5000 rpm. As previously, the particle was homogenised and resuspended. The supernatants were mixed and centrifuged for another 20 minutes at 15,000 rpm (Fatima et al. 2021). According to Chang et al. 2021, the activities of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Gamma Glutamyl transferase (-GT) were measured. The levels of urea and creatinine were analyzed using tissue homogenates. Serum creatinine and Serum urea was measured by Berthelot's method were determined by spectrophotometric methods as described by (Rahman et al. 2021). The statistical software programme version (SPSS) 7.0 was used to examine the data. The significance of differences between male albino rats was determined using the student's t-test. All results were reported as mean SD for a total of n=6 people. At $p<0.05$, $p<0.01$, and $p<0.001$, differences were declared significant.

Table 1. Changes in body weight of rats treated with Potassium Dichromate (PDC/K2Cr2O7)

Days	Control	K2Cr2O7 (10 mg/kg)
0th Days	170 ± 6.02	180 ± 6.38
10th Days	176 ±6.23	178 ± 6.30
20th Days	184 ± 6.51	172 ± 6.08
30th Days	198 ± 7.01	169 ± 5.98
42nd Days	215 ± 7.61	157 ± 5.56

Figure 1: Distribution of body weight rats treated with Potassium Dichromate



RESULTS AND DISCUSSION

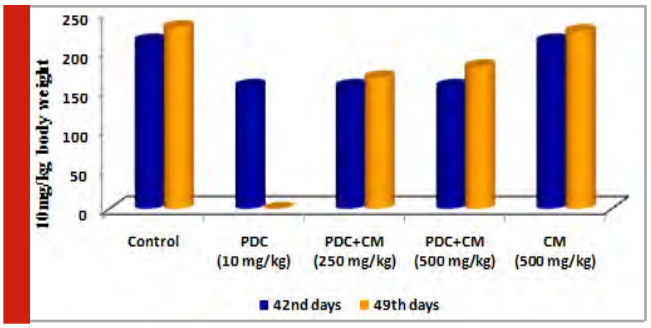
Changes in body weight of rats due to PDC on the 0th, 10th, 20th, 30th, and 42nd days were recorded and are shown in table 1 and figure 1. It may be noted that initially there was

a gradual increase in body weight in the control animals starting from 0 to 42 days, whereas the administration of PDC on 10th, 20th,30th, and 42nd day a significant reduction in body weight was observed from 20th days when compared with the control and further it may be noted that on 42nd day the decrease was much more which may be due to PDC toxicity.

Table 2. and Figure 2: Changes in body weight of rats treated with PDC and Cucumis melo L. (CM)

Days	Control	PDC (10 mg/kg)	PDC+CM (250 mg/kg)	PDC+CM (500 mg/kg)	CM (500 mg/kg)
42nd days	215 ± 7.61	157 ± 5.56$	157 ± 5.56$	157 ± 5.56$	215 ± 7.61NS
49th days	232 ± 7.61	_	168 ± 5.94$	182 ± 6.44$	227 ± 8.03NS

PDC-Potassium DiChromate; CM- Cucumis melo L. (CM)
Values represent mean± SD of six animals - @P<0.05, $P<0.01,
* P<0.001 when compared to 49th days animals.

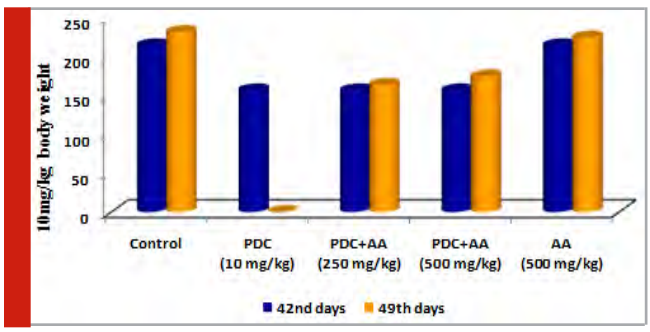


The current study discovered the presence of physiologically active phytocompounds in *Cucumis melo*, tasty oval-shaped fruit with high nutritional content that is widely consumed in many tropical nations. Many secondary metabolites, including as polyphenolics and flavonoids, are found in the fruits, and they have powerful pharmacological properties such as antibacterial, antioxidant, anti-inflammatory, and anticancer properties (Moustafa et al. 2020; Nash et al. 2020). *Cucumis melo* whole fruit extract has the greatest gallic acid and rutin concentration, according to Xudong et al. (2020). In agreement with this fruit, the extract is more combat to the external environmental stresses when compared to the seeds (Cunha et al. 2020). Therefore our present study infers that *Cucumis melo* 500mg of fruits aqueous extract was confirmed to modify the chromium VI induced male albino rat body weight as well as their organ weight when compared to the control animal (Cunha et al. 2020).

Table 3 and Figure 3: Changes in body weight of rats treated with PDC and Ascorbic Acid

Days	Control	PDC (10 mg/kg)	PDC+AA (250 mg/kg)	PDC+AA (500 mg/kg)	AA (500 mg/kg)
42nd days	215 ± 7.61	157 ± 5.56$	157 ± 5.56$	157 ± 5.56$	215 ± 7.61NS
49th days	232 ± 7.61	_	164 ± 5.81$	175 ± 6.19$	225 ± 7.24NS

PDC-Potassium DiChromate; AA- Ascorbic Acid
Values represent mean± SD of six animals - @P<0.05, $P<0.01,
* P<0.001 when compared to 49th days animals.



When compared to control animals, rats on PDC therapy exhibited a drop in body weight as the days of treatment progressed, and the changes in body weight were significant at the P<0.01 level. When the same rats were fed *Cucumis melo* L (250mg/kg body weight and 500mg/kg body weight) for 42 days, the body weight steadily increased and reached near-normal weight with control rats, which was statistically significant at the P<0.01 level. On the 49th day when the animal was treated with *Cucumis melo* L (500mg /kg body weight) the increase in body weight was gradual and this was significant indicating the role of *Cucumis melo* L

which could have helped to bring back the normal weight acting as an antidote to PDC (Table 2 and Fig 2) (Cunha et al. 2020).

Rats on treatment with PDC showed a decrease in body weight as the days of treatment increased and the changes in the body weight were significant at P<0.01 level when compared with the control animals. After the 42nd day when the same rats were fed with ascorbic acid (250mg/kg body weight and 500mg/kg body weight), the body weight was found to increase gradually and reached near-normal weight with control rats, and the increase was statistically significant at P<0.01 level. On the 49th day when the animal was treated with ascorbic acid (500mg /kg body weight), the increase in body weight was gradual and this was significant indicating the role of ascorbic acid which could have helped to bring back the normal weight acting as an antidote to PDC (Table 3 and Fig 3) (Wu et al. 2021).

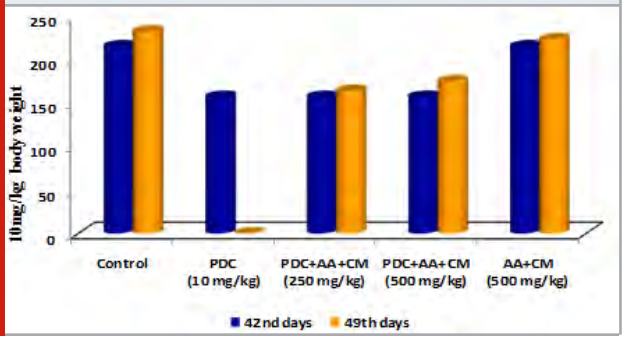
Rats after treatment with PDC for 42 days when fed with *Cucumis melo* L and Ascorbic acid (250 and 500 mg/kg body weight) for 7 days and (49th day) the result on 49th day showed that the bodyweight of rats showed a slight increase in 250 and 500 mg/kg body weight (Table 4 and Fig 4) when compared with the control animals [6.05± 0.53 for Liver and 1.75± 0.16 for kidney]. The increase was statistically significant at P<0.01 level respectively. The combined role of *Cucumis melo* L and Ascorbic acid for 500 mg/kg body weight dose was found to be more effective in bringing back the bodyweight of rats to normal than the 250 mg/kg body weight dose (Wu et al. 2021).

Table 4. Changes in body weight of rats treated with PDC and Ascorbic Acid (AA) along with *Cucumis melo* L

Days	Control	PDC (10 mg/kg)	PDC+AA+CM (250 mg/kg)	PDC+AA+CM (500 mg/kg)	AA+CM (500 mg/kg)
42nd days	215 ± 7.61	157 ± 5.56$	157 ± 5.56$	157 ± 5.56$	215 ± 7.61NS
49th days	232 ± 7.61	_	164 ± 5.81$	175 ± 6.19$	223 ± 7.24NS

PDC-Potassium DiChromate; CM- *Cucumis melo* L. (CM); AA-Ascorbic Acid
Values represent mean± SD of six animals - P<0.05, P<0.01, P<0.001 when compared to 49th days animals.

Figure 4: Body weight changes



Chromium (VI) is a toxic metal that is widely utilized in industry. It has harmful effects on the liver, kidneys, and other organs. (A.J. Machado et al., 2021) As a result, several studies show that chromium (VI) causes considerable oxidative stress in both the liver and the kidney, although the kidney appears to be more prone and sensitive to Chromium (VI) poisoning than the liver. Because of its role in xenobiotic metabolism, the liver is particularly vulnerable to damage. Potassium dichromate's toxicity was demonstrated at the cellular level (Wu et al. 2021). After 24 and 48 hours of treatment, cells exposed to 10 M potassium dichromate (K2Cr2O7), a dangerous Cr (VI) compound, had significantly lower viability and increased intracellular ROS generation. 2021 (Wu and colleagues) In K2Cr2O7-exposed HK2 cells, the expression levels of indicators that initiate the apoptotic cascade, such as cleaved caspase 3 and poly (ADP-ribose) polymerase, were considerably elevated. HK2 cells treated to K2Cr2O7 also demonstrated activation of intrinsic and extrinsic apoptotic markers. (Wu et al. 2021).

The effect of PDC after 42 days followed by 7 days of treatment with *Cucumis melo* L on two different organ weights of rats' viz., liver and kidney are represented in table 5. When compared to the control, there is a substantial rise in liver weight and a reduction in kidney weight, both of which are significant at the P0.01 level. There was a decrease in the weight of the liver and an increase in the weight of the kidney in rats given *Cucumis melo* L (250 and 500 mg/kg body weight). When *Cucumis melo* L was given individually (500 mg/kg body weight), the findings were comparable, suggesting a change that was statistically significant at the P0.05 level. On feeding the rats with Ascorbic acid (250 and 500 mg/kg body weight) for 7 days the rats showed a significant decrease in the weight of liver and increase in the kidney and the changes were so observed significant at P<0.05 when compared with the PDC toxicity alone (Pinero et al. 2021).

Whereas animal fed with ascorbic acid (500 mg/kg body weight) above showed results which were near equivalent to control, indicating that ascorbic acid would have detoxifies the effect of PDC. Treatment with *Cucumis melo* L and ascorbic acid to rats already treated rats with PDC showed reduced liver weight and the weight increased in the kidney weight and these changes were significant at P<0.05 level

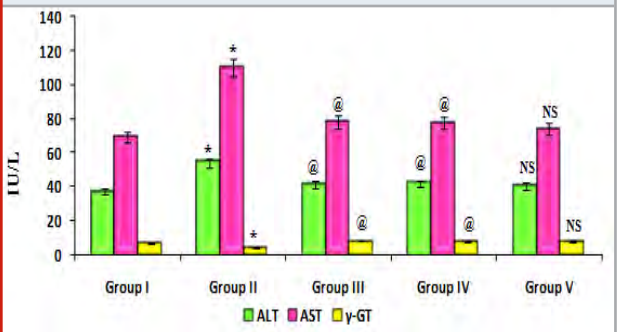
when compared with the PDC toxicity rats. Treatment with 500 mg/kg body weight of *Cucumis melo* L, ascorbic acid and its combination was more significant than the 250 mg/kg of body weight when compared with the PDC toxicity rats, were 500 mg/kg body weight of *Cucumis melo* L and ascorbic acid combined dose was able to detoxify the effect of PDC (Pinero et al. 2021).

Table 5. Changes in organ weight of rats treated with PDC, *Cucumis melo* L, and ascorbic acid

	PDC+CM			PDC+AA			PDC+CM+AA	
	Liver	Kidney		Liver	Kidney		Liver	Kidney
PDC (10 mg/kg)	7.12±0.41$	0.83±0.06$	PDC (10 mg/kg)	7.12±0.41$	0.83±0.06$	PDC (10 mg/kg)	7.12±0.41$	0.83±0.06$
PDC+CM (250 mg/kg)	6.63± 0.34@	1.35± 0.13@	PDC+AA (250 mg/kg)	6.78±0.38@	1.28± 0.12@	PDC+AA+CM (250 mg/kg)	6.68±0.46@	1.32±0.11@
PDC+CM (500 mg/kg)	6.01±0.36@	1.35± 0.13@	PDC+AA (500 mg/kg)	6.13±0.36NS	1.71± 0.14NS	PDC+AA+CM (500 mg/kg)	6.07±0.44NS	1.74±0.18NS
CM (500 mg/kg)	6.14±0.41NS	1.35± 0.13@	AA (500 mg/kg)	6.11±0.41NS	1.75± 0.15NS	AA+CM (500 mg/kg)	6.12±0.4NS	1.76±0.12NS

PDC-Potassium DiChromate; CM- *Cucumis melo* L. (CM); AA-Ascorbic Acid
Values represent mean± SD of six animals
P<0.05, P<0.01, P<0.001 when compared to 49th days animals

Figure 5: Quantify the Liver marker enzymes such as ALT, AST, and γ-GT on rats treated with PDC, *Cucumis melo* L, and ascorbic acid



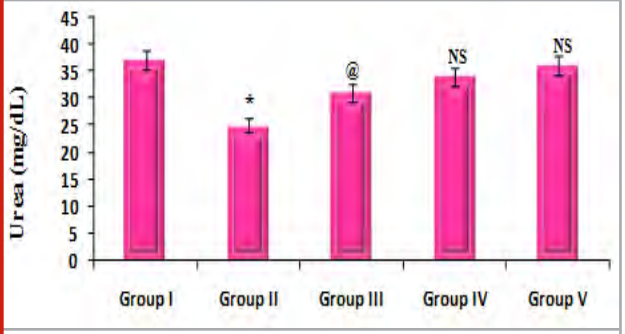
Group I: Control; Group II: K2 Cr2 O7/PDC; Group III: K2 Cr2 O7/PDC+ Ascorbic acid; Group VI: K2 Cr2 O7/PDC + *Cucumis melo* L; Group V: K2 Cr2 O7/PDC+ Ascorbic acid + *Cucumis melo* L
Values represent mean± SD of six animals
@P<0.05, $P<0.01, * P<0.001 when compared to 49th days animals

Figure 5 depicts the effect of PDC toxicity and *Cucumis melo* L therapy on liver marker enzymes in rats. When compared to control rats, PDC-treated rats showed a substantial drop in -GT, which was statistically significant at the P0.001 level, however there was a significant rise in the levels of ALT and AST, which was statistically significant at the P0.001 level. When compared to the PDC treated animals, those given *Cucumis melo* L (500 mg/kg) and Ascorbic acid (500 mg/kg) or a combination of *Cucumis melo* L (500 mg/kg) and Ascorbic acid (500 mg/kg) showed an increase in -GT that was statistically significant at P0.05, P0.05, and P-Non significant levels, as well as a significant decrease in the levels of ALT and AST. *Cucumis melo* L has an antidote effect on PDC toxicity, however it is not considerable. According to reports by Das et al. (2020), ascorbic acid has the capacity to alter gene expression, apoptosis, and other cellular activities in living systems exposed to heavy metals, such as nickel (Das et al. 2020).

The effect of PDC toxicity and treatment with *Cucumis melo* L on kidney marker enzymes in rats is shown in fig 6. PDC-treated rats showed a decrease in Urea by 32% which was statistically significant at a P<0.001 level when compared with the control animals. These animals on treatment with *Cucumis melo* L (500 mg/kg) and ascorbic acid (500 mg/

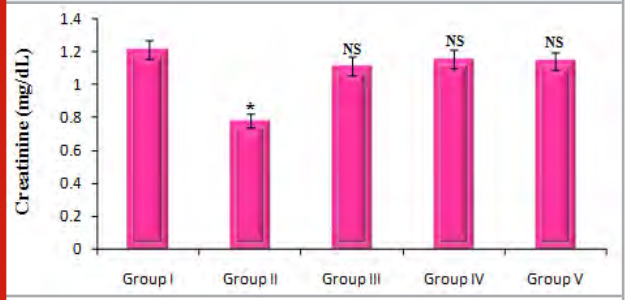
kg) and a combination of *Cucumis melo* L (500 mg/kg), Ascorbic acid (500 mg/kg), showed an increase in Urea by 16%, 8% and 3% which was statistically significant at P<0.05, P-Non significant and P-Non significant level, when compared with the PDC, treated rats, indicating that the antidote effect of *Cucumis melo* L on PDC toxicity (Pinero et al. 2021).

Figure 6: Quantify the Kidney marker enzymes Urea on rats treated with PDC, *Cucumis melo* L, and ascorbic acid



Group I: Control; Group II: $K_2 Cr_2 O_7$/PDC; Group III: $K_2 Cr_2 O_7$/PDC+ Ascorbic acid; Group VI: $K_2 Cr_2 O_7$/PDC + *Cucumis melo* L; Group V: $K_2 Cr_2 O_7$/PDC+ Ascorbic acid + *Cucumis melo* L
Values represent mean± SD of six animals
P<0.05, P<0.01, P<0.001 when compared to 49th days animals

Figure 7: Quantify the Kidney marker enzymes Creatinine on rats treated with PDC, *Cucumis melo* L, and Ascorbic acid



Group I: Control; Group II: $K_2 Cr_2 O_7$/PDC; Group III: $K_2 Cr_2 O_7$/PDC+ Ascorbic acid; Group VI: $K_2 Cr_2 O_7$/PDC + *Cucumis melo* L; Group V: $K_2 Cr_2 O_7$/PDC+ Ascorbic acid + *Cucumis melo* L;
Values represent mean± SD of six animals
P<0.05, P<0.01, P<0.001 when compared to 49th days animals

Hepatocytes must maintain a delicate balance between cellular oxidants and antioxidant defences even when they are at rest (Pinero et al. 2021). Due to the generation of inflammatory cytokines, the activation of numerous signalling pathways, and cellular changes, a breakdown of this equilibrium may lead the cells to enter an inflammatory state, resulting in harm to both the cells involved and the surrounding tissues. Although the activities of AST and ALT are the most widely used and recognised enzyme indicators of liver damage, they only vary in the late stages of many liver illnesses and frequently lack sensitivity in the early stages (Asdaq et al. 2021).

The effect of PDC toxicity and treatment with *Cucumis melo* L on kidney marker enzymes in rats is shown in fig 7. PDC-treated rats showed a decrease in Creatinine by 36% which was statistically significant at a P<0.001 level when compared with the control animals. These animals on treatment with *Cucumis melo* L (500 mg/kg) and ascorbic acid (500 mg/kg) and a combination of *Cucumis melo* L (500 mg/kg), ascorbic acid (500 mg/kg), showed an increase in creatinine by 8%, 5%, and 6% which was statistically significant when compared with the PDC treated rats, indicating that the antidote effect of *Cucumis melo* L on PDC toxicity (Asdaq et al. 2021).

CONCLUSION

The findings of this study suggest that the beneficial effect of *Cucumis melo* L is to alleviate liver and kidney function impairment due to the presence of their phytonutrients, which may render the toxic effect of Chromium (VI) induced liver damage via the assessment of significant increases in AST and ALT levels, which may render the toxic effect of Chromium (VI) induced liver damage via the assessment of significant increases in AST and ALT levels. *Cucumis melo* can also assist with damaged kidney enzymes such as urea and creatinine. In the future, it is hoped that the findings of this study may aid in the development of effective therapies for acute Cr (VI) poisoning.

Conflict of Interests: Authors declare no conflict of interests.

Ethical Clearance: Siddha Central Research Institute, Chennai (Central Council for Research in Ayurveda and Siddha, New Delhi, Ministry of Health & Family Welfare, Government of India), Reference No: C14022001M, verified the fruits.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Aquatic Weeds as an Encouraging Resource of Alternative Feed for the Tilapia *Oreochromis mossambicus*

Mukti Pada Bag
Department of Zoology, Bankura Sammilani College, Bankura, West Bengal, India

ABSTRACT

A series of experiments were carried out to ascertain the nutritional value of commonly available aquatic weed i.e., Lemna (AWL), aquatic hyacinth (AWW) and Azolla (AWA) as alternative feed sources for decreasing the expenditure of feed. Using those aquatic weeds as principal ingredient three (3) isonitrogenous (crude protein-30%) and isocaloric feeds were made. Three batches of juvenile fish of thirty (30) numbers per batch (Average weight-5.1 gm; L- 5.6 cm) were provided with 3different types of prepared feeds with AWL, AWW and AWA. Weight gain, PER (Protein Efficiency Ratio) and GSI (Gonado Somatic Index) are significantly high in the AWL, AWW and AWA fed fish than the NOR. The AWL has suitable amino acid and fat that enhances yield and quality of flesh. The better ω-3/ ω-6ratio is obtained from food supplied experiment trial comparison to control treatment.

KEY WORDS: AQUATIC WEED, FISH FEED, GONADOSOMATIC,ISOCALORIC, ISONITROGENOUS,JUVENILE

INTRODUCTION

In fish farming, fish feed occupies 55-65% farming expenses for fish culture (Singh et al. 2006). This traditional food is utilised for fish farming was costly as well as inadequate to meet the demand. Not only that, in some cases feeds are adulterated and non-hygienic. So, there is an urgent need to develop of hygienic and low-cost fish feed for fish farming. For producing alternative, low cost and fresh fish feed from non-conventional feed ingredients i.e., terrestrial and aquatic macrophytes play an important role for this purpose (Edwards et al. 1985; Wee and Wang 1987; Mondal and Ray 1999; Mmanda et al. 2020).

Aquatic weeds have been utilised as alternative fish foods for aquaculture from very ancient time and now perform a key substitute of fish food for culture. These alternative sources exhibited encouraging amount for amino acids as well as other vital elements as studied in the past (Bardach et al. 1972; Edwards 1987; Ray and Das 1995; Debnath et al. 2018). However,these elements are not evaluated in respect of lipid profile earlier. From fish feed which is consumed by fish, the beneficial fatty acids are synthesized (Horrobin and Manku 1990; Mulokozi et al. 2020). The advantageous influence of fatty acid from fish particularly upon heart patients has by now been well recognized. So, great effort was given for establishing any relation between fish feed and fatty acid deposition as well as yield (Cengiz et al 2003; Mukhopadyay 2009). Mossambique tilapia (*O.mossambicus*), the cultured fish was selected for its tolerance to unfavourable environment and diseases, (Suresh 2003; Mulokozi et al. 2020). The main aim for these trials are to establish the effect of such non-conventional as well as alternative low-cost feed ingredient on yield and fatty acid deposition of the fishes.

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MATERIAL AND METHODS

All the experiments were conducted in tanks (1200 l) of aquaculture Engineering section of Indian Institute of Technology, Kharagpur, India. In total 12 cemented tanks were used. In each tank, 25 fingerlings of tilapia were cultured. Initial weights and lengths were recorded for growth calculation. Three prepared (AWL, AWW, AWA) and one control feed (NOR) were supplied to the fish. Each batch of fish was cultured with one type of feed. The nitrogen content and energy value of all feeds were similar. Except normal feed (NOR) in each formulated feed, one type of non-conventional source (aquatic weed) was used for protein sources. Besides aquatic weed which was the main ingredient of each feed, MOC, RB (rice bran) and coarse flour were used for preparing feed for tilapia.

By using different ingredients with proper ratio feeds were made as a pellets form (AOAC 1990). Then pellets were dried under sunlight or in hot air oven. After drying, feeds were kept in polythene bags for preventing moisture. Using two calculation formulae different biochemical compositions of feed were recorded (Maynard et al. 1979; Giri et al. 2000). By adopting formula of Chakraborty et al. (2010), growth

performance and feed consumption are calculated (table 2). Lipids from all samples were collected (Bligh and Dyer 1959). Then MUFA was prepared (Christie 1982). Next FAME was made (Mangold 1969; Dahlgren 1979; Misra et al. 1984). ANOVA was run to determine the effect of food. GSI and HSI of the experimental fishes were measured by removing gonads and liver from the body respectively.

RESULTS AND DISCUSSION

The maximum growth in weight of (93.44g) was recorded from AWL supplied foods. The growth of other tanks was recorded as follows AWW (74.55g) AWA (70.11g) and NOR (58.32g) (Table 2). The maximum (13.8cm) and minimum (10.8cm) length of fish was observed from NOR and AWA supplied trial respectively. The food consumption value of different trials was calculated. As desire maximum (3.19gm) food intake was observed from AWL and lowest (2.01gm) from NOR. The total amount of feed intake (in g) in three treatments is not differed significantly. So, feed acceptability is almost similar in all treatments but it is significantly less in NOR treatment. As feed acceptability is low in NOR so feed conversion ratio (FCR) is highest in control experiment which is not desirable. The lowest (0.52) and highest (0.98) specific growth rate were observed in NOR and AWL fed treatment respectively. The protein efficiency ratio (PER) was significantly differed ($P<0.05$) from different treatments. The Value of PER (highest-2.56 in AWL and lowest-0.75 in NOR) was recorded during the experiment. The obtained value of HSI (highest-2.81 in AWL and lowest 2.41 in NOR) presented in table 3. The recorded GSI value shows same tendency as HSI values.

Table 1. Chemical ingredient of three aquatic weeds (lemna, water hyacinth and azolla)

Composition	AWL	AWW	AWA
DM	91.61	91.42	91.31
Organic matter	82.82	81.58	82.65
Crude protein	25.14	20.17	20.36
Crude lipid	8.07	6.95	8.80
Ash	8.79	9.84	8.66
Nitrogen free extract	41.23	44.55	44.07
Fibre	8.38	9.91	9.42
Calorific value	4.76	4.69	4.65

For description of fatty acid profile minimum 14 fatty acids (FA) are required, yet 15 FAs recorded (Ackman 2000). At running trail, 26 FAs were recorded from different samples (carcass and food). Outcome was identical to the findings of temperate and tropical aquaculture (Zenebe et al. 1998; Ackman et al. 2002). Regarding fatty acids, experimental fish showed variation with IMC. The fatty acid profile of present fish had a great relation with other findings (Andrade et al.1995; Zenebe et al.1998; Mmanda et al. 2020; Bag 2021). Particularly 20:3n-9 FA indicated the FA deficiency by its presence (Watanabe 1982; Bag 2021).

The fatty acid (saturated) was estimated greater in amount when compared to MUFA, DUFA and PUFA. In AWA fed fish, the saturated fatty acid (SFA) content (%) was maximum (55.2) and lowest value (47.4) was recorded from NOR fish. As far as MUFA (Mono Unsaturated Fatty Acid) content (%) was concerned the highest value (26.9) also obtained from AWW fed fish. In case of PUFA (Poly Unsaturated Fatty Acid) content highest value was observed in NOR (15.06) followed by AWA (14.8), AWW (13.43) and AWL (14.33). The ω3fatty acid which was vital for human health, recorded highest (13.8) in AWL fed fish. On the contrary ω6 fatty acid was measured highest (9.5) in NOR. The ω3/ ω6 proportion was maximum (1.94) from AWL fed treatment succeeded by AWA (1.54), AWW (1.42) and NOR (1.20).

During trials, main parameters of water for the experimental tanks were in tolerable range for tilapia (Ballarin and Hatton 1979). The fish fed with AWL feed obtained highest weight gain. This refers the AWL feed accepted by cultured fish was fine. For this reason, highest feed acceptability was observed in AWL fed treatment. The low FCR value was desired for any fish culture. Low FCR indicates low loss of fish feed which reduced the cost of feeding. It can also make fish farming more profitable. Another important parameter i.e., PER which was appreciably superior in AWL feed treatment as compared to the others which implied that better and balanced quality of protein must be available in AWL feed where Lemma was the key ingredient (Bag 2021).

Table 2. Yield of tilapia using experimental feeds (After Duncan 1955).

	NOR	AWL	AWW	AWA
I W	6.12±.12	6.10±0.11	6.12±0.12	6.25±0.11
FW	58.32±0.62	93.44±0.72	74.55±0.12	70.11±0.58
I L	5.6±0.15	5.7±0.21	5.4±0.20	5.7±0.23
F L	10.8±0.22	13.8±0.20	11.5±0.20	10.8±.20
F I	2.01±0.30	3.19±0.30	3.10±0.30	3.08±0.30
S G R (%day-1)	0.52±0.06	0.98±0.18	0.77±0.19	0.72±.18
F C R	3.31±0.19	3.26±0.27	3.73±0.13	3.88±0.11
P E R	0.75±0.05	2.56±0.06	1.80±.07	1.77±0.07
H S I	2.41±0.05	2.81±0.05	2.62±0.06	2.60±.05
G S I	.85±.03	2.70±0.05	2.08±0.07	2.05±0.06

In our experimental fish the above-mentioned FA (fatty acid) was absent. This finding reflected that those fish were not lack of fatty acid. For other fish like Bass in America, the same observation was reported (Gatlin and Nematipour 1993; Bag 2021).When ω3/ω6 ratio was low then it enhanced the thrombogenicity. Linolenic and linoleic acid require almost similar type of catalyst (Li et al. 1999). The ω3PUFA was comparatively beneficial than ω6 fatty acids. ω3 fatty acids prevent thrombogenesis whereas ω6 fatty acids prevent atherogenesis (Christensen et al. 2001; Dewailly et al. 2001; Bag 2021). The ω3/ω6fatty acid ratio of experimental creatures placed the fish in Type II lean

fish category. In the present trial, fish fed with AWL feed shows the tendency of increasing ω3 FAs. This increasing tendency of ω3FAs enhanced value of ω3/ω6 ratio which provides health benefits to human particularly heart patients (Bag 2021).

Table 3. Fatty acid profile of NOR, AWL, AWW and AWA fed fish

Composition	NOR	AWL	AWW	AWA
S F A				
13:0	4.1	2.7	4.3	3.7
14:0	2.0	1.2	2.0	1.5
16:0	31.0	28.9	28.9	30.6
17:0	0.98	2.1	2.8	2.3
18:0	7.9	9.0	8.9	8.4
20:0	0.5	0.3	00	0.5
22:0	0.3	8.1	7.8	6.8
24:0	0.7	1.1	1.6	0.5
Σ SFA	47.4	54.4	54.4	55.2
Monoene				
14:1	0.8	0.5	0.7	0.5
15:1	0.2	0.1	0.3	0.4
16:1	7.6	7.8	7.9	7.9
17:1	0.4	0.9	1.0	1.1
18:1ω9	12.7	12.1	12.6	14.0
20:1ω9	1.2	1.3	1.6	0.8
22:1ω11	1.4	0.6	0.7	0.7
24:1	1.6	2.1	2.1	1.4
Tot.MUFA	25.9	25.4	26.9	26.8
Dien				
16:02	1.1	00	1.3	1.4
18:2n6	5.9	4.8	4.5	4.7
20:02	00	00	00	2.3
Tot. DUFA	7.0	4.8	6.1	5.3
Poly				
18:3n6	1.2	1.4	1.1	1.3
18:3n3	2.0	3.0	3.4	2.9
20:3ω6	1.2	1.1	0.8	1.1
20:3ω3	0.06	0.03	0.03	0.1
20:4ω6	0.98	0.9	0.5	1.8
20:5ω3	1.7	1.9	1.4	1.1
21:5ω3	0.6	0.1	0.1	1.3
22:5ω6	0.3	0.1	0.3	1.1
22:5ω3	2.7	2.0	1.9	2.3
22:6n3	5.5	4.8	4.9	4.9
Σ PUFA	15.06	15.40	13.43	14.8
Total ω3	11.22	13.80	11.62	14.6
Total ω6	9.5	7.1	7.4	7.5
ω3/ω6	1.20	1.94	1.43	1.54

CONCLUSION

The findings of the present study has shown that generally aquatic weeds create different severe problems in water bodies. By using these non-conventional sources, the rapid growth of those unwanted weeds must be controlled to some extent. It created local employment generation and reduced the cost of fish feed. The feed prepared from aquatic weeds particularly Lemna (*Lemna minor*) enhanced yield as well as production.Feeds prepared by using aquatic weeds by magnifying ω-3PUFA in carcass get better value of fish

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Factors Affecting Satisfaction Among Diabetic Patients Seeking Orthodontic Treatment in Saudi Arabia

Hana O AlBalbeesi,[1] Sahar M BinHuraib,[1] Eman I Alshayea,[2] Afnan M AlAssiri[3] and Abdallah B Abu-Amara[3]

[1]Dental University Hospital, King Saud University, Riyadh, Saudi Arabia.

[2]Department of Pediatric Dentistry and Orthodontics, College of Dentistry, King Saud University, Riyadh, Saudi Arabia.

[3]King Saud University, Riyadh, Saudi Arabia.

ABSTRACT

The objectives of this study were to assess diabetic patients' satisfaction when seeking orthodontic treatment, and to identify the factors and causes that may influence or prohibit their orthodontic treatment. A total of 206 diabetic participants were randomly selected for a cross-sectional study by allocating a satisfaction closed-ended questionnaire. All young and adult patients with three different types of diabetes mellitus, seeking orthodontic care, were included in the study. Comparison between three groups of diabetic patients were performed using chi square statistical analytical method. A significant association was found between diabetes mellitus type and patient satisfaction with access to care provided to them ($p<0.0001$). In addition, there was a significant association between diabetes mellitus type and the satisfaction level during or after treatment. Generally, the level of satisfaction among Orthodontic diabetic patients in all studied categories was medium, with lower satisfaction level among older age type II diabetic patients than younger age type I diabetic patients. Orthodontists should be aware of the importance of diabetes in relation to the patients' susceptibility to periodontitis, especially if uncontrolled. Periodontal health and proper oral hygiene should be strictly observed during treatment.

KEY WORDS: DIABETES MELLITUS, ORTHODONTIC TREATMENT, PATIENT SATISFACTION, TYPES.

INTRODUCTION

The need for orthodontic care, as one of the most important specialties in current dental practice, centers around fixing malocclusions that have a great social and psychological impacts on the quality of life for both healthy and compromised young people (Littlewood and Mitchell 2019; Baidas et al. 2020). However, as medical science continues to make advances in helping to improve the quality of life for patients with chronic diseases, dentists are seeing an increasing number of medically compromised patients seeking orthodontic treatment (OT) for some types of malocclusions. Thus, it is crucial that orthodontists increase their awareness of basic working knowledge of these diseases with all possible clinical implications on the course of the treatment. This also requires orthodontists to be in close contacts with the physicians who should be regularly informed about the type of planned dental procedures ahead of time (Rizvi et al. 2014; Ahmad et al. 2015). The growing concern of dentists in attracting new patients and keeping them satisfied with the treatment is the main cause of the emerging and increasing trend to study and identify patient's perceptions towards different dental specialties in general and orthodontic care in particular (Farishta 2015). More importantly in this regard is the medically compromised patients such as the diabetic patients, as diabetes mellitus (DM) is rapidly becoming one of the main health issues in the 21st century (Baidas et al. 2020).

The number of diabetic patients in the Kingdom of Saudi Arabia is increasing rapidly, 18.3% out of the 34.8 million population, according to International Diabetes Federation (IDF), which is around 7 million affected individuals. This number ranks Saudi Arabia as the seventh country globally and the second- highest among Middle Eastern countries (Bergman and Newman 1987). According to the Saudi Ministry of Health (MOH), the number of reported cases of DM in the hospitals and medical centers in (2016) was 485,754, and this number has been increasing to 730,775 in 2018. This is very high increase in just two years (Dawish et al. 2016; Alotaibi 2020). IDF expects that by year 2045, 51% or about 700 million individuals of the whole world

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population will develop diabetic conditions (Alotaibi 2020; Tran et al. 2020).

Diabetes mellitus has four major classifications: Type I resulting from destruction of beta-cells in the islets of Langerhans of the pancreas which occurs early in life, type II DM due to insulin resistance, and III Gestational diabetes mellitus (GDM) arise during pregnancy, and type IV caused by pancreatic disease, hormonal disorders and drugs. It is a chronic disease characterized by an impaired production or utilization of insulin, leading to high amounts of blood glucose causing the blood vessels, nerves, and body organs destruction (Muhamad et al. 2015). The five classic complications of diabetes are microangiopathy, neuropathy, nephropathy, macrovascular diseases, and wound-healing delay. The World Health Organization (WHO) added periodontal disease as a sixth classic complication in 1993 (Loe 1993). Due to an impaired immune system and Xerostomia, individuals with DM have a higher incidence rate of dental caries, periodontal disease, acetone smell, burning mouth syndrome, candida, and oral infections (Nirmala and Saikrishna 2016; Najeeb et al. 2017). The increased risk of periodontitis in diabetic patients is associated with multiple factors, including the patient's age, the duration of the diabetes, the presence or absence of metabolic controls, and the level of bacterial plaque (Rizvi et al. 2014; Muhamad et al. 2015).

Table 1. Demographic data and medical information of the participants.

Variable		N	%
Age group	< 10	2	1.0
	10 - 14	10	4.9
	16 - 20	64	31.1
	21 - 25	36	17.5
	26 - 30	28	13.6
	31 - 35	12	5.8
	36 - 40	11	5.3
	> 40	43	20.9
Gender	Male	80	38.8
	Female	126	61.2
Nationality	Saudi	189	91.7
	Non Saudi	17	8.3
Marital status	Single	117	57.6
	Married	80	39.4
	Divorced	4	2.0
	Separated	2	1.0
Is your diabetes controlled?	Yes	99	48.1
	No	107	51.9
Diabetes type	Type I	109	52.9
	Type II	77	37.4
	Gestational	20	9.7
Medication Type	Pills	52	25.2
	Insulin injection	128	62.1
	Dietary program	25	12.1
	Pills + Insulin injection	1	.5
Other accompanied medical problems	Yes	59	29.8
	No	139	70.2
variable		Median	Quartile
		(25,75)	
How long did you have diabetes?	7	(4,13)	

The emerging trend in the field is to attempt to identify patient's perceptions towards different dental specialty treatment in general and OT in particular. Medically compromised patients, specifically diabetic patients, have been gaining more attention recently, since their numbers are tremendously increasing among younger generations globally, and especially in Saudi Arabia (Alotaibi et al. 2016; Dawish et al. 2016). The aim of this study is to assess the satisfaction levels of diabetic patients' seeking OT and to identify the factors and causes that may influence or prohibit their treatment. To the authors' knowledge, there is no previously performed study with this aim in Saudi Arabia.

MATERIAL AND METHODS

A total of 206 diabetic patients were randomly selected for a cross-sectional study. The participants in this study included young and adults' diabetic patients with three different diabetes types (I, II, III) who were seeking orthodontic care during 2021 (Muhamad et al. 2015). The data was collected by distributing a close ended questionnaire to all participants in person or through online google site. The participants completed a survey consisted of three parts. The first was the demographic and medical history information, the second consisted of questions about orthodontic treatment data, and the third contained questions intended to assess the satisfaction level of OT. The study was approved by the Institutional Review Board (IRB), King Saud University {E-15-1657}, and the objectives were thoroughly explained to all participants and an informed consent form was obtained. Data was analyzed using Statistical Package for the Social Science (SPSS), software version 21. For data analysis descriptive statistics was applied for all variables. Analytic statistics was applied in the form of chi square to compare between the three groups of diabetic patients (type I, type II, and GDM). Statistical significance was considered at P –value < 0.05 and Confidence interval of (95%).

RESULTS AND DISCUSSION

A total of 206 diabetic patients participated in this study consisting of 126 females and 80 males, with a response rate of 80%. Demographic characteristics of the participants were presented in Table 1. The majority of the participants were in the age group of 16-20 years (31.1%). More than half of the participants were females, and the majority were Saudi (91.7%). More than half of the participants stated that their sugar level is under control (51.9%). Moreover, the higher percentage was type I (52.9%), followed by type II (37.4%), while the remaining (9.7%) were gestational type III (GDM). The majority of the participants (62.1%) receive insulin injections as the source of medication to control their glucose level. Less than third of the participants reported other accompanied medical problems (29.8%). The median score of the duration of being diabetic were 7 (Table 1).

The results presented in Table 2 revealed a significant association between DM type and orthodontic data, where the rate of fixed appliance was higher among type I (90.0%), while the rate of removable appliance type was higher among type II (82.4%) (p=0.020). Two thirds of the participants (66.5%) stated that their oral health was good, 21.4% stated poor oral health, while only 12.1% of the participants stated an excellent oral health. The evaluation of oral health showed that it was excellent among type III (60%), followed by type II (54%), and the worst oral health was among type I (93.2%) (p<0.0001). In regard to previous OT, more than one third (39.8%) of the participants reported having had previous treatment with the majority of the responses were from type I (45.1%, p=0.001). On the other hand, 27.2% of the participants reported that they are currently undergoing OT, with the majority were from type II (57.1%, p<0.0001).

In addition, more than two third of the participants were planning to receive orthodontic treatment in the near future (71.4%), with the majority were from type II DM (51%, p<0.0001). Concerning the main reasons behind the lack of previous orthodontic treatment among type I DM patients, the results showed the following responses that are statistically significant: bad experience from previous treatment (93.3%, p=0.002), appearance satisfaction (84%, p=0.003), associated medical conditions (78.9%, p=0.014), financial problems (77.4%, p=0.0001), fear of treatment (70.6%, p=0.039). On the other hand, "not convinced to receive OT" was the most significant response among type II DM (83.3%, p<0.0001). When the responses of all 3 types of DM were assessed in regard to the main reasons for not having orthodontic treatment, the highest percentage of answers referred to the high expenses of treatment (25.7%, p<0.0001), followed by the patient's satisfaction with appearance (12.1%, p=0.003), and lastly associated medical problems were cited as reasons for not having previous OT (9.2%, p=0.014) (Table 2).

The main reasons for seeking orthodontic treatment among type I DM participants, with significant association, were Protrusion of upper and lower teeth (p=0.0001), Gummy smile (p=0.001), Crowded teeth (p=0.002), and to be socially accepted (p=0.013). Even though other reasons were mentioned among the groups, they were not of significance such as spaces between teeth, speech problems, TMJ problems, open bite, deep bite, biting and chewing problems (Table 2). According to the survey, significant responses of type I diabetic patients regarding the orthodontist's refusal to perform OT was due to uncontrolled blood sugar levels (p=0.0001). On the other hand, the lowest was no OT is required and poor oral hygiene of the patients (p=0.009) (Table 2).

Table 3 shows that there is statistically significant association between DM type and patient satisfaction with access to the dental services provided to them. The results revealed strong disagreement among type II DM patients, while the rate of "disagreed" and "neutral" responses were higher among type I DM patients in all questions (p<0.0001). This indicates low satisfaction level with adult patients over young patients regarding access to services (Table 3). According to the survey, responses regarding the relation between DM type and satisfaction level of diabetic patients during and after OT revealed significant association as shown in Table 4. A significant higher rate of positive responses was found among type II DM patients for three questions (dietary habits, running blood sugar test, and recurrent candida infection). This is in contrast to type I DM patients where the rate of negative response was higher for the same questions (p<0.0001). No significant association was found between the DM type and the experience of fainting in the orthodontic clinic (p=0.852). Most of the participants (75%) reported no fainting experience in the orthodontic clinic although their orthodontists didn't perform regular blood glucose level checkup before treatment (64.6%).

More than half of the participants reported that they never been infected with candida infection during treatment (63.3%), and 43.8% of the responses showed that OT

didn't affect their dietary habits, indicating medium level of satisfaction among diabetic patients during treatment. In addition, the result revealed a significant association between diabetes mellitus type during or after treatment, where a significant higher rate of positive response was found among type II patients for four questions (endodontic treatment, having an abscessed tooth, gum problems, and root damage during OT). On the other hand, the rate of negative response was higher among type 1 patients for the same four questions (p=0.31, p<0.0001, p=0.005, and p<0.0001, respectively). The rate of positive response was higher among type I patients for the question of "Did you suffer from dental caries during or after OT?" (p=0.032). However, no association was found when the participants were asked if they have any pain in their teeth during OT (p=0.275).

Table 2. The relation between DM type and orthodontic data

Variable		Diabetes Mellitus				P- value
		Type I	Type II	Type III	Total	
Type of orthodontic appliance	Fixed	72(90.0%)	7(8.8%)	1(1.3%)	80(63.5%)	0.020*
	Removable	3(17.6%)	14(82.4%)	0	17(13.5%)	
	Combined	23(79.3%)	5(17.2%)	1(3.4%)	29(23.0%)	
Evaluation of oral health?	Excellent	9(36%)	1(4.0%)	15(60%)	25(12.1%)	0.0001**
	Good	59(43.1%)	74(54.0%)	4(2.9%)	137(66.5%)	
	Poor	41(93.2%)	2(4.5%)	1(2.3%)	44(21.4%)	
Receiving any previous orthodontic treatment	Yes	37(45.1%)	28(34.1%)	17(20.7%)	82(39.8%)	0.001*
	No	72(58.1%)	49(39.5%)	3(2.4%)	124(60.2%)	
Current orthodontic treatment	Yes	8(14.3%)	32(57.1%)	16(28.6%)	56(27.2%)	0.0001
	No	101(67%)	45(30%)	4(2.7%)	150(72.8%)	
Planning for orthodontic treatment for future	Yes	54(36.7%)	75(51.0%)	18(12.2%)	147(71.4%)	0.0001
	No	55(93.2%)	2(3.4%)	2(3.4%)	59(28.6%)	
Do you think you need orthodontic treatment?	Yes	50(34.5%)	76(52.4%)	19(13.1%)	145(70.4%)	0.0001
	No	59(96.7%)	1(1.6%)	1(1.6%)	61(29.6%)	
If no orthodontic treatment was done this is because?	Satisfied with your appearance	21(84.0%)	3(12%)	1(4%)	25(12.1%)	0.003
	No orthodontic problem	8(44.4%)	10(55.6%)	0	18(8.7%)	0.934
	Parents disagreement	1(100%)	0	0	1(0.5%)	0.392
	Not convinced to have ortho tx.	3(16.7%)	15(83.3%)	0	18(8.7%)	0.0001
	Afraid to have treatment	24(70.6%)	8(23.5%)	2(5.9%)	34(16.5%)	0.039
	Your medical condition	15(78.9%)	4(21.1%)	0	19(9.2%)	0.014
	Doctor refused to treat	2(28.6%)	5(71.4%)	0	7(3.4%)	0.553
	Bad experience from previous tx	14(93.3%)	1(6.7%)	0	15(7.3%)	0.002
	-ve impact of friends for ortho. Tx.	2(100%)	0	0	2(1%)	0.225
	Financial problems	41(77.4%)	11(20.8%)	1(1.9%)	53(25.7%)	0.0001
	Other	1(50%)	1(50%)	0	2(1%)	0.884
Doctor refused to treat.	Your uncontrolled diabetes condition	26(100%)	0	0	26(12.6%)	0.0001
	Chronic Gingival inflammatory	12(100%)	0	0	12(5.8%)	0.002
	Repeated ulcers and fungi	2(100%)	0	0	2(1%)	0.225
	I'm not interested of treatment	5(100%)	0	0	5(2.4%)	0.63
	No orthodontic problem	9(100%)	0	0	9(4.4%)	0.009
	Severe decayed teeth	5(100%)	0	0	5(2.4%)	0.225
	Cannot afford Tx. fees	5(83.3%)	1(16.7%)	0	6(2.9%)	0.133
	Sensitivity of used materials	0	0	0	0	
	Pregnant	1(100%)	0	0	1(0.5%)	0.392
	Cannot maintain good OH	13(86.7%)	2(13.3%)	0	15(7.3%)	0.009
	Medically compromised patient	4(100%)	0	0	4(2%)	0.084
	Lack of experience	19(100%)	0	0	19(9.2%)	0.0001
	Other	1(33.3%)	2(66.7%)	0	3(1.5%)	0.796
	Did not follow the doctor instructions	2(100%)	0	0	2(1%)	0.225
If you have orthodontic treatment answer the following questions:	Crowded teeth	29(76.3%)	8(21.1%)	1	38(18.4%)	0.002
	Pronunciation and speech problems	7(77.8%)	2(22.2%)	0	9(4.4%)	0.111
	Protruded lower teeth	19(95.0%)	1(5%)	0	20(9.7%)	0.0001
	Space between teeth	16(47.1%)	18(52.9%)	0	34(16.5%)	0.711
	Protruded upper teeth	37(100%)	0	0	37(18%)	0.0001
	TMJ pain and clicking	4(50%)	4(50%)	0	8(3.9%)	0.768
	gummy smile	13(100%)	0	0	13(6.3%)	0.001
	Being teased by your colleagues	9(75%)	3(25%)	0	12(5.8%)	0.088
	Open bite	7(26.9%)	19(73.1%)	0	26(12.6%)	0.181
	To be socially accepted	12(85.7%)	2(14.3%)	0	14(6.8%)	0.013
	Deep bite	1(100%)	0	0	1(0.5%)	0.392
	A transfer from another dentist	2(100%)	0	0	2(1%)	0.225
	Other	0	0	1(100%)	1(0.5%)	0.095
	biting and chewing Problems	9(52.9%)	8(47.1%)	0	17(8.3%)	0.528

Generally, most of the cases reported equal percentage concerning "no root damage during OT", and "no abscessed teeth" (60.5%). Furthermore, 38.9% of the responses revealed "no root canal treatment" during OT, while 67.9% of cases reported that having toothache, 53.8% suffering from dental caries, and 45.4% reported gum problems during and after OT. Most of the responses were from type I DM, indicating medium level of satisfaction during or after treatment (Table 4). Furthermore, the results revealed no significance among different types of DM after treatment completion, even though the rate of approval was higher among type I patients ($p > 0.05$) indicating medium level of satisfaction after treatment among diabetic groups as shown in Table 5. According to the survey, more than third of the participants (39.8%) reported OT discontinuation, where more than half of them reported that it was their decision (54.9%) as presented in Table 6.

Table 3. The relation between DM type and participants' satisfaction level (Measuring patient satisfaction with access to services provided to them).

Variable		Diabetes Mellitus				P value
		Type I	Type II	GDM	Total	
Q1-Was it easy to get an appointment?	Strongly disagree	2(3.8%)	44(83.0%)	7(13.2%)	53(28.2%)	0.0001
	Disagree	24(52.2%)	22(47.8%)	0	46(24.5%)	
	Neutral	33(89.2%)	2(5.4%)	2(5.4%)	37(19.7%)	
	Agree	16(80.0%)	3(15.0%)	1(5%)	20(10.6%)	
	Strongly Agree	29(90.6%)	3(9.4%)	0	32(17%)	
Q2- Were you giving a clear direction to the clinic?	Strongly disagree	2(3.4%)	55(94.8%)	1(1.7%)	58(32.8%)	0.0001
	Disagree	44(80.0%)	11(20%)	0	55(31.1%)	
	Neutral	20(90.9%)	0	2(9.1%)	22(12.4%)	
	Agree	18(78.3%)	4(17.4%)	1(4.3%)	23(13%)	
	Strongly Agree	16(84.2%)	3(15.8%)	0	19(10.7%)	
Q4- Have you been greeted and welcomed with a good manner & offered appointment time that was convenient to you?	Strongly disagree	2(5.1%)	37(94.9%)	0	39(23.4%)	0.0001
	Disagree	37(68.5%)	17(31.5%)	0	54(32.3%)	
	Neutral	36(97.3%)	1(2.7%)	0	37(22.2%)	
	Agree	11(61.1%)	4(22.2%)	3(16.7%)	18(10.8%)	
	Strongly Agree	15(78.9%)	4(21.1%)	0	19(11.4%)	
Q6- Did you receive your treatment in right time?	Strongly disagree	1(3.2%)	30(96.8%)	0	31(19.6%)	0.0001
	Disagree	33(66%)	17(34%)	0	50(31.6%)	
	Neutral	28(96.6%)	1(3.4%)	0	29(18.4%)	
	Agree	19(86.4%)	2(9.1%)	1(4.5%)	22(13.9%)	
	Strongly Agree	19(73.1%)	6(23.1%)	1(3.8%)	26(16.5%)	
Q7- Did your doctor allocate enough time to listen to your problems and personal health, answer all your questions and explain to you what you need to know?	Strongly disagree	3(8.8%)	31(91.2%)	0	34(20.9%)	0.0001
	Disagree	22(52.4%)	20(47.6%)	0	42(25.8%)	
	Neutral	33(97.1%)	1(2.9%)	0	34(20.9%)	
	Agree	21(80.8%)	2(7.7%)	3(11.5%)	26(16.0%)	
	Strongly Agree	22(81.5%)	5(18.5%)	0	27(16.6%)	
Q8- Did your doctor explain the treatment options, the risks and side effects resulting from your treatment clearly?	Strongly disagree	2(6.1%)	31(93.9%)	0	33(21.4%)	0.0001
	Disagree	28(68.3%)	13(31.7%)	0	41(26.6%)	
	Neutral	31(96.9%)	1(3.1%)	0	32(20.8%)	
	Agree	22(81.5%)	2(7.4%)	3(11.1%)	27(17.5%)	
	Strongly Agree	16(76.2%)	5(23.8%)	0	21(13.6%)	
Q12- Have you given the opportunity to choose the type of treatment?	Strongly disagree	3(9.7%)	28(90.3%)	0	31(20.5%)	0.0001
	Disagree	21(58.3%)	15(41.7%)	0	36(23.8%)	
	Neutral	41(97.6%)	1(2.4%)	0	42(27.8%)	
	Agree	23(88.5%)	2(7.7%)	1(3.8%)	26(17.2%)	
	Strongly Agree	11(68.8%)	3(18.8%)	2(12.5%)	16(10.6%)	
Q13- Did you feel comfortable during the duration of treatment?	Strongly disagree	2(7.1%)	26(92.9%)	0	28(18.9%)	0.0001
	Disagree	22(62.9%)	13(37.1%)	0	35(23.6%)	
	Neutral	36(94.7%)	2(5.3%)	0	38(25.7%)	
	Agree	24(85.7%)	2(7.1%)	2(7.1%)	28(18.9%)	
	Strongly Agree	15(78.9%)	4(21.1%)	0	19(12.8%)	
Q14- Are you confident with the treatment provided to you & satisfied with your doctor?	Strongly disagree	2(5.9%)	32(94.1%)	0	34(22.5%)	0.0001
	Disagree	31(75.6%)	10(24.4%)	0	41(27.2%)	
	Neutral	32(97%)	1(3%)	0	33(21.9%)	
	Agree	20(87.0%)	2(8.7%)	1(4.3%)	23(15.2%)	
	Strongly Agree	14(70%)	5(25%)	1(5%)	20(13.2%)	
Q15- Was treatment fee reasonable and acceptable to you?	Strongly disagree	2(6.7%)	28(93.3%)	0	30(19.9%)	0.0001
	Disagree	15(46.9%)	14(43.8%)	3(9.4%)	32(21.2%)	
	Neutral	38(95%)	2(5%)	0	40(26.5%)	
	Agree	22(91.7%)	2(8.3%)	0	24(15.9%)	
	Strongly Agree	22(88%)	3(12%)	0	25(16.6%)	

The result showed that the main reasons for discontinuing OT, with significant association to Diabetes Mellitus type, were due to bad oral hygiene and treatment expenses (23.2%), followed by teeth mobility and transportation difficulty (15.9%), and lastly were due to relocation to different place (9.8%), and painful procedure (8.5%). In addition, the main reasons of the participants not advising others to undergo orthodontic treatment were "painful treatment" (11.2%, p=0.0001), less satisfaction about treatment results (9.2%, p=0.0001), and due to difficulty of maintaining good oral hygiene with fixed orthodontic appliances (8.7%, p=0.001) (Table 6). Majority of the responses were among type I DM patients.

Table 4. The relation between DM type and participants' satisfaction level (Measuring the satisfaction of diabetics during and after OT).

Variable		Diabetes Mellitus				P value
		Type I	Type II	GDM	Total	
Did your orthodontic treatment affect your dietary habits?	Yes	21(45.7%)	24(52.2%)	1(2.2%)	46(35.4%)	0.0001
	No	53(93%)	4(7%)	0	57(43.8%)	
	Not a concern	24(88.9%)	1(3.7%)	2(7.4%)	27(20.8%)	
Did your orthodontists do regular blood glucose level test before treatment?	Yes	4(14.3%)	23(82.1%)	1(3.6%)	28(22.0%)	0.0001
	No	81(98.8%)	1(1.2%)	0	82(64.6%)	
	Not a concern	13(76.5%)	2(11.8%)	2(11.8%)	17(13.4%)	
Have you ever fainted in the orthodontic clinic?	Yes	2(13.3%)	13(86.7%)	0	15(11.7%)	0.852
	No	90(93.8%)	5(5.2%)	1(1%)	96(75.0%)	
	Not a concern	6(35.3%)	9(52.9%)	2(11.8%)	17(13.3%)	
Have you ever been infected with candida infection or ulcers during treatment?	Yes	8(28.6%)	20(71.4%)	0	28(21.9%)	0.0001
	No	74(91.4%)	6(7.4%)	1(1.2%)	81(63.3%)	
	Not a concern	16(84.2%)	1(5.3%)	2(10.5%)	19(14.8%)	
Did you have any pain in your teeth.	Yes	61(68.5%)	27(30.3%)	1(1.1%)	89(67.9%)	0.275
	No	22(91.7%)	2(8.3%)	0	24(18.3%)	
	Don't know	15(83.3%)	1(5.6%)	2(11.1%)	18(13.7%)	
Have you received root canal treatment?	Yes	41(61.2%)	25(37.3%)	1(1.5%)	67(51.1%)	0.031
	No	46(90.2%)	5(9.8%)	0	51(38.9%)	
	Don't know	11(84.6%)	0	2(15.4%)	13(9.9%)	
Did you have an abscessed tooth?	Yes	14(37.8%)	22(59.5%)	1(2.7%)	37(28.7%)	0.0001
	No	72(92.3%)	6(7.7%)	0	78(60.5%)	
	Don't know	12(85.7%)	0	2(14.3%)	14(10.9%)	
Did you have gum problems ?	Yes	34(57.6%)	24(40.7%)	1(1.7%)	59(45.4%)	0.005
	No	46(90.2%)	5(9.8%)	0	51(39.2%)	
	Don't know	18(90%)	0	2(10%)	20(15.4%)	
Did you have a root damage during orthodontic treatment?	Yes	7(23.3%)	22(73.3%)	1(3.3%)	30(23.3%)	0.0001
	No	72(92.3%)	6(7.7%)	0	78(60.5%)	
	Don't know	19(90.5%)	0	2(9.5%)	21(16.3%)	
Did you suffer from dental caries?	Yes	44(62.9%)	25(35.7%)	1(1.4%)	70(53.8%)	0.032
	No	40(90.9%)	4(9.1%)	0	44(33.8%)	
	Don't know	14(87.5%)	0	2(12.5%)	16(12.3%)	

The fundamental requirement of quality healthcare services is the adoption of a system that is 'patient orientated'. In any health care set up, patient satisfaction with regards to quality of services and treatment provided is a very important indicator and a sensitive issue. It is a determining factor since patients choose the healthcare providers who can respond to their needs and meet their expectations (Khan et al. 2014). Patient level of satisfaction has been shown to correlate positively with the success of treatment provided. There is variation in patients' expectations of orthodontic treatment and these differences arise commonly from factors such as age, gender, satisfaction with facial appearance, as well as influence from peers, parents, and others. Understanding the patients' expectations and attitude is a prerequisite for appropriate behavioral and clinical management. Increasingly, patient-centered measures aim to improve health services and are used to assess these subjective attributes in assessing orthodontic need and in determining the outcomes of orthodontic care. Assessment of patients' expectations is central to understanding the oral health needs, patient satisfaction with the treatment, and ultimately the perceived overall quality of health systems (Farishta 2015; Afrashtehfar et al. 2020).

The results revealed that the level of satisfaction was lower among type II than type I diabetic patients. This could be explained by the fact that type II diabetic patients' satisfaction levels with the care they received is affected by many factors such as age, gender, and education levels (Othman et al. 2015; Jalil et al. 2017). In addition, adults with type II DM, especially those in middle age, do care more about treatment cost, convenience, duration, and results. Hence, they develop more practical expectations, and do approach to an Orthodontist for consultation more than patients with type I DM. Several studies reported that the key to any orthodontic treatment for a patient with diabetes is good medical control. OT should not be performed in a patient with uncontrolled diabetes (Chauhan et al. 2018). Similar result was reported in the current study, where the main reason for orthodontists' refusal of providing treatment for diabetic patients was due to uncontrolled blood sugar. Previous studies reported that it is essential to pay attention to maintaining good oral hygiene, especially when fixed appliances were used. Diabetes related microangiopathy can affect the peripheral vascular supply, resulting in unexplained toothache, tenderness to percussion and even loss of vitality. Furthermore, applying

light forces during OT is recommended, where uncontrolled or poorly controlled diabetic patients have an increased tendency for periodontal breakdown (Rizvi et al. 2014; Muhamad et al. 2015).

Table 5. The relation between DM type and participants' satisfaction level (After completion of OT).

Variable		Diabetes Mellitus				P value
		Type I	Type II	GDM	Total	
Q1- Have you had a bone resorption?	Yes	10(83.3%)	2(16.7%)	0	12(11.9%)	0.298
	No	58(96.7%)	2(3.3%)	0	60(59.4%)	
	Don't know	25(86.2%)	2(6.9%)	2(6.9%)	29(28.7%)	
Q2- Have you had TMJ pain and clicking?	Yes	20(83.3%)	3(12.5%)	1(4.2%)	24(24%)	0.812
	No	54(94.7%)	3(5.3%)	0	57(57%)	
	Don't know	16(84.2%)	1(5.3%)	2(10.5%)	19(19%)	
Q3- Did you feel mobility in your teeth?	Yes	32(97%)	1(3%)	0	33(34%)	0.067
	No	38(90.5%)	4(9.5%)	0	42(43.3%)	
	Don't know	19(86.4%)	1(4.5%)	2(9.1%)	22(22.7%)	
Q4- Have you had root resorption ?	Yes	11(84.6%)	2(15.4%)	0	13(13%)	0.506
	No	56(94.9%)	3(5.1%)	0	59(59%)	
	Don't know	25(89.3%)	1(3.6%)	2(7.1%)	28(28%)	
Q5- Has your speech improved after treatment?	Yes	36(94.7%)	2(5.3%)	0	38(38.4%)	0.288
	No	23(88.5%)	3(11.5%)	0	26(26.3%)	
	Don't know	32(91.4%)	1(2.9%)	2(5.7%)	35(35.4%)	
Q6- Did you remove or lose any of your teeth?	Yes	46(93.9%)	2(4.1%)	1(2%)	49(46.7%)	0.151
	No	32(88.9%)	4(11.1%)	0	36(34.3%)	
	Don't know	17(85%)	1(5%)	2(10%)	20(19%)	
Q7- Has your chewing ability improved after treatment?	Yes	42(97.7%)	1(2.3%)	0	43(43.9%)	0.103
	No	19(82.6%)	4(17.4%)	0	23(23.5%)	
	Don't know	29(90.6%)	1(3.1%)	2(6.3%)	32(32.7%)	
Q8- Are you satisfied about your appearance?	Yes	47(88.7%)	5(9.4%)	1(1.9%)	53(53.5%)	0.481
	No	25(96.2%)	1(3.8%)	0	26(26.3%)	
	Don't know	17(85%)	1(5%)	2(10%)	20(20.2%)	
Q9- In general are you convinced with your treatment results?	Yes	53(89.8%)	5(8.5%)	1(1.7%)	59(57.8%)	0.398
	No	21(95.5%)	1(4.5%)	0	22(21.6%)	
	Don't know	18(85.7%)	1(4.8%)	2(9.5%)	2120.6%()	
Q10- Is your appearance improved significantly?	Yes	54(91.5%)	4(6.8%)	1(1.7%)	59(57.8%)	0.190
	No	23(92%)	2(8.0%)	0	25(24.5%)	
	Don't know	15(83.3%)	1(5.6%)	2(11.1%)	18(17.6%)	
Q11- Have you noticed improvement on your self-confidence?	Yes	55(91.7%)	4(6.7%)	1(1.7%)	60(58.3%)	0.356
	No	15(88.2%)	2(11.8%)	0	17(16.5%)	
	Don't know	23(88.5%)	1(3.8%)	2(7.7%)	26(25.2%)	
Q12- Do you noticed better social acceptance after treatment?	Yes	49(94.2%)	3(5.8%)	0	52(51.5%)	0.217
	No	15(88.2%)	2(11.8%)	0	17(16.8%)	
	Don't know	29(90.6%)	1(3.1%)	2(6.3%)	32(31.7%)	
Q13- Are you satisfied about your smile?	Yes	56(90.3%)	5(8.1%)	1(1.6%)	62(60.8%)	0.289
	No	20(95.2%)	1(4.8%)	0	21(20.6%)	
	Don't know	16(84.2%)	1(5.3%)	2(10.5%)	19(18.6%)	
Q14- Is your doctor has given you the dates for future follow-up?	Yes	40(90.9%)	4(9.1%)	0	44(43.6%)	0.220
	No	36(97.3%)	1(2.7%)	0	37(36.6%)	
	Don't know	17(85%)	1(5%)	2(10%)	20(19.8%)	
Q15- Can you repeat the experience to undergo orthodontic treatment again?	Yes	48(96%)	2(4%)	0	50(49.5%)	0.093
	No	20(87.0%)	3(13%)	0	23(22.8%)	
	Don't know	25(89.3%)	1(3.6%)	2(7.1%)	28(27.7%)	
Q16- Would you recommend diabetic patients to undergo orthodontic treatment?	Yes	60(92.3%)	4(6.2%)	1(1.5%)	65(62.5%)	0.208
	No	10(90.9%)	1(9.1%)	0	11(10.6%)	
	Don't know	24(85.7%)	2(7.1%)	2(7.1%)	28(26.9%)	

Table 6. The relation between DM type and orthodontic discontinuation.

Variable	Diabetes Mellitus				P value
	Type I	Type II	GDM	Total	
If your orthodontic treatment had to be discontinued this was:					
Your decision	43(95.6%)	0	2(4.4%)	45(54.9%)	0.898
Parents decision	10(90.9%)	1(9.1%)	0	11(13.4%)	
Orthodontist decision	14(93.3%)	1(6.7%)	0	15(18.3%)	
Physician decision	8(88.9%)	1(11.1%)	0	9(11%)	
Others	2(100%)	0	0	2(2.4%)	
If you answered the previous question, the cause of discontinuing treatment was due to:					
Bad oral hygiene	19(100%)	0	0	19(23.2%)	0.0001
Mobility of teeth	12(92.3%)	1(7.7%)	0	13(15.9%)	0.006
Recurrent fainting on dental chair	1(100%)	0	0	1(1.2%)	0.392
Teeth invitality	3(100%)	0	0	3(3.7%)	0.136
Severe inflammation of the gums	3(75%)	1(25%)	0	4(4.9%)	0.334
Transition to another place	8(100%)	0	0	8(9.8%)	0.014
Lacking cooperation and attention from me	2(100%)	0	0	2(2.4%)	0.225
Transportation	13(100%)	0	0	13(15.9%)	0.001
Treatment expenses	18(94.7%)	0	1(5.3%)	19(23.2%)	0.001
Caries	4(80%)	1(20%)	0	5(6.1%)	0.210
Physician recommendation	1(50%)	1(50%)	0	2(2.4%)	0.884
Recurrent mouth ulcers	0	0	0	0	
Uncontrolled sugar level	6(85.7%)	1 (14.3%)	0	7(8.5%)	0.085
eRoot resorption	2(100%)	0	0	2(2.4%)	0.225
I cannot tolerate the pain	7(100%)	0	0	7(8.5%)	0.021
There are no progress in the treatment	0	0	0	0	
Orthodontist transferred	4(80%)	1(20%)	0	5(6.1%)	0.396
Others	4(100%)	0	0	4(4.9%)	0.084
Other health problems adversely affect the treatment	4(100.0%)	0	0	4(4.9%)	0.084
If you advice others not to undergo orthodontic treatment this is because:					
You are not satisfied about treatment results	19(100%)	0	0	19(9.2%)	0.0001
Risks of the results is more than the benefits	9(100%)	0	0	9(4.4%)	0.009
It was difficult to maintain good oral hygiene with orthodontic appliances	17(94.4%)	1(5.6%)	0	18(8.7%)	0.001
Changing in the dietary habits	8(100.0%)	0	0	8(3.9%)	0.014
Recurrent mouth ulcers and fungus	1(100.0%)	0	0	1(0.5%)	0.392
Bad mouth breath after treatment	6(100.0%)	0	0	6(2.9%)	0.034
Teeth mobility and bone resorption	8(88.9%)	1(11.1%)	0	9(4.4%)	0.035
Treatment was stressful	8(80.0%)	2(20.0%)	0	10(4.9%)	0.073
Several carious lesions	5(83.3%)	1(16.7%)	0	6(2.9%)	0.133
Root resorption	2(100%)	0	0	2(1%)	0.225
Painful Treatment	23(100%)	0	0	23(11.2%)	0.0001
gingival recession	0	0	1(100%)	1(0.5%)	0.031
TMJ problems	4(100%)	0	0	4(2%)	0.084
Other	2(100%)	0	0	2(1%)	0.225

The results in the present study showed that even though the orthodontists dealt with of these issues, patients reported medium level of satisfaction during treatment and after treatment. Most of the responses showed that there was no root damage, no endodontic treatment, and no abscessed teeth during OT. On the other hand, they reported having toothache, suffering from dental caries, and having gum problems during OT. Various previous studies have established that patients and dentists' interaction is the most important factor that can influence satisfaction levels among dental patients. Patients judge dentist skills and the quality of care they receive on the basis of their personal interaction with dentist. Behavior of the dentist towards the patient, which must include showing empathy to patients needs and reassuring them regarding their expecta¬tions and demands, needs to be given top priority by all dental professionals (Khan et al. 2014; Luo et al. 2018).

In the present study, the findings revealed low levels of satisfaction with adult patients over young patients regarding access to services provided to them. Financial cost is a key factor from patients' perspective. High dental treatment costs are one of the important factors that can hinder patients' visits to dental clinics and their decision to seek dental treatment. Many previous studies have reported that patients prefer to visit teaching dental hospitals due to a good source of quality, reduced-cost dental treatment as most of these teaching facilities have clinics that allow dental students to gain experience treating patients while providing care at a reduced cost (Khan et al. 2014; Khan et al. 2017). In this study, only 25.7% of patients visiting Orthodontic department found that the cost of OT to be reasonable. Similar result was reported in previous studies (Al-Hussyeen 2010). In the present study, most of the responses showed that the appointment time was not convenient, and they didn't receive their treatment on time. This finding is consistent with the conclusion drawn by other researchers who found that long waiting times in dental clinics and lack of proper waiting areas are the main reasons of disappoint¬ment and dissatisfaction among dental patients (Al-Hussyeen 2010; Khan et al. 2014; Yong et al. 2021).

The satisfaction level in the present study was higher among type I DM patients in all variables than type II DM patients. This finding could be attributed to the fact that responsibilities and life stress among younger individuals are less than those among adults. In addition, their increased exposure to dental care facilities and their modest expectations and demands are more likely to be attained. Finally, it is necessary to mention that the present study has some limitations. Most importantly is the small sample

size and the subjective nature that is difficult to quantify. In addition, the long-term nature of OT, and the results that involve both functional and aesthetic components limit the generalization of the results to all Saudi community. Therefore, further studies are required to increase the sample size and to evaluate the level of satisfaction among Orthodontic diabetic patients to improve the quality of provided services.

CONCLUSION

The findings of the present study has shown that the level of satisfaction among orthodontic diabetic patients with access to services provided to them, patient satisfaction during and after completion of OT and causes of treatment discontinuation was medium in the present study. Regular feedback and evaluation of patient satisfaction level is essential in order to further improve quality of services. Orthodontist should be conscious about the importance of diabetes in relation to the patients' susceptibility to periodontitis, especially if uncontrolled. Periodontal health and proper oral hygiene should be strictly observed during treatment.

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Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Inhibition of α Amylase Activity by some Bacterial and Medicinal Plant Extracts *In vitro*

Nehal A Alqahttani,[1] Sohair M. Khojah[1]* and Majdah MA Aburas[2]

[1]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

[2]Biology Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

ABSTRACT

Inhibition of carbohydrate digestive enzymes by natural and non-toxic secondary products had less adverse effects than synthetic drugs. This study was aimed to inhibition of α-amylase activity to a significant level by some bacterial and plant extracts which decrease the digestion of carbohydrates, obesity, and diabetes side effects. Bacteria were isolated from soil and fermented milk and the most active isolate in inhibition of α-amylase was selected and identified. Also, more than ten plants were collected, extracted and screened for inhibition of α-amylase. Out of 30 bacterial isolates were tested as inhibitor for α-amylase *in vitro,* ten isolates showed inhibition of the α-amylase. Furthermore, different aqueous and organic plant extracts were investigated as inhibitors of α-amylase. The active plants in α-amylase inhibition were rosemary, garlic, lepidium, white been, cumin, coffee peel, linseed, green tea, cinnamon, and chili pepper. The isolate *Lactobacillus* and *Streptococcus* extracts showed the highest enzyme inhibition compared to the other bacterial isolate while *Bacillus* sp. had the lowest inhibitory enzyme activity. Also, coffee peel aqueous extracts cause the highest inhibition of α-amylase enzyme and the lowest activity compared to the other plant extracts (70.23%). In addition, it was found that the methanolic extracts rosemary, cumin and green tea completely inhibited the enzyme (100%) while linseed and cinnamon had lower inhibitory activity of α-amylase compared to the other plant extracts (2.38% and 3.57%, respectively). In conclusion, the increase uptake of sugars cause obesity and some plant and bacterial extracts can be used to inhibit α-amylase which treat obesity, and diabetes with minimal side effects.

KEY WORDS: INHIBITION, A-AMYLASE, MEDICINAL PLANTS, EXTRACTS, LACTOBACILLUS.

INTRODUCTION

Human consumption of increased amounts of fats and polysaccharides which contained low micronutrients like vitamins and minerals and, at the same time, low physical activity had significant risk factors and led to obesity. Starch, bread, rice and sweets provide the body with the greatest amount of energy and in the developing courtiers, most of human diets is polysaccharides. The high uptake of these materials causes obesity which makes the body at risk of diabetes and other diseases. The famous health disorders, overweight and obesity are obtained due to an imbalance between energy intake and uptake and had significant risk factors for many chronic diseases as heart disease and cardiovascular diseases, type 2 diabetes, dyslipidemia, hypertension, and certain types of cancer like breast, and colon cancer (Barrett and Udani, 2011, Mahmood, 2016, Buchholz and Melzig, 2016, Moses et al., 2020).

Amylase history began in 1811 by Kirchhoff, when it was discovered as starch degrading enzyme α-amylase and α- glucosidase, which are the key enzymes in food digestion. Various studies have reported different materials for the significant inhibition of these enzymes to decrease digestion of carbohydrates. Inhibition of carbohydrate digestion can decrease their absorption and promote weight loss. The endoenzyme α-amylase is called 1,4-glucan-4-glucanohydrolase and considered one of the major products of the pancreas (about 5–6%) and salivary glands and acts on internal α-glycosidic bonds of polysaccharides. The amylase enzyme has been detected in different microorganisms and many plants and have a significant role in the digestion of starch and glycogen through the hydrolysis of α-1,4-glycosidic linkage in starch, amylose, amylopectin, glycogen, and various malto oligosaccharides (Barrett and Udani, 2011; Feng et al., 2011; Prakash and

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Jaiswal, 2010; Sales, 2012; Mahmood, 2016; Sales, 2012, Mahmood, 2016).

Alpha-amylase inhibitors are widely used by the pharmaceutical and agricultural industries. In comparison, this enzyme's inhibitors can be used to lower blood glucose and in the treatment of obesity, adipose, hyperlipemia, diabetes, pre- diabetes, gastric ulcer, duodenal ulcer, and as insect control. Natural α-amylase inhibitors are distributed in microorganisms, higher plants, or animal's secretions. Microorganisms represent a vast resource of novel compounds with many agricultural and medical applications. Natural inhibitors of α-amylase attract considerable interest as excellent sources of bioactive compounds due to their longtime clinical practice and reliable therapeutic efficacy. Some extracts from the microbial origin like members of the family Bacillaceae and Streptomycetaceae were reported as α-amylase inhibitors (Obiro et al., 2008; Feng, et al., 2011; Liu et al., 2015; Sun et al., 2015; Moses et al., 2020).

Moreover, from the yeast cells, a functional α-amylase inhibitor was detected and purified from culture the culture supernatant using a two-step chromatographic method and can be used for biotechnological and pharmaceutical applications. Similarly, some plant extracts are sources of α-amylase inhibitor, including White been, Cumin and Rosemary. There are many types of plants reported to show α-amylase inhibitory activity, and so maybe relevant to the treatment of type 2 diabetes and obesity. About 800 plant species have been antidiabetic properties. A wide range of plant-derived principles belonging to compounds, mainly alkaloids, glycosides, galactomannan gum, polysaccharides, hypoglycins, peptidoglycans, guanidine, steroids, glycopeptides, and terpenoids, have demonstrated bioactivity against hyperglycemia (Sales, 2012; Brain-Isasi et al., 2017; Moses et al., 2020).

Among the new chemical structure approved as drugs by the U.S. Food and Drug administration, a total of 46% are relevant to natural products, including 4% natural products, 22% natural-product derivatives, and 20% natural mimic compounds (Liu et al., 2015). In a survey conducted in 2017, the results indicated that α-amylase could inhibit glycoprotein in common beans (*Phaseolus vulgaris* L). Another study in 2016 performed on Basil seed was found to contain bioactive peptides, which have antioxidative and a-amylase inhibitory activities, and three novel inhibitor peptides successfully identified. These peptides can use therapeutic agents to reduce the risk of oxidative stress and prevent type-2 diabetes (Saufi, 2016). In another study performed on the essential oil from *Cedrus deodara* cones in 2017 reported that this evergreen tree was distributed in western Himalaya also found abundantly in Asia, including China, Afghanistan, Pakistan, India, and Nepal. The results indicated that cones essential oils act as a potential α-amylase inhibitors due to the presence of long pinene which is the primary active component in the essential oil from *Cedrus deodara* cones (Xu et al., 2017). Another study was conducted in 2017, it discover the bioactive peptides derived from cumin seed, with antioxidative and antidiabetic activities (Siow et al., 2017). In summary, most of the studies approved that there are several plants that have chemical structures and bioactive compounds that inhibit amylase enzyme that leads to development for food and pharmaceutical applications (Moses et al., 2020).

The present research aimed to analyze the inhibition of amylase enzyme by specific bacterial and some medicinal plant extracts, and to help the selection of the most active one that inhibits the enzyme.

MATERIAL AND METHODS

α-amylase enzymes Kit was purchased from α-amylase liquid color Human Gesell schaft, Germany Kit and it is stored at 2-4°C.

Soil and fermented milk samples: In this study, 5 soil samples of agricultural soil, approximately 5 g each, were obtained from different sites which included: Qurayyat Governorate, Taif, and Abha, Saudi Arabia in addition to Jumeirah Beach in Dubai, United Arab Emirates. of each sample was collected in a sterile plastic package and transferred to the Microbiology laboratory at King Abdulaziz University to isolate bacteria. Five samples from the fermented milk were collected from the supermarket to isolate lactic acid bacteria.

Plants samples: Ten kinds from plants were selected and used to inhibit amylase enzyme, which included: rosemary, garlic, Lepidium, white been, cumin, coffee peel, linseed, green tea, cinnamon, and chili pepper. They were collected from the local market in Jeddah, Saudi Arabia. Approximately 10 g of each plant was taken to be extracted with water or methanol. The extracts were saved in the refrigerator at 4°C before testing.

Isolation and characterization of bacteria: The medium used for bacteria isolation was nutrient agar medium which containing (g/l) peptone (5.0), beef extract (3.0), and sodium chloride (5.0), pH 7.4 at 25°C. Agar 15.0 g/L was added to prepare solid medium (Gowsalya et al., 2014). *Lactobacillus* was isolated from AL-Maraee yogurt using de Man, Rogosa and Sharpe medium (MRS) at pH 6 under anaerobic condition (10% CO2) and 37°C. All isolated bacteria were stored on Slant agar in a refrigerator at 4ºC to be used a later (Carrim, 2006). Finally, ten pure bacteria samples were isolated, and they were used for inhibition of amylase. The morphology, physiology and biochemistry of the bacterial colonies like shape, color microscopic appearances and Gram's type were recorded as described by Cheesbrough (2005). API20 was used for Gram negative bacterial isolates. Electron microscope and molecular method using 16SrRNA were used to study the bacterial isolate B11(Al-Haik et al., 2017a, b).

Bacterial growth in liquid medium: Nutrient broth or MRS broth media (50 ml) were inoculated with 0.1 ml (4x104 CFU/ml) of each bacterial suspension in 250 ml Erlenmeyer flasks, incubated at 37°C for 2 days at 120 rpm. At the end of the fermentation period, each bacterial filtrate was collected, sterilized by 0.45 µm bacterial filter and preserved at 4°C until used for amylase assay.

Preparation of plants extract: The selected plants, used for inhibition of amylase enzyme, included: rosemary, garlic, *Lepidium sativum*, white been, cumin, coffee peel, linseed, green tea, cinnamon, and chili pepper. Plants were ground to a fine powder. Then, they were stored at room temperature and protected from light until extraction. Extraction of samples with water was carried out where 5g of the powdered plant parts were mixed with 50mL of boiling deionized water for 24h at room temperature. Then, the aqueous solution was filtrated and lyophilized immediately and stored dry at 4°C. It was protected from the light. A refluxed condenser was then prepared with methanolic extracts by extracting 5g powdered plants with 50ml of methanol for 24 h. After that, the extract was filtrated, dried and stored in the refrigerator at 4°C until used for amylase assay.

Enzyme assay procedure: Inhibition of α-amylase was measured based on the hydrolytic cleavage of a modified starch derivative material (Buchholz and Melzig, 2016). By micropipette, 1000 µl of enzyme reagent kit was put in the cuvette and mixed with 10 µl of the sample solution (bacteria filtrate, aqueous or methanolic plant extracts). The mixture was incubated for 2-3 min at 37°C and finally, the absorbance was detected at 405 nm (A405 nm). Distilled water was used as a positive control and all testes were carried out in triplicate and mean value was calculated. The enzyme activity was determined by the increase of fluorescence per minute. The inhibitory activity of the extracts was the difference between the enzyme activity in the 100% activity control (no inhibitor added) and the enzyme activity in the reaction mixture containing the bacteria filtrate or plant extract and was expressed as a percentage of the enzyme activity of the positive control. Then, each extract was tested for the inhibitory activity. The results were expressed as the average ± standard deviation (Buchholz and Melzig, 2016).

$$(\%)\ \text{amylase inhibition} = \frac{\text{absorbance of control at 504nm} - \text{absorbance of samples at 504 nm}}{\text{absorbance of the control at 504nm}} \times 100$$

Statistical Analysis: The results were obtained as the average (mean value) and standard deviation. The results are expressed as mean ± SD (n =3) of three experiments. Statistical Analysis was performed using S.P.S.S. for Windows, the means of results were compared by one-way Analysis of variance (ANOVA), and the significant level was P<0.05 (Orhan, and Akincioglu, 2020).

RESULTS AND DISCUSSION

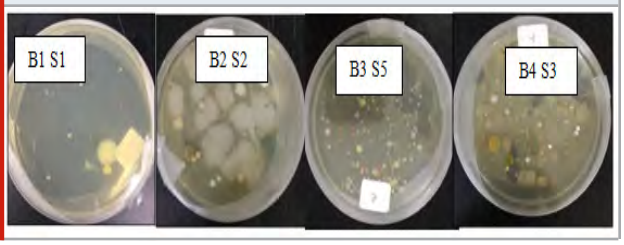
Alpha-amylase is an enzyme which hydrolyzes carbohydrate into monosaccharides then into glucose, and in advance, it is easily absorbable. From one of current methods for prevention of obesity is reduction of caloric intake during the food digestion and absorption, and inhibition of α-amylase is important for prevention of starch hydrolysis to small parts such as maltose, maltotriose, and maltotetraose. Inhibition of α-amylase enzymes is one of the most widely studied for the treatment of obesity that was became one of the most common problems in the world (Kellogg et al., 2014, Mahmood, 2016; Buchholz and Melzig, 2016; Rasouli et al., 2017).

Analyzing the inhibition of α-amylase is required because they stimulate the absorption of glucose and the associated postprandial hyperglycemic spike. Alpha amylase inhibition is a policy in diabetes administration as it can manage the serum glucose level (Hidayati et al., 2018). In this study, a colorimetric method, based on 2-cloro-4-nitrofenil-maltotriosido (CNPG3) which mainly react with α- amylase, forming 2, 2 dichloro-4-nitrophenol from the substrate, resulting in the increase in the absorbance/ minute. There is a directly proportional between the measured absorbance and the concentration of α-amylase, found in the tested sample.

In this study, the selected bacteria on nutrient agar were characterized based on cell shape and colony shape, elevation, size and diameter (Figure1). Some bacterial colonies were white, yellowish white, orange or pink on nutrient agar while on MRS agar they were white or yellowish white. The elevation of the colony was smooth, flat, or serrated. The colonies diameter was ranged from 1.0 to 8.0 mm, and some the edge was round or irregular. Similarly, five bacterial isolates were obtained from fermented milk on MRS agar. All bacterial isolates were selected for further screening for inhibition of amylase enzyme. Table 1 showed the source of the isolated bacteria, colony color, cell shape, presence or absence of endospore and gram reaction.

Based on the data presented in Table 2, the filtrates of the ten tested bacterial isolates had considerable potential in producing α- amylase inhibitor and each isolate of bacteria inhibited enzyme at different rates. Some bacterial filtrates showed good inhibition of enzyme activity like the isolate B1S1 which had the highest inhibition (13.4 ± 0.07) while lower inhibition were recorded for the isolates B1S5 (8.75 ± 2.19), B2S3 (8.8 ± 0.28), B3S5 (9 ± 1.13), B5S3 (9.3± 0.07), B2S2 (9.5 ± 0.42), B4S3(10 ± 1.83), B5S1 (10.2 ± 0.42), B1S4 (10.5 ± 1.06) and B2S4 (12.3 ± 3.04). Based on analysis of variance (ANOVA) results, significant differences between the values of enzyme inhibitory significant differences between the values of enzyme inhibitory for all bacteria isolates compared to the control or isolate B1S1(P<0.05).

Figure 1: The isolated bacteria obtained from collected soil on nutrient agar medium



The bacterium that showed the highest inhibitory enzyme activity compared to 9 other isolates was selected and identified. Morphological characterization of the bacterial

isolate B1S1was determined. It was gram negative long chain bacilli, microaerophilic and non spore forming bacteria. The identification was confirmed using molecular methods. Bacterial DNA was separated and 16Sr RNA was analyzed and the obtained sequence was compared to related genera at Gene bank data base. The isolate B1S1 was confirmed as species belonging to the genus *Lactobacillus*. It was identified as *L. plantarum* B1S1 with similarity level of 95% to strains *L. plantarum* SSK03 and *L. plantarum* strain TS1and of 90% to *Bacillus subtilis* strain S12, *Bacillus subtilis* ZAA, *Bacillus* sp. strain Pg 12-1.

Table 1. The selected bacterial isolates from different sources

Soil sample	Source	Shape	Color	Medium used	Endospore	Gram stain	Isolate identification
B1S1	Fermented milk	Bacilli	White	MRS agar	Absent	-ve	*Lactobacillus*
B1S4	Taif soil	Bacilli	Orange	Nutrient agar	Present	+ve	*Bacillus*
B1S5	Jumeirah Beach soil	Bacilli	White	Nutrient agar	Absent	-ve	*E. coli*
B2S2	Taif soil	Bacilli	Yellow	Nutrient agar	Absent	-ve	*Pseudomonas*
B2S3	Abha soil	Bacilli	Pink	Nutrient agar	Present	+ve	*Bacillus*
B2S4	Abha soil	Bacilli	White	Nutrient agar	Present	+ve	*Bacillus*
B3S5	Fermented milk	Bacilli	White	MRS agar	Absent	-ve	*Lactobacillus*
B4S3	Qurayyat soil	Cocci	Yellow	Nutrient agar	Absent	-ve	*Proteus vulgaris*
B5S1	Fermented milk	Bacilli	White	MRS agar	Absent	-ve	*Lactobacillus*
B5S3	Fermented milk	Cocci	White	MRS agar	Absent	-ve	*Streptococcus*

Table 2. Inhibition percentage of α-amylase enzyme by the isolated bacterial filtrates. Data represent means ± SD; P<0.05 (n=3).

Bacteria	α-Amylase inhibition (%)	Bacteria	α-Amylase inhibition (%)
B1S1	13.4 ± 0.07*	B2S4	12.3 ± 3.04*
B1S4	10.5 ± 1.06*#	B3S5	9.0 ± 1.13*#
B1S5	8.7 ± 2.19*#	B4S3	10.0 ± 1.830*#
B2S2	9.5 ± 0.42*#	B5S1	10.2. ± 0.42*#
B2S3	8.8 ± 0.28*#	B5S3	9.3± 0.07*#
Control	8.4 ± 0.14		

*: significant results compared to control, #: significant results compared to isolate B1S1

Figure 2: The percentage of inhibition (%) of α-amylase by the selected bacterial filtrates.

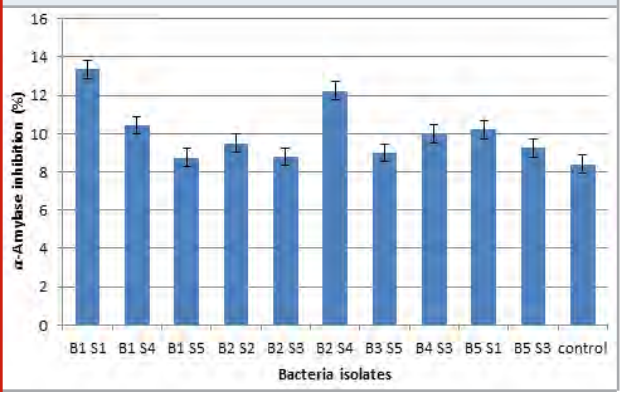


Figure 3A: The growth of the isolate B1S1 on MES agar medium, D: examined under electron microscope, C: After Gram staining

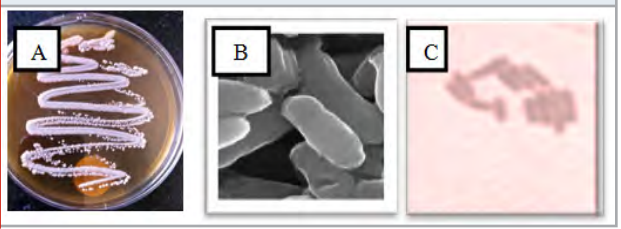
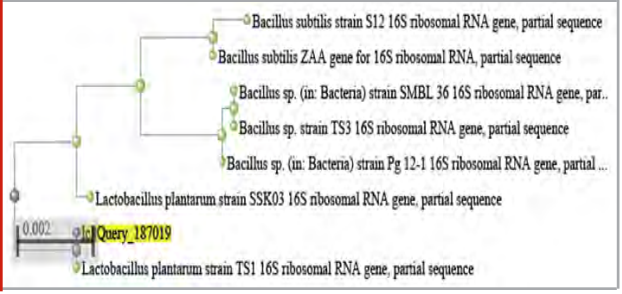


Table 3. The biochemical characters of the isolate B1S1

Tests	Substrate degradation	Results
ADH	Araginine	+
LDC	Lysine	-
ODC	Ornithine	-
CIT	Citrate	-
H2S	Na Thiosulfate	-
URE	Urea	-
TDA	Tryptophan	-
VP	Na Pyruvate	-
GEL	Charcoal gelatin	+
INO	Inositol	-
AMY	Amygdalin	+
ARA	Arabinose	-
OX	Oxidase	-
IAA	Indole production	-

+: positive results, -: negative results

Figure 4: The polygenetic tree of the isolate B1S1 and the closely related strains.



The bacterial filtrates were evaluated as inhibitors of α-amylase. From bacteria, *Lactobacillus* isolate showed the highest inhibitory enzyme compared to other isolates. The two isolates, *Bacillus* sp. and *E. coli* had the lowest inhibitory enzyme activity. *Lactobacillus* belong to Gram positive, microaerophilic, long rod, non spore forming bacteria. Genus *Lactobacillus* had more than 25 taxonomic genera and 250 species. *Lactobacillus* is the main agent of human and animal microbiota as it was recorded in the female genital organ like vagina in addition to digestive system. It is a probiotic bacteria can be isolated from yogurt, cheese and fermented milk and has varied application where it protects human body against pathogens and treat diarrhea, vaginal infections, and skin disorders (Allam et al., 2016; Al-Zahrani et al., 2019).

The utilization of microbes in the production of metabolites to cure diabetes is urgent and necessary. The potentially used microbes, in this case, are some fungi responsible in the biosynthesis of secondary metabolites that inhibit alpha-amylase. They used ethyl acetate extract of these fungi to inhibit alpha-amylase activity and three of them had high inhibition percentages (14.385%, 12.849%, and 39.246%). In another study, α amylase inhibitory assay showed that whey of both cow milk and buffalo milk fermented by *L. delbeurkii* had higher antidiabetic activity than *L. lactis* (Vankudre et al., 2015). Moreover, eleven different α-amylase inhibitors with maximum activity of 72% were recorded (Sri Pujiyanto et al., 2018; Hidayati et al., 2018). They added that presence of starch or lactose in growth medium or adjusting the initial pH at pH 5.0 or 6.0 affect significantly the production of α-amylase inhibitors ($P < 0.05$).

The used plants for amylase inhibition, their common names, scientific names and used parts were summarized in Table 4. The plants were extracted with boiling water or with methanol. The inhibition rates of the two extracts of the tested plants for α-amylase have been determined. Inhibition of α-Amylase is expressed in percentage (%). Based on Figure 5, the results of amylase inhibition indicated that all aqueous extracts of the tested plants showed that α -amylase was inhibited in different proportions and the values were in the range of 1.19 % to 70.23%. Also, it was found that the aqueous extract of coffee peel had the highest inhibitory enzyme activity (70.23%) compared to the other plant extracts and it is followed by green tea (68.45%) and white beans (65.47%). Rosemary was found to have lower inhibitory enzyme activity compared to other plant extracts (1.19 %). Furthermore, Lepidium and Linseed extracts showed partial inhibition. The results in Figure 6 showed that the methanol extract had results completely different as it was found that three plant extracts showed complete inhibition of the enzyme (100%), so that there was no apparent hydrolysis of the starch.

Table 4. The used plants for amylase inhibition, their common and scientific names and the used parts.

Common name	Scientific name	Used part
Rosemary	*Salvia Rosmarinus*	Leaves
Garlic	*Allium sativum*	Powder
Lepidium	*Lepidium sativum*	Seeds
White beans	*Phaseolus vulgaris*	Seeds
Cumin	*Cuminum cyminum*	Seeds
Coffee peel	*Coffea arabica*	Peel of the seeds
Linseed	*Linum usitatissimum*	Seeds
Green tea	*Camellia sinensis*	Leaves
Cinnamon	*Cinnamomum zeylanicum*	Park
Chili pepper	*Capsicum annuum*	Dried fruit

Figure 5: Inhibition of α amylase enzyme by some aqueous plant extracts.

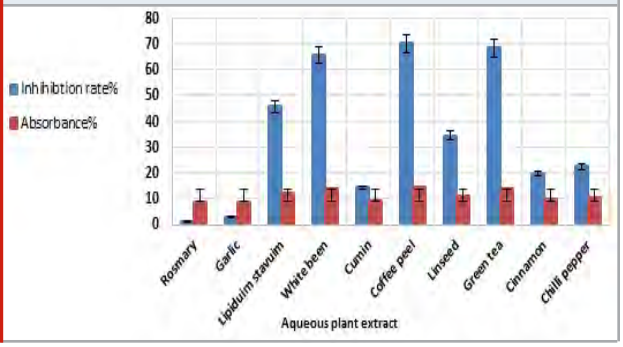
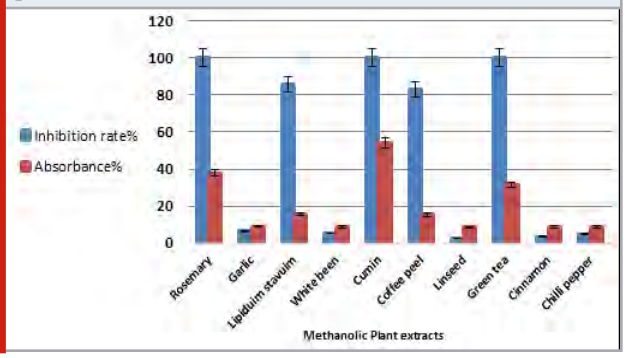


Figure 6: Inhibition of amylase enzyme by some methanolic plant extracts



They were rosemary, cumin and green tea. Linseed and cinnamon had lower inhibitory enzyme activity compared to the other plant methanol extracts (2.38%) and (3.57%). α-Amylase inhibition by water extract and methanol extracts

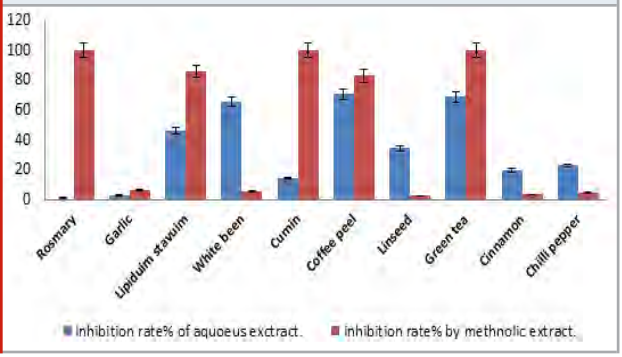
were compared. The data from methanolic extracts of the tested plants have a strong inhibitory effect than aqueous extract (Table 5. Figure 7). Also, it was noticed that generally methanolic extracts were more active and extracts of rosemary and cumin showed the highest activities while water extracts of Green tea, Coffee peel and White beans showed excellent α-Amylase inhibition. It was found that coffee peel in the aqueous extract was the best compared to other extracts (70.23%), while in methanol extract it was not the best. Rosemary, cumin, and green tea were the most enzyme inhibitors in methanol extract (100%). Moreover, rosemary is lowest inhibitory in the aqueous extract (1.19%).

Table 5. Percentage of inhibition of α-amylase (%) by the tested aqueous and methanolic plant extract, data represent means ± SD.

Medicinal plants	Aqueous extract (control)		Methanol extract	
	Absorbance (%)	α-Amylase inhibition (%)	Absorbance (%)	α-Amylase inhibition (%)
Rosemary	8.5± 2.68	1.19	37.95 ± 1.35	100*
Garlic	8.65 ± 1.48	2.97	8.95 ± 0.85	6.54*
Lepidium sativum	12.25 ± 0.77	45.83	15.6 ± 2	85.71*
White beans	13.9 ± 0.70	65.47	8.85 ± 0.95	5.35*
Cumin	9.6 ± 0.98	14.28	54.85 ± 6.25	100*
Coffee peel	14.3 ± 0.28	70.23	15.35 ± 1.85	82.73*
Linseed	11.3 ± 3.25	34.52	8.6 ± 0.9	2.38*
Green tea	14.15 ± 2.89	68.45	31.45 ± 3.25	100*
Cinnamon	10.05 ± 1.21	19.64	8.7± 0.2	3.57*
Chili pepper	10.3 ± 3.81	22.61	8.8 ± 1.5	4.67*

* indicates significant difference at P <0.05 compared to the control

Figure 7: Inhibition of α-amylase enzyme by aqueous and methanol plant extracts.



The inhibition rate of green tea in both extracts was high (in methanol extract was 100% while in aqueous extract was 68.45%). Based on the results of the analysis of variance (ANOVA), significant differences were indicated between the values of enzyme inhibitory for all water or methanolic plant extracts (P<0.05(. Also, significant differences were found between water and methanol extracts of all tested plants. Nowadays, people still believe that plants can be used as alternative medicines as bay leaves cure diabetes. Also, many researcher and nutritionist are extremely interested to fabricate a novel nutritional approach to perfectly control the postprandial glycaemia without inducing negative circumstances on the digestive system. Thus, medicinal plants were gained special importance for the treatment of diabetes (Gislin et al., 2018). In many third world countries, plant extracts are traditionally used and have been accepted by the users for the treatment of diabetes (Hidayati et al., 2018, Orhan and Akincioglu, 2020).

In this study, the 10 plants extracts either aqueous and methanol extracts were screened using fluorescence-based in vitro enzyme assays to provide plants with potential α-amylase inhibitory activities. It was found that coffee peel had the highest inhibitory for enzyme in aqueous extract (70.23%), while in methanol extracts rosemary, green tea and cumin gave complete inhibition of the enzyme (100%). As it is well known, the most popular and consumed beverage in the world is coffee. In 2014, amount consumed approximately 17.8 billion packages of coffee bought in common food stores. Coffee peel or coffee husk is one of the main components of coffee which is a thin layer that directly covers the coffee seeds. Coffee peel composed high amount of dietary fiber which contain mainly of cellulose, and hemicellulose and the last composed mainly of xylose. It is also rich in protein and minerals such as potassium, magnesium and calcium and many other components (Bessada et al., 2018).

Coffee peels contributed significantly to the inhibitory potential of the extract due to the presence of bioactive compounds such as: chlorogenic acids (1-6%), caffeine (0.8-1.25%), melanoid (17-23%) and other antioxidants. The presence of the bioactive compounds was proven vital activity in prevention skin aging. Moreover it considered anti-microbial, anti-inflammatory, anti-hair loss activities and protection against UV damage. There is another use for

coffee peel, as in 2014 there was a study of an antioxidant beverage for body weight control made on a powdered coffee peel which consists of 1.37% caffeine and 1.1-3.0% chlorogenic acids with low concentrations of reducing sugars and high fiber and also it had low glycemic index. This beverage was suitable for obese and diabetic people (Bondesson, 2015; Bessada, et al., 2018).

Different secondary plant metabolites had potential inhibition activity for α-amylase and hence maybe used as therapeutic drugs. Some species of the genus Teucrium showed excellent α-amylase inhibitory activities compared to the standard inhibitor, Acarbose. The inhibitory activities of α amylase was higher for Teucrium polium extract compared to *T. oliverianum* extract which showed more inhibition actively than *T. Orientale*. The IC50 of the alcoholic extracts of *T. polium, T. oliverianum* and *T. orientale* were 3.63, 3.86 and 13.93 mg/ml, respectively. Moreover, the organic plant extracts had strong inhibitory activity on the α-amylase activity. However, the dichloromethane extract showed the lowest inhibitory strength. Thus, α-amylase inhibition by these plant extracts could provide a successful use of these plants as useful drugs. Also, Hussain et al. (2018) concluded that aqueous extracts of Amomum subulatum and Amomum tsako dry fruit constitutes (seeds and rind) have the significant inhibitory activity of α-amylase due to the occurrence of plant secondary products like polyphenol, flavonoids, alkaloids, saponins, and tannins.

Numerous α-amylase inhibitors were purified from some medicinal plants and are used as alternative drugs with good activity and less side effects compared to synthetic products. Active compounds like phenolic substances derived from the medicinal plants are a source of α-amylase and α-glycosidase inhibitors. The seeds of the terrestrial herb, Amomum which belongs to family Zingiberaceae and found in tropical area are used in traditional medicine. The methanol extract of *Amomum tsaoko* had markedly influence on plasma glucose and thiobarbituric acid reactive substances and antioxidant potential. So, some plant extracts and some types of bacteria are considered useful factors for preventing or treating obesity. However, more studies are needed to develop new plant and bacterial resources to treat obesity with minimal side effects (Matsuda et al., 2002; Longguan 2008; Cicero et al., 2013; Pujiyanto et al., 2018; Moses et al., 2020).

Inhibition of α-amylase by natural resources is a successful method in preventing obesity and its associated diseases more than synthetic compounds and slimming drugs. Therefore, traditional medicines, including herbal medicine, have a great way in this regard (Mahmood, 2016, Buchholz and Melzig, 2016). In summary, it could be stated that the advantage of carbohydrate digestive enzyme inhibitors by plant extracts consists in not causing severe side effects compared to synthetic compounds and slimming drugs. So, it may be beneficial in weight loss in individuals consuming large amounts of starch, or had overweight, obesity or diabetes (Mahmood, 2016). Based on the results of Gulati et al., (2018), natural products are particularly best compared to the other oral drug or anti-diabetes agents currently available which recorded many side effects. In this study, Lactobacillus and/or some water or organic extracts of some medicinal plants showed excelled inhibition of α-amylase, thus they must be chosen for more detail studies and detection of the active product which can be used safely to treated obesity.

CONCLUSION

The metabolic disorder, Diabetes mellitus is associated with high level of blood glucose which is obtained due to insufficiency of insulin production or action. Inhibition of α-amylase can be an essential strategy in the management of postprandial blood glucose levels in patients with type II diabetes. Inhibition of α-amylase using some plant extracts increases levels of undigested starch in the colon and prevents the degradation of polysaccharides which decreases the blood sugar. These data may be beneficial in further exploring natural sources having pharmaceutical applications, as natural products have the advantage of being safe in their activity and have lower side effects.

Conflict of Interest: There is no conflict of interest.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Comparing Anti-Inflammatory, Anti-protease Activities and Untargeted Metabolite Profiling Based on Ultra-Performance Liquid Chromatography-Mass Spectroscopy of Five *Memecylon* species from Western Ghats Karnataka, India

Bharathi T R,[1] Ramith Ramu,[2] Shrisha Naik Bajpe[3] and H.S. Prakash[1]

[1]Department of studies in Biotechnology, University of Mysore, Manasagongothri, Mysore, Karnataka, India

[2]Department of Biotechnology and Bioinformatics, School of Life Sciences, JSS Academy of Higher Education & Research, Sri Shivarathreeshwara Nagara, Mysuru, Karnataka, India.

[3]Department of Biotechnology, Sri Dharmasthala Manjunatheshwara College (Autonomous), Ujire, Karnataka, India

ABSTRACT

Memecylon umbellatum, M. edule, M. talbotianum, M. malabaricum, and *M. wightii* species belong to the family Melastomataceae. The genus is well-known traditional medicinal herb used to treat skin diseases. The goal is to compare the metabolite makeup of the five *Memecylon* spp. and to evaluate the extract's anti-inflammatory and antiprotease activities. UPLC-ESI-QTOF-MS was used to profile untargeted metabolites in a water/methanol extract, followed by statistical analysis with PCA and HCA. Anti-inflammatory and antiprotease activity were determined by inhibiting soybean 15-lipoxygenase and Protease. Phenols and flavonoids are the most abundant secondary metabolites in *Memecylon* spp. Principal component analysis (PCA) was used to identify marker chemicals from five species, tocopherol, isorhamnetin 3-glucoside, isothalic acid, stearoylglycerol, and pyrrolidine. The *Memecylon* spp. maybe clustered into two groups based on principal component analysis, with *M. malabaricum* & *M. wightii* clustered together and M. umbellatum, *M. edule* & *M. talbotianum* forming another clustered. The anti-inflammatory (Soybean 15-lipoxygenase inhibition) and antiprotease activities (Trypsin and thrombin Inhibition) of crude extracts suggested that *M. malabaricum* and *M. talbotianum* extracts exhibited higher inhibition compared to the other three species. These data suggest that differences in metabolite profiles might be connected to differences in the bioactivity of the five plant extracts examined. The untargeted UPLC-ESI-QTOF-MS technique is efficient for identifying bioactive components of *Memecylon* spp.

KEY WORDS: MEMECYLON SPP, METABOLITES PCA, UNTARGETED.

INTRODUCTION

Memecylon spp. of the Melastomataceae family are small trees found in Western and Eastern Ghats and are extensively renowned for treating herpes and skin allergies, as well as exhibiting a wide variety of biological activity (Bharathi et al. 2015; Bharathi et al. 2016a; Bharathi et al. 2016b). Phytoconstituents found in *Memecylon* spp. includes umbelactone, oleanolic acid, sitosterol, α-tocopherol, epigallocatechin gallate, myricetin, and quercetin-7-O-rhamnoside. The extract of *Memecylon* spp leaves has antioxidant, anti-diabetic, antibacterial, and anti-inflammatory activities. The biochemical analysis and inductively coupled plasma-mass spectrometry were performed on the fruits of M. grande, *M. randerianum,* and *M. umbellatum*, it was discovered that they were rich in phenolic, alkaloids, flavonoids, and terpenoids, as well as various trace metals (Sree et al. 2021). GC-MS study of *M. Umbellatum* contained α-Tocopherol, Campesterol, Stigmasterol, β-Sitosterol, β-Amyrin which a potent inhibitor of enzymes related to diabetes, steroid metabolism, and cancer (Perumal et al. 2021). Ursolic acid extracted from *M. edule* was tested for anti-proliferative action against human leukemic monocyte lymphoma (U-937) and human acute promyelocytic leukaemia (HT-60) cell lines, which

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showed significant growth suppression and Topoisomerase II inhibition (Srinivasan et al. 2021). New species of such as *M. viswanathanii* have been identified in Kalakkad-Mundanthurai Tiger Reserve, and *M. pachaimalayanum* Pachaimalayanum, in the Eastern Ghats of India (Rajesh et al. 2021a; Rajesh et al. 2021b).

Plant products from the same species may have mass spectral properties that are comparable. Metabolite study and multivariate statistical profiling of undiscovered Mass Spectroscopic (MS) features provides fine resolution for differentiating closely similar plant species (Patil et al. 2021). Metabolite findings might be used as crucial supplemental evidence for taxonomic classification of plants with high medicinal potential and close genetic similarities (Xin et al. 2014; Li et al. 2016, Ramasetty et al. 2016). Plant bioactive research is essential in the identification of new medications. Highly advanced chromatographic methods Liquid Chromatography-MS(LC-MS) and Gas Chromatography-MS(GC-MS) are required for metabolite profiling from plant extracts (Antunes et al. 2020; Kiran et al. 2020; Pushpa et al. 2021).

According to the literature review and prior research, *Memecylon* spp exhibits a wide range of biological activities. Screening of metabolites based on activity from biochemical assays and standard isolation methods results in the identification of just a few biomolecules. As a result, in the current work, a high number of potentially bioactive compounds were identified utilizing MS, untargeted metabolite composition profiling, compound identification, and multivariate statistical analysis of the five *Memecylon* spp. (*M. wightii, M. talbotianum M. umbellatum, M. edule,* and *M. malabaricum*) was performed. Anti-inflammatory using Soybean 15-lipoxygenase (LOX-15) and antiprotease using trypsin and thrombin inhibition was conducted to investigate the potential anti-viral bioactivity of *Memecylon* spp.

MATERIAL AND METHODS

Five of *Memecylon* spp (*M. umbellatum, M. edule, M. talbotianum, M. malabaricum,* and *M. wightii*) were gathered from the Western Ghats of India in April-May 2014 and authenticated (voucher specimens #IOELP0001a, #IOELP0002, #IOELP0001h, # IOELP0003, # IOELP0004). Fresh leaves were collected, washed with distilled water, and then immersed in liquid nitrogen. For the extract's preparation, *Memecylon* samples that had been frozen and crushed were lyophilized (Telstar, Thermo scientific).

16 mL of Hydro-methanol [80:20 (v/v)] was added to 0.5gm of lyophilized *Memecylon* samples and kept in a sonicator for 30 minutes at room temperature. In a round bottom flask, the supernatant was collected after centrifugation for 15 minutes at 4000 rpm. A rotary evaporator was used to evaporate the solvent under vacuum at 40°C. 500 ul of Hydro-methanol was added to the dry residue and vortexed to completely dissolve the extract. The extracts were filtered and kept at 20 °C using a 0.2 m syringe filter. For the MS analysis, the LC system was connected to a Waters Acquity Series UPLC/SYNAPT G2 HDMS (Milford, MA, USA) with electrospray ionization for qualitative analysis of the metabolites. At a drying temperature of 350 °C, the nebulizer pressure was 60 psi, and the nitrogen flow rate was 10 L/min. A 5ul aliquot of the hydro-methanol extract of leaves was evaluated following the protocol described in previous studies (Stark et al. 2015). The data was analyzed, and the correct mass was determined using MassLynx 4.1 SCN 9.16 (Waters, Manchester, UK).

The pentapeptide leucine enkephaline (m/z-554.2615) was used to lock mass in a solution (1 ng/L) of MeCN/0.1 percent HCO_2H (1:1, v/v). Progenesis QI was used to handle the raw data of all samples and replicates acquired from MS analysis. The following peak picking settings were used: all runs, limits automated, sensitivity 3, and retention time limits 0.59 minutes. An ANOVA with a p-value of 0.05 and a fold change of 2 was used to filter data. The matrix was analyzed using PCA and Pareto scaling after the data was transferred to EZinfo. Hierarchical clustering analysis was carried out using neighbor-joining and Pearson's coefficient matrix (HCA) (Deng et al. 2014; Martucci et al. 2014; Ramu et al. 2016). Soybean 15-lipoxygenase (LOX-15) was used in the anti-inflammatory experiment. Inhibition experiments employing 0.2 M linoleic acid as the substrate and product in a solubilized form in 0.2 M borate buffer (pH 9.0) were used to evaluate the loss of soybean -LOX- 15activity (5g). A UV-Vis spectrophotometer (Beckman Coulter, DU 730 Life Sciences) was used to record inhibition tests in the presence of various doses of extracts (20 to 40 g/ml) and quercetin is used as a reference.

The IC_{50}, or the concentration required to block LOX-15 activity by 50%, was also calculated. (Rackova et al. 2007). For protease inhibition assay, the substrate N-benzoyl-DL-arginine-paranitroanilide hydrochloride (BApNA) was dissolved in DMSO (20 mg/mL). Enzyme Stock solution was prepared by dissolving 2 mg each of trypsin and thrombin was in 10 mL of 1.0 mM HCl. The enzymes (0.3 mL) and 100 µ L of plant extracts in the concentration range of 10 to 50 g/mL were incubated at 37oC for 15 minutes subsequently, 0.6 mM substrate was added and volume was adjusted to 2.5 mL with Tris buffer (100 mM, pH 7.5). The reaction mixture was incubated at 37°C for 30 minutes. The enzyme reaction was stopped by adding 100 uL of 30% acetic acid. Phenyl methane sulfonyl fluoride (PMSF) was employed as a positive inhibitor. A UV/Vis spectrophotometer was used to detect absorbance at 410 nm (Jedinak et al. 2010; Ramu et al. 2015).

RESULTS AND DISCUSSION

Putative identification of peaks by UPLC-ESI-QTOF-MS: The primary components of *Memecylon* spp. analyzed in the current study were phenolic acid derivatives, flavonoid derivatives, and hydroxyl derivatives (Table1). The compounds Enoxolone, Stearamide, and Dibutyl phthalate were identified in *M. talbotianum, M. umbellatum,* and *M. edule*. Methyl 9,10-Dihydroxystearate, Stearoyl glycerol, isorhamnetin 3-glucoside, Deiten, Myricetin were identified in *M. malabaricum* and *M. wightii*.

Table 1. Putative metabolites identified

Sl.No	Metabolite	MU	ME	MT	MM	MW
1	(-)-Cholesteryl acetate	0	0	1	1	0
2	(-)-Vindoline	0	0	0	0	1
3	(â˜)-Epigallocatechingallate	1	1	1	1	0
4	(E)-Diethylstilbestrol	1	0	0	0	0
5	2,3,23-Trihydroxyurs-12-en-28-oic acid	1	1	1	1	0
6	3,6-diacetyl-9-isopropylcarbazole	1	0	0	0	0
7	Alpha-tocopherol	1	1	1	1	1
8	Baicalin	1	0	1	1	0
9	Daphnoretin	1	0	0	0	0
10	Deiten	0	0	0	0	1
11	D-Glucosaminide	0	0	0	0	1
12	Enoxolone	1	1	1	1	0
13	Fucoxanthinol	0	0	1	1	0
14	Fustin	0	0	1	1	0
15	Iso-Quercitrin 6"-acetate	0	0	1	1	0
16	Isorhamnetin 3-glucoside	1	1	1	1	1
17	Iso-Scopoletin	0	1	0	0	0
18	Kaempferol-3-Glucoside-3"-Rhamnoside	0	1	0	0	0
19	Kaempferol-3-O-beta-D-galactoside-7-O-alpha-L-rhamnoside	0	1	0	0	0
20	Kojic acid	0	0	1	1	0
21	Methyl 9,10-Dihydroxystearate	1	1	1	1	1
22	Myricetin	0	0	0	0	1
23	Myrtillin	0	1	0	0	0
24	N-(b-Pyrrolidinoethyl) phenothiazine	1	0	0	0	0
25	Nicotianamine	1	0	0	0	0
26	Peltatoside	0	1	0	0	0
27	P-hydroxyphenylacetamide	0	0	1	1	0
28	Procyanidin B1	0	0	1	1	0
29	Pyrrolidine, 1-oleoyl-	1	0	0	0	0
30	Pyrrolidine, 1-stearoyl-	1	1	1	1	1
31	Quercetin-7-O-rhamnoside	0	1	0	0	0
32	R-(-)-Phenylephrine	0	1	0	0	0
33	Razoxane	0	0	0	0	1
34	Stearamide	1	1	1	1	0
35	Stearoylglycerol	1	1	1	1	1
36	Syringetin-3-glucoside	0	0	1	1	0
37	Trimethylsilyl (9Z)-9-hexadecenoate	0	0	1	1	0

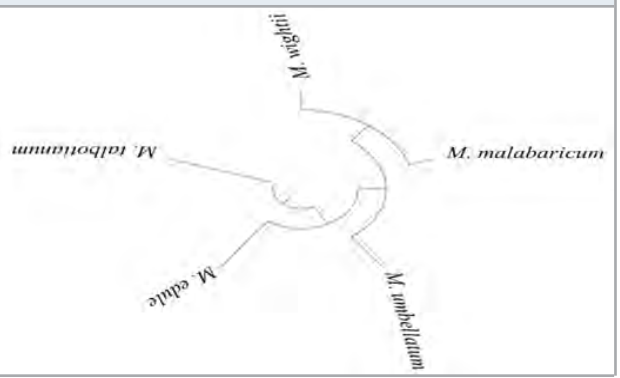
M. umbellatum = MU, M. edule= ME, *M. talbotianum*=MT, *M. malabaricum*= MM and *M. wightii*=MW. 0=Absent,1=Present.

All five *Memecylon* spp. included marker chemicals such as α-tocopherol, Pyrrolidine, 1-stearoyl-, Stearoylglycerol, and isorhamnetin 3-glucoside (Table 1). The variations in chemicals found from one species to the next may, however, be reflected in the PCA analysis, indicating that all five *Memecylon* spp. are differentiated from one another. Some of the compounds identified from Memecylon spp. such as Kaempferol-3-Glucoside-3-Rhamnoside, isorhamnetin 3-glucoside, Quercetin-7-O-rhamnoside, tiliroside, *Myricetin* are also reported by several other workers in several medicinal plants such as *Bidens pilosa*, Cucumber, *Citrullus lanatus* (Chiang et al. 2004; Abu-Reidah et al. 2012; Abu-Reidah et al. 2013). α-tocopherol identified from *Memecylon* spp. is also identified in other

plants such as *Cyamopsis tetragonoloba, Moringa oleifera,* Stevia rebaudiana, *Millingtonia hortensis*, and *Jasminum sambac* (Tomar and Rijhwani 2015). The identified compounds Kaempferol-3-Glucoside-3-Rhamnoside has Anti-inflammatory activity; isorhamnetin 3-glucoside and Quercetin-7-O-rhamnoside has Anti-inflammatory, hepatoprotective, antiviral against influenza virus, Myricetin has Anti-microbial, Anti-diabetes, Cardio-cerebrovascular protection, Anti-inflammatory, Anti-tumor activities (Lee et al. 2019; Nile et al. 2020; Ahn et al. 2020; Hu et al. 2021; Kumar et al. 2021; Song et al. 2021). α-tocopherol, Kojic acid (present in *M. talbotianum, M. malabaricum*) are well known anti-aging metabolite which further validated *Memecylon* spp use in folk medicine for skin diseases (Świątek, et al. 2021).

Multivariate PCA analysis: By using an ANOVA with a p-value of 0.05 and a fold change of 2, the number of compounds utilized for PCA was reduced, resulting in the PCA shown in Fig 2. The repeatability of the UPLC-ESI-QTOF-MS profiling approach was confirmed after analysis of each species in three replicates revealed a satisfactory clustering of each duplicate in the vicinity. The PCA result revealed that the greatest differences were found between all five *Memecylon* spp. along PC1, but that all five *Memecylon* spp. could be clearly distinguished along PC2. In addition, hierarchical cluster analysis (HCA) (Figure 1) demonstrates that *M. umbellatum, M. edule,* and *M. talbotianum* clustered together (Świątek, et al. 2021).

Figure 1: *Memecylon* spp. Hierarchical cluster analysis (HCA)



The chemical constituents present in different *Memecylon* spp. differ significantly when metabolite profiling and multivariate analysis results are compared but grouping in PCA and HCA analysis showed that plants like M. umbellatum, *M. talbotianum*, and *M. edule* are grouped together, as are *M. malabaricum* and *M. wightii*. The grouping is in accordance with to morphotypes as described by Saldhana (1996) and Gamble (1967) in their floras. This confirms that variations also occur at metabolic level (Martucci et al. 2014). Similar type of conclusions were drawn while studying, characterizing and comparing the metabolite profiles of the medicinal plants such as *Panax* ginseng, *Lonicera* spp., *Fritillaria bulbs*, genus *Vernoni*, *Garcinia buchananii*, *Garcinia oblongifolia* and genus *Panax* (Kim et al. 2011; Gao et al. 2012; Xin et al. 2014; Stark et al. 2015; Li et al. 2016; Nguyen et al. 2016; Świątek, et al. 2021).

Anti-inflammatory and antiprotease potential of *Memecylon* spp. extracts: Lipoxygenase inhibitory activity was tested at different doses of extracts ranging from 20 to 40 g/mL. At 40 g/mL, the LOX inhibitory activity of hydro-methanol extracts ranged from 66 to 95%. *M. talbotianum* (95%) and *M. malabaricum* (86%) hydro-methanol extracts had the strongest LOX inhibitory efficacy (Table 2).

Quercetin was utilized as a control, with a 95.7 % inhibition in a 40 g/reaction concentration and an IC_{50} of 20 1.2 g for LOX. The reference standard PMSF had an inhibitory activity of 84.8 % at 50 g/reaction concentration and an IC_{50} of 97% 1.2g/ml for trypsin and thrombin. Data were expressed as mean ± SD (n=3). Antiprotease activity was found in hydro-methanol extracts of five *Memecylon* spp. at 50 g/mL, hydro-methanol extract inhibited trypsin and thrombin in the range of 55 and 83% for trypsin and 53 and 80% for thrombin, respectively. The hydro-methanol extracts of *M. malabaricum* and *M. talbotianum* had the greatest antiprotease activity, with trypsin activity of 68% and 83% and thrombin activity of 66% and 80%, respectively (Table 2). *M. malabaricum* and *M. talbotianum* hydro-methanol extracts had the strongest anti-inflammatory and antiprotease efficacy compared to other *Memecylon* spp. extracts. Crude methanol leaf extracts of *Memecylon* spp. substantially suppressed the LOX and COX in vitro (Bharathi et al. 2014; Aliter and Al-Horani 2021).

Table 2. Hydro-methanol extracts Inhibition of LOX, trypsin, and thrombin enzyme by five Memecylon spp extracts.

Plant	M U	ME	MT	MM	MW
Lipoxygenase enzyme%	66 ± 0.9	78 ± 08	95 ± 0.3	86 ± 0.5	72 ± 0.6
Trypsin %	55 ± 0.3	63 ± 0.8	83 ± 0.4	68 ± 0.6	62 ± 0.2
Thrombin enzyme%	53 ± 2.1	64 ± 2.2	80 ± 2.1	66 ± 1.2	58 ± 2.1
IC_{50} values in µg	30, 2,63, 60.7	20.8,71.8, 75.5	20,95, 91.5	20,77.8, 73.2	20, 71, 66.4

Herpes simplex viruses stimulate thrombin synthesis to make cells more susceptible to infection via a process requiring PAR1-mediated cell regulation (Sutherland et al. 2007). Thrombin was also discovered to exacerbate the inflammation caused by the influenza virus. In reality, influenza viruses can cause thrombin production, which can lead to platelet activation-mediated lung inflammation (Keller et al. 2006). Human metapneumovirus and respiratory syncytial virus, two enveloped, negative-sense, single-stranded RNA viruses that cause respiratory infections, have also been connected to thrombin. Thrombin was shown to promote the replication of these viruses and to aggravate the accompanying inflammation in these experiments (Aliter and Al-Horani 2021).

CONCLUSION

The findings of the present study have shown that the UPLC with ESI-QTOF-MS approach is a viable chromatographic method for denoising and identifying phytochemicals from the genus *Memecylon*. The use of Waters Progenesis QI software to analyze the data resulted in the discovery of more phytoconstituents in the plant extracts than could have been discovered using the method used. The results obtained using ESI-QTOF-MS, PCA, and HCA show that the discovered molecules may be linked to variations in bioactivity of the plant species. This study validates the ethno-medicinal use of *Memecylon* spp in the treatment of skin diseases mainly viral infection.

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Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Design of Heterocyclic Compounds as Epidermal Growth Factor Receptor Inhibitors Using Molecular Docking and Interaction Fingerprint Studies

G. Rajitha,[1]* M. Vidya Rani,[1] V. Umakanth Naik[2] and A. Umamaheswari[2]

[1]Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati, 517502 Andra Pradesh India

[2]Department of Bioinformatics, Sri Venkateswara Institute of Medical Sciences, Tirupati, 517502 Andra Pradesh India

ABSTRACT

EGFR (Epidermal Growth factor receptors) expressed in different type of cancers, such as breast, esophageal, lung cancer etc. Because of their multifaceted role in cancer progression, EGFR and its related receptors have been considered as attractive targets for developing anticancer agents, i.e. EGFR inhibitors consisting molecules targeting EGFR ATP binding pocket and monoclonal antibodies targeting EGFR ligand binding domain. For patients with EGFR-mutant non-small-cell lung cancer, acquired resistance to drugs obstructs long-term therapeutic efficacy of targeted therapy. Even though third-generation medicines targeting EGFR T790M mutation have shown promise in overcoming acquired resistance to EGFR-tyrosine kinase inhibitors, fourth-generation drugs targeting acquired resistance to 3rd generation inhibitors are still needed. Hence, in present study, EGFR (PDB: 1M17) was selected as a target to perform molecular docking studies for existing ligands from literature. Interaction fingerprint analysis was applied for docked complexes. Docking studies revealed that compound **3** exhibited good binding affinity towards the selected target with XPG score-9.439Kcal/Mol among existing ligands which is comparable with that of standard drug erlotinib -9.192 Kcal/Mol. Interaction fingerprint analysis further confirmed that best docked compounds showed H-bond interaction with backbone residue of Met 769 and Cys 773 in an identical manner with that of standard drug erlotinib. Present study concludes that among selected existing compounds, the ligands containing quinazoline nucleus exhibited good binding affinity and similar binding interactions when compared with that of standard drug erlotinib and these ligands can be further optimized to increase binding affinity and interactions with the selected target.

KEY WORDS: EGFR, HETEROCYCLIC, INTERACTION FINGERPRINT, DOCKING.

INTRODUCTION

Cancer is the major cause of death worldwide, accounting for nearly a crore death in 2020 (Sung et al. 2021). EGFR (Epidermal Growth factor receptor) is a major factor in epithelial malignancies, and EGFR activity increases tumor growth, invasion and tumor metastasis. In normal tissues, the EGFR ligands availability is strictly controlled to make sure that the cell proliferation kinetics absolutely meet the requirements of tissues to maintain homeostasis. In the tumor microenvironment, EGFR is constantly stimulated because of continuous production of EGFR ligands or as a result of EGFR mutation that sticks receptor in a continuous activation state. Hence, EGFR has been considered as an important target for anticancer agents (Sasaki et al. 2013; Tsubata et al., 2021).

Attempts were made to the structural modification of heterocyclic compounds to discover powerful anticancer agents. Pyrazole & fused pyrazoles, viz., pyrazolpyrimidine derivatives and pyranopyrazolesare promising scaffolds for numerous anticancer agents (Saleh et al. 2020). Pyrimidine based, third generation irreversible EGFR inhibitors are WZ4002 and osimertinib (Engel et al. 2015; Greig et al. 2016; Wu et al. 2020). Various thiophene based derivatives were reported as dual EGFR or HER2 (human EGFR-related receptor 2) inhibitors (Xiao et al. 2020). Quinazoline-based EGFR tyrosine kinase inhibitors such as erlotinib, lapatinib etc. are available in the market for treatment of nearly 30% of Non-small cell lung carcinoma cases (Bhatia et al. 2020). Hence, in our present study, existing ligands containing heterocyclic compounds were retrieved from literature for molecular docking studies against EGFR to determine the binding affinity and interactions of existing ligands containing different nucleus with the selected target (Tsubata et al., 2021).

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MATERIAL AND METHODS

Existing EGFR inhibitors with heterocyclic nucleus were retrieved from literature (Li et al. 2011; Wang et al. 2016; Tu et al. 2016; Toolabi et al. 2019). Structure of co-crystal ligand AQ4 (Erlotinib) was collected from PDB database. Ligands were prepared using LigPrep application with Schrödinger Epik module, such as enhancement of stereochemical nature and protonation state of ligand, development of tautomers and minimization of energy using force field OPLS-3 at pH7.0±2.0 (Friesner et al. 2004; Pradeep et al. 2015). The best resolute X-ray structure of EGFR tyrosine kinase domain with 4-anilinoquinazoline inhibitor erlotinib (1M17) was selected in present study to propose EGFR antagonists by molecular docking & protein-ligand interaction fingerprint analysis (Jennifer et al. 2002). Literature report indicated that 4-anilinoquinazolines like erlotinib inhibits EGFR by binding to site which is occupied by ATP during phosphotransfer. EGFR crystal structure was imported to Maestro and protein structure was prepared using protein preparation wizard. The optimization of H-bonding network was done by reorientation of hydroxyl groups and thiol groups in protein; other operations were performed which were not part of refinement process of X-ray crystal structure (Sudheer et al. 2021).

Protein optimization was done at neutral pH and then minimization of energy was done by applying OPLS-3 force field for all atoms. Grid was generated around target key residues using Glide v7.1. Removal of the undesirable water molecules was done using Protein preparation wizard from the inhibitor binding site of target (Sudheer et al. 2021). GLIDE XP (grid-based ligand docking with energetic extra precision) docking procedure was used for determining target & ligand binding affinity (Duch et al. 2007). The prepared ligands were docked flexibly into grid box which was generated around the inhibitor binding site residues of EGFR using Monte Carlo-based simulated algorithm minimization method (Sudheer et al. 2021). Binding,energy, binding orientation and ranking were represented by Glide Score. During XP docking, ten poses were generated for each ligand and after post-docking minimization the best pose was retained.

Selected existing ligands were subjected to QikProp module of Schrödinger suite for prediction of the pharmacological descriptors and ADME properties. In addition, SASA and related values of docked ligands were also determined using Schrödinger suite (Thirupathi et al. 2016). Docking interactions were further analyzed by interaction fingerprint studies to determine whether the test compounds showed similar interactions like standard drug. Interaction fingerprint was generated for the best docked compounds and erlotinib docked complexes (Deng et al. 2004). Value 1 was given for established interactions and 0 for absence of specific interaction (Rácz et al. 2018).

Table 1. Docking scores of EGFR with erlotinib and best docked existing compounds

S.No	Compound code	XPG Score (Kcal/Mol)
1.	Erlotinib	-9.192
2.	3	-9.439
3.	7	-9.402
4.	24	-8.863
5.	33	-8.291

Table 2. Pharmacological descriptors of erlotinib and best docked compounds

Comp. Code	M.W	SASA	WPSA	FOSA	PISA	Vol	HBD	HBA	Glob	IP
Erlotinib	393.441	714.826	0	374.94	279.021	1278.941	1.5	7.4	0.797135	8.396
3	609.451	856.13	114.902	338.114	311.952	1620.635	2	8.95	0.779379	8.382
7	594.396	848.926	114.901	188.262	356.093	1549.14	2	8.45	0.762703	8.591
24	574.408	852.538	114.901	183.498	385.154	1545.105	2	8.95	0.758153	8.542
33	424.879	597.812	111.28	46.685	337.15	1138.137	2	5	0.881855	8.72

MW = Molecular weight(130/725); SASA = Total solvent accessible surface area(300/1000); WPSA= Weakly polar solvent accessible surface area(0/175); FOSA = Hydrophobic solvent accessible surface area(0/750); PISA = Carbon Pi solvent accessible surface area (0/450); Vol = Molecular volume (A^3)(500/2000); HBD = Hydrogen bond Donor (0/6); HBA = Hydrogen bond acceptor (2/20); Glob = Globularity (0.75/0.95); IP (eV) = Ionization potential (7.9/10.5)

RESULTS AND DISCUSSION

The structures of 77 EGFR inhibitors were collected from literature, and erlotinib structure was retrieved from PDB database and prepared using LigPrep application with Schrödinger Epik module. The PDB id 1M17 was selected to determine the antagonist behavior of existing ligands. The protein preparation wizard was used for minimizing energies of prepared crystal structure of EGFR. Inhibitor binding site residues were defined around the grid generated within the EGFR. The EGFR-AQ4 (Erlotinb) complex inhibitor binding site comprises residues such as Leu 694, Gly 695, Ser 696, Ala 719, Lys 721, Glu 738, Leu 764, Thr 766, Glu 767, Leu 768, Met 769, Pro 770, Phe 771, Gly 772, Cys 773, Leu 820, Thr 830, Asp 831, Phe 832 within the 4 Å region surrounding AQ4. The binding site residues

of EGFR were defined 4 Å around co-crystal ligand using PDBsum.

About 77 existing ligands from literature and Erlotinib were docked with EGFR. Among all the docked complexes, Compound 3 exhibited least XPGscore of -9.439 Kcal/mol with good interactions with key amino acid residues (Table 1). Pharmacological descriptors and ADME/T profile of erlotinib and best docked ligands were determined using epic module of schrodinger. Results indicated that the selected existing ligands showed favorable ADME/T properties within the range of 95% FDA approved drugs. Results were represented in table 2 & table 3 (Pradhan et al. 2013).

Table 3. ADME/T properties of erlotinib and best docked compounds

Compounds	Rule of 5	Log P o/w	Log BB	Log KhSa	Log KP	Rule of 3
Erlotinib	0	4.053	-0.738	0.157	-0.602	0
3	2	5.738	-0.758	0.776	-1.141	1
7	1	4.854	-1.871	0.714	-2.897	1
24	1	4.795	-1.657	0.602	-2.413	1
33	0	4.319	-0.405	0.473	-1.648	0

Rule of 5: Violations of Lipinski's Rule of 5 (Max 4); Log P o/w = log P octanol/water (1.80/6.50); Log BB = log BB brain/blood (-3/1.2); Log KhSa = serum protein binding (-02.5/01.5); Log KP = skin permeability (KP in cm/h); Rule of 3: Violations Jorgensen Rule of 3 (Max 3)

Figure 1: Interactions of erlotinib with EGFR

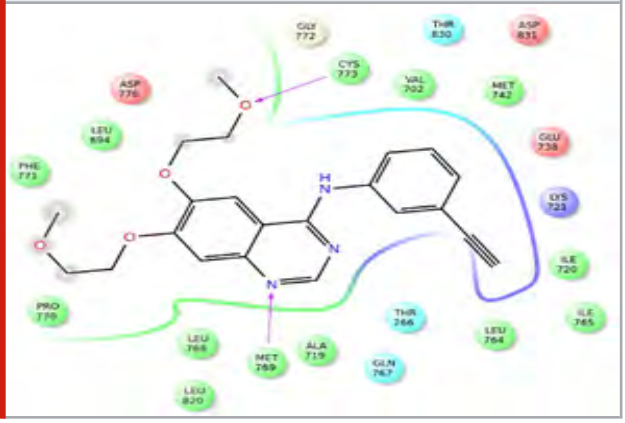


Figure 2: Interactions of compound 3 with EGFR

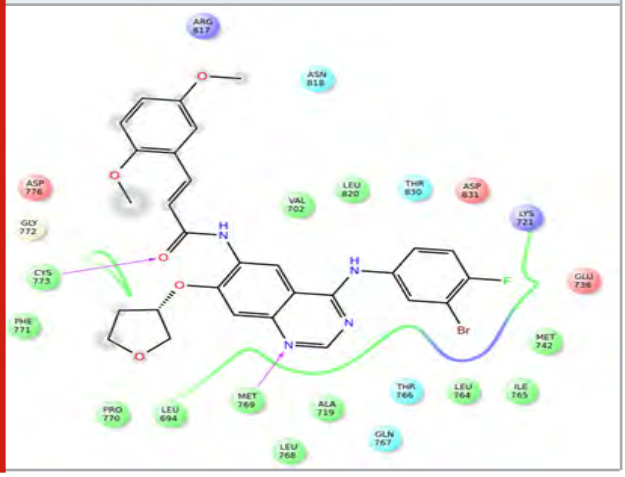


Figure 3: Interactions of compound 7 with EGFR

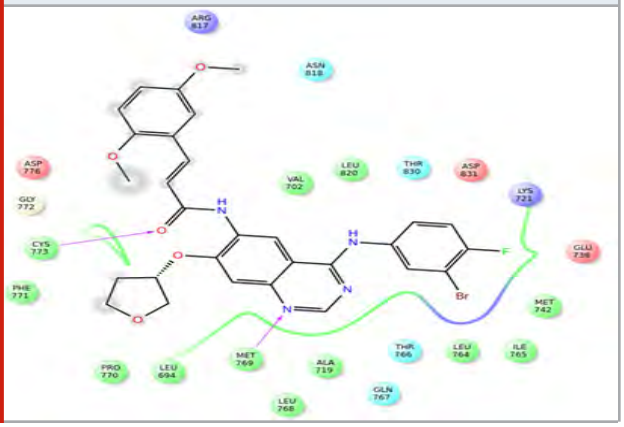


Figure 4: Interactions of compound 24 with EGFR

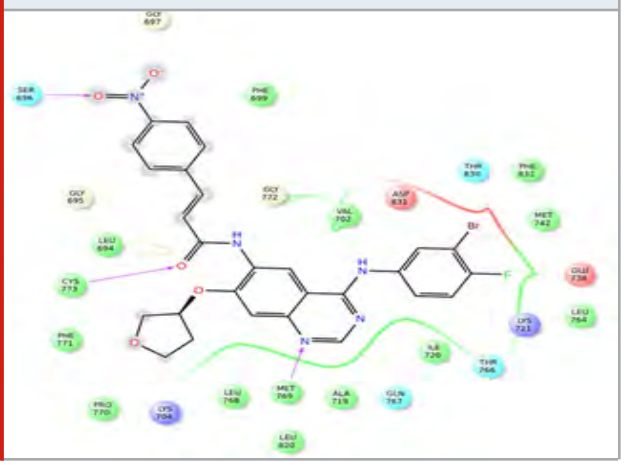
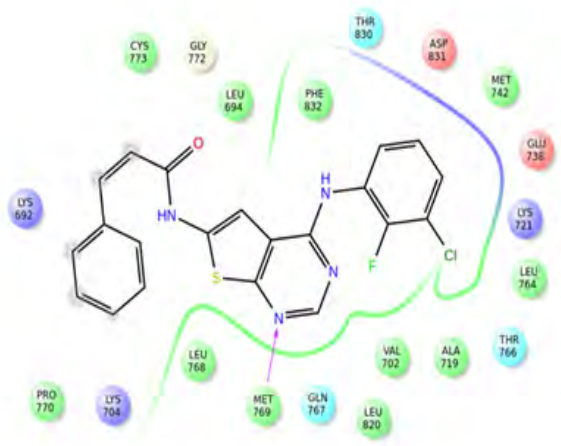


Figure 5: Interactions of compound 33 with EGFR



The co-crystal ligand erlotinb exhibited two hydrogen bond interactions with back bone residues of Met 769 and Cys 773 respectively. Among all the selected heterocyclic compounds, the compounds containing quinazoline heterocyclic nucleus (compounds 3, 7 and 24) exhibited good interactions with key aminoacid residues and two H-bonds with backbone residues of Met 769 and Cys 773 with docking scores -9.439Kcal/Mol, -9.402Kcal/Mol and -8.863Kcal/Mol respectively which is comparable to that of standard drug erlotinib. In addition to the interactions with Met 769 and Cys 773, compound 7 showed one H-bond interaction with backbone residue of Ser 696 indicating good EGFR inhibition. Among the compounds containing thienopyrimidine nucleus, compound 33 showed moderate binding affinity with XPG score -8.291 Kcal/Mol and exhibited only one H-bond interaction with Met 769 (Fig 1-5). All the best docked compounds exhibited good interaction with "hing region key residue" Met769 of EGFR, which is involved in the anticancer treatment strategies.

Table 4. Interaction fingerprint of the erlotinib and best docked compounds

Compound	Met 769									Cys 773								
	Any contact	H- bond(Backbone)	H-bond (Sidechain)	Salt Bridge	Pi-Pi stacking	Hydrophobic	Polar	Charged (Negative)	Charged (Positive)	Any contact	H- bond(Backbone)	H-bond (Sidechain)	Salt Bridge	Pi-Pi stacking	Hydrophobic	Polar	Charged (Negative)	Charged (Positive)
Erlotinib	1	1	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0
3	1	1	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0
7	1	1	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0
24	1	1	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0
33	1	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0

Best docked compounds 3, 7 and 24 exhibited H-bond interaction with backbone residues of key amino acid Cys-773 which is positioned within the EGFR ATP binding pocket (Fry et al. 1998; Nasab et al. 2018; Sudheer et al. 2021). A 9 bit interaction fingerprint was generated to determine interaction of best docked compounds in the inhibitor binding site of hERα (Table 4) and compared with standard drug erlotinib (Rajitha el al. 2021).. Results indicated that compounds with quinazoline nucleus exhibited similar interactions with that of erlotinib.

CONCLUSION

The findings of present study can help in designing the novel EGFR inhibitors with better binding affinity and interactions by using molecular docking studies of existing EGFR inhibitors against EGFR (1M17) and interaction fingerprint analysis. Among all the selected existing ligands, compound 3 exhibited good binding affinity with XPG score -9.439 Kcal/Mol which is comparable with that of standard drug erlotinib. Interaction fingerprint analysis further confirmed that the best docked compound showed two H-bond interactions with backnone residues of Met 769 and Cys 773 similar to that of standard Erlotinib. Results of the present study concludes that these derivatives properly fit into the ATP binding pocket in EGFR crystal structure with good binding affinity, indicating that they may act as potential EGFR inhibitors.

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Conflict of Interests: Authors declare no conflicts of interests to disclose.

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Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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A Survey on the Postpartum Depression Among Young Mothers in Kerala, India

Pooja Prasad and Balakrishnan Kalamullathil
Department of English and Languages, Amrita Vishwa Vidyapeetham, Kochi Campus, India

ABSTRACT

The increase in the number of cases of Postpartum Depression (PPD) in Kerala increases day by day. This makes a study on the awareness of PPD significant in this scenario. There are not many studies conducted on this area particularly a survey collecting details from young mothers. This study tries to quantify the awareness women in Kerala have about PPD and it also covers how they tackled the issue – various methods used by them to cope up with the issue. The study is conducted using an online-survey method. A prepared questionnaire is circulated online among 150 young mothers who were born and brought up in Kerala. The questionnaire consists of 8 questions about PPD and baby blues. Each question is provided with options from which the participants can choose one. The results of the survey are analysed to arrive in a conclusion. There was active participation from the side of the participants. The interpretation of the statistical data shows that even though most of the participants faced symptoms of Postpartum Depression, and 35.9% of the participants faced difficulty in bonding with the baby, only negligible percentage of them went to seek medical help. The results show that the percentage of participants who are well aware of PPD is very low. The study helped to interpret the situation in Kerala as far as PPD is considered. The study brought to light that Postpartum Depression in Kerala is an unaddressed issue and much attention and activities are needed to make changes in the current scenario. Most of the women who suffer PPD are reluctant to seek medical help. This situation has to be changed through proper campaigns and other related activities. As this study deals with a prevalent problem in the society it is significant in every aspect. This study prompts researchers to delve deeply in to the problems faced by women related to child birth and pregnancy and find out new ways to reduce the stigma associated with problems like Postpartum Depression.

KEY WORDS: ANXIETY, AWARENESS, COPING MECHANISM, POSTPARTUM DEPRESSION, TREATMENT.

INTRODUCTION

Postpartum Depression is a serious issue that is faced by many women in present-day society. Postpartum Depression (PPD) is a medical condition that can be cured with proper care and treatment. In women, depression can occur during and after pregnancy. Depression after delivery can occur as "baby blues" that last only for one or two weeks after childbirth. It has mild symptoms like mood swings, anxiety, and insomnia. A more severe condition is Postpartum Depression. It is long-lasting than baby blues and shows intense symptoms including anxiety and panic attacks, sadness, irritability, severe mood swings, problems in appetite, difficulty in bonding with your baby, thoughts about harming baby or yourself, severe anger, etc. The lack of awareness about Postpartum Depression increases the depth of the problem. Studies show that the lack of early detection of PPD also worsens the condition. The awareness about the real problem – that is, PPD is a serious issue that can affect the female's later life – can bring some changes in the present situation (Zauderer 2009; Miller 2002; Jayarajan 2021).

The purpose of the study is to bring the problem of PPD to the forefront and thus reduce the risk women face nowadays. Such a study is relevant where people are unaware of the seriousness of PPD and when no proper care is given to women suffering from PPD. The study tries to quantify women's awareness of the issue (Miller 2002).

MATERIAL AND METHODS

An in-depth analysis of Postpartum Depression was conducted using a survey. The study was conducted by circulating the prepared questionnaire among 150 young mothers. The sample of the study was selected after much research on the topic. The ages of the participants were in the range of 25 – 40. Mothers who gave birth in the last 10 years, and who were born and brought up in Kerala were

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considered for this study. This was done to examine about the recent developments in the area, especially in Kerala. The survey mainly aimed at checking the knowledge/ awareness women in Kerala had about PPD. Moreover, the study checked how they came to know about PPD and how far they are aware about the issue. It also helped to collect information about their personal experience of PPD, how they overcame it. The problem of bonding with the baby was also included as one question (Jayarajan 2021).

The questionnaire consisted of 8 questions about PPD and baby blues. These were intended to collect information about the level of awareness women had about PPD, how did they come to know about it, have they attended any awareness programs etc. The survey also tried to collect information about the coping mechanism the participants chose to overcome the situation. Responses to this particular question revealed how worse are the condition and what percentage of women seeks medical help. There were questions about the symptoms they had to suffer and about the span of time they experienced it.

Each of the questions was given options from which the participants chose one. The number of the options varied from question to question, that is, from 2 – 5. The research subjects were verified to be cognizant in English and all the questions were in English. The participants were well informed about the intension of the survey. They were informed to read the instructions clearly and answer the questions. It is also assured that their personal details will be kept confidential (Jayarajan 2021).

RESULTS AND DISCUSSION

The intensity of the psychic problems faced by women suffering from PPD needs to be discussed in detail, and a long-lasting solution has to be found out. An important part in reducing the percentage of patients can be done by giving proper awareness. Not only females but also males and people of other genders have to be educated about the mental condition of women during and after pregnancy. Medical professionals suggest providing proper awareness can considerably reduce the problem. This can contribute a lot to the healthy development of the baby and mother (Zauderer 2009; Jayarajan 2021).

Figure one shows that only about 53.9% of the participants heard about baby blues. This revealed the intensity of the situation. The participants included in the other section, that is, in the 46.1%, might be the ones who have been gone through the same. But it is understood that unawareness of the real problem worsens the condition. As baby blues last only for two or three weeks it is not a dangerous problem as PPD. But even in the case of PPD a considerable percentage of people who are unaware of PPD is found. Figure 2 shows that 14.8 % of the participants are unaware of postpartum depression. And the results showed that people are not much aware of a serious problem like PPD as they are about Baby Blues. This condition has to be changed through continuous practices to make the general public aware of the PPD and its symptoms (Jayarajan 2021).

There exists a social stigma in India, especially in Kerala, to consult a psychiatrist or a psychologist. This is another reason which prevents the cure of PPD through proper treatment. People are reluctant to admit the fact that they are facing some mental problems. The same kind of reluctance is there in the case of PPD also. They fear a kind of 'Othering' from the society and its roots can be traced back to the stigma towards madness that existed in the Middle Ages. This tendency limits the possibility to take proper medical care. This can also be controlled by conducting awareness programs and thus normalizing mental problems and depression (Foucault 1988; Kuriakose et al. 2020).

Figure 1: Pie chart showing the awareness of participants about Baby Blues

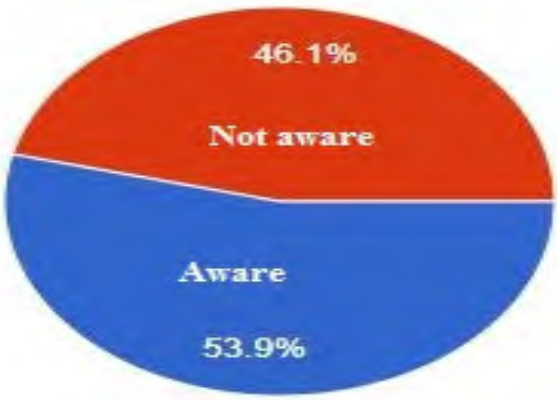


Figure 2: Pie chart showing the awareness of participants about PPD

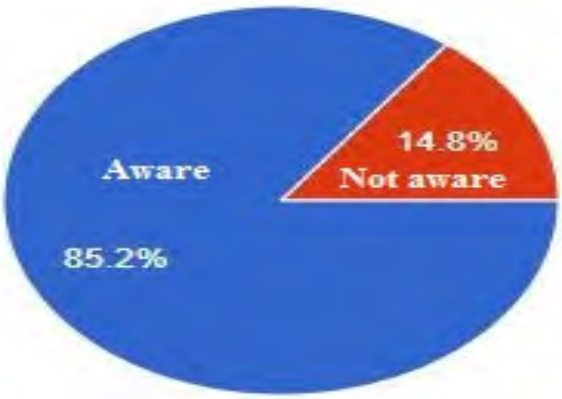


Figure 3. Pie chart showing the response of participants to whether they faced symptoms of PPD

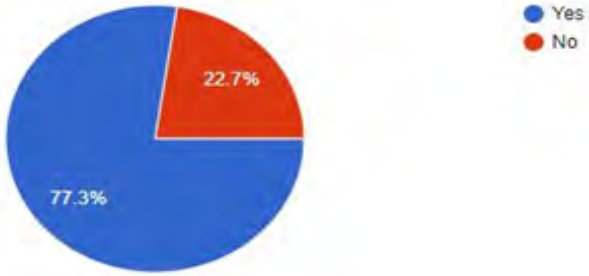


Figure 3 and 4 brings out the seriousness of the situation. In Figure 3, one can see that about 77.3% of participants faced the symptoms of PPD. This is proof of the problem faced by women after delivery. Figure 4 gives the period for which they faced these difficulties. 19.2% faced symptoms of PPD up to one year after delivery/C-section. For 28.3% it lasted for six months, and for 24.2% it lasted up to one month. Only 28.3% experienced it for two weeks after childbirth (Kuriakose et al. 2020).

Figure 4: Pie chart showing how much time did the participants felt the symptoms

Figure 5 shows that 8.6% of the participants found it difficult to create a bond with the baby and 27.3% of them felt the same for a short period. This is a period that is highly dangerous and may lead up to harming the child and oneself. Recent news reports give evidence to this where mothers in their depressive state harmed babies and some violent acts ended up in their death (Jayarajan 2021). The results of the survey indicate that most of the participants have gone through severe Postpartum Depression and all of them needed medical help. But Figure 6 shows the real scenario of Kerala's treatment of a serious mental issue like PPD (Jayachandran 2021).

Figure 5: Pie chart showing the level of difficulty the participants faced in bonding with the baby

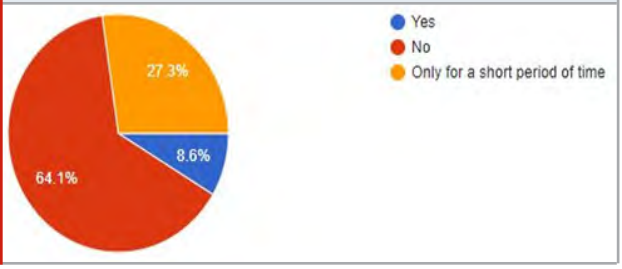
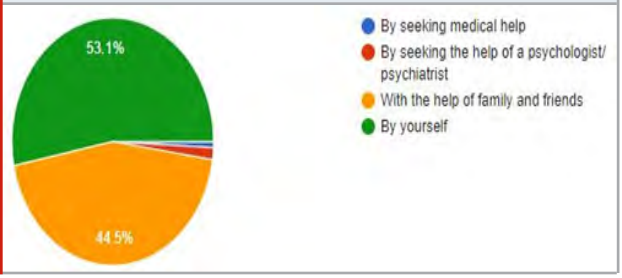


Figure 6: Pie chart showing how participants with PPD cope up with it



Even though most of the participants faced severe PPD related problems only 0.8% of them sought medical help and only 1.6% of them were ready to seek the help of a psychologist or psychiatrist. This reveals the pathetic condition of Kerala concerning PPD. This situation can be changed by giving proper awareness about the issue. The collected data shows that only 7% of the participants had attended any awareness programs related to PPD. Most of them learned about this issue through social media. And some of them learned through articles and only 1.7% of them got informed through the newspapers. This shows that the government, health department, as well as the public, have to play a significant role in reducing the difficulties faced by mothers and newborns (Kuriakose et al. 2020).

CONCLUSION

The findings of the present study has quantified the awareness young mothers in Kerala have about PPD. As this study deals with a health-related issue which is related to the well-being of the society, it has significance in the present scenario. This study prompts studies in the future that may lead to the change in current situation. Proper awareness about the issue can be given through newspapers, news channels, social media and other media. Constant active participation from the public and initiatives from the government are needed to improve the situation.

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Conflict of interests: There was no conflict of interest among the authors. Moreover, all the authors provided equal input for the paper.

Ethical Statement: The present study has been approved by the Institutional Ethics Committee of **Amrita Vishwa Vidyapeetham, Coimbatore**. (Name of Institute/ University) All due permissions have been taken by the concerned authorities including consent etc.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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On the Diversity of Beetles in the Baghdad Campus, Islamia University of Bahawalpur, Pakistan

Khansa Nadeem,[1] Nuzhat Sial,[1] Mariyam Javed,[1] Sobia Abid,[1]* Fozia Afzal[1] and Aqsa Yasin[2]

[1]The Islamia University of Bahawalpur, Department of Zoology, Pakistan

[2]Department of Entomology, National Chung-Hsing University, Taichung, Taiwan

ABSTRACT

Coleoptera fauna in Baghdad Campus was investigated from July 2020 to April 2021. Three habitats included inter dunal sandy area, core desert area, and area in and around the hostel which have been named as H-1, H-2 and H-3 respectively. Techniques like hand picking, plant jerking and pitfall trapping were adopted for sampling in order to explore distribution and diversity of Coleoptera fauna. 1037 samples were collected and preserved in 70% alcohol. Samples were identified on the basis of morphological characters by using bug guide. 7 families and 16 genera were sorted out. The family Tenebrionidae include the genera *Blaps*, *Eleodes*, *Pimelia*, *Gonocephallu*, and *Eusattus* was present. The family Coccinellidae includes genera Coccinella was also found. The family Carabidae includes Anthia, Scarites and Calosoma. The family Elateridae, Carculionidae, and Chrysomelidae contains genera Agriotes, Rhynchophorus, and Altica respectively. The Family Scarabaeidae contains the Thyce, Heteronychus, Tomarus, Phyllophagaa genera. In total count, maximum diversity and abundance of Altica was reported. Genus Anthia, Scarites, Agriotes, Rhynnchophorus, Heteronychus, and Tomarus were found in minimum but equal number. In habitat-3, maximum diversity was observed as compared to other habitats. It is concluded that these findings seemed to be helpful in ecological management of the beetles .

KEY WORDS: OLEOPTERA, MORPHOLOGY, DIVERSITY, ABUNDANCE, ECOLOGICAL MANAGEMENT.

INTRODUCTION

Coleoptera is an order of insects commonly called beetles. The word “coleoptera” is derived from two Greek words; kelos, meaning “sheath” and pteron, meaning “wing” so called “sheathed wing”. The reason for naming is that most beetles have two pairs of wings. The front pair is known as “elytera” which is hard and thick like a sheath. It is for protection of rear pair and for the rear part of body (Hunt et al. 2007: Wagner et al. 2021). In animal kingdom, Coleoptera is the largest order (Nieto and Alexander 2010). This order has more species than any other order, consisting of almost 25% of all known life forms (Ruchin et al. 2021). Identified and described species of beetles are about 40% (about 400,000 species) and new species are also being discovered. According to some estimates, the total number of species are as high as 100 million (Ruchin et al. 2021).

Beetles are adorned with bright metallic coloration. Though, they may also be dull black or brownish. Some are gorgeously coloured like ladybugs (García et al. 2021). In size, they range from the smallest (10.25 mm) to the largest (cerambycids 150 mm long). The beetles have variations in their habitats. They have wide range of distribution and adaptations. They exist in soil, stored grains, humus, rotten wood, flowers, decaying organic matter, furniture, bark of trees and museum specimens; some are also present in water and near water (Rossa and Goczał 2021).

Approximately, ¾ of beetle species feed on plants in both immature and mature stages. Beetles are important as they play vital role in the ecosystem. Some beetles act as decomposers in ecosystem. They feed on dead plants and fungi (Bug guide). Most of them destroy crops, forests, and other economically important plants. Thus, the beetles are known as pest (Souza et al. 2021). However, members of subfamily Epilachninae feed on plants and are considered as serious pests of important agricultural crops like pea and sunflower (Ćurčić et al. 2021). Some beetle species are invasive. They have capacity to destroy 30.3% of the urban trees (Nowak et al. 2001). The beetle’s capability to attack multiple genera of healthy hardwood trees could intensely change urban and forest ecosystems. Initially, precise recognition of this invasive pest is important to determine swarms before they become unmanageable (Biffi et al. 2021).

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Beetles are not only pest but also beneficial. They have a role in controlling the populations of pests. One of the well-known examples is the ladybug (family: Coccinellidae). Both the larvae and adults eat aphids and soft bodied insects to assist in regulating pest population. Some feed on mealybugs and insects (Biffi et al. 2021).

If food is less available, they may feed on other sources like nectar (Brown et al. 2010; Čurčić et al. 2021). Beetles have ability to mimic for their protection. Mimicry provides one of the clearest examples of significance of natural selection (Banerjee 2014). The mimetic system is being investigated on different levels. It includes the heredities and evolution of warning coloration and the factors causing distastefulness. Discrepancy in chemical defense as a result of individual differences in physiology, rather than differences in the host plant, has been less investigated. It was due to the trouble normally experienced in precise quantifying toxic components (Wang et al. 2021).

MATERIAL AND METHODS

Abundance and species richness of beetles were studied in three different habitats of Baghdad-ul-Jadeed Campus, The Islamia University of Bahawalpur. The experimental sites were named as Site H-1, interdunal sandy area near human habituation, Site H-2, core desert area and Site H-3, area in and around hostel. The field survey was carried out on weekly basis from July 2020 to April 2021. In experimental habitats, beetles were collected by hand picking, trapping through pitfall and plant jerking techniques. Hand picking was done for random picking of beetles in selected sites and plant jerking was used to capture beetles from flowers. After collection, the experimental samples were preserved by using 10% formalin solution or 70% ethanol. The specimens were identified on basis of morphometric characters. It was done by using online keys, diagrams, and the bug guide. Shannen's weiner and Simpson's diversity index is calculated for the available research data in MS. Excel.

RESULTS AND DISCUSSION

Mean temperature and rainfall were observed during study period. Highest temperature was noticed in July i.e., 92°F and minimum temperature was in January i.e., 54.5°F (Accweather). The sites showed maximum diversity in June and July (monsoon season). This season is resourceful and fortunate for growth and survival of Coleoptera fauna. It is true that insects avoid harsh winter through diapause, thus diversity of beetles in all sites are least in winter months. (Banerjee 2014). Similar results were found in the present research.

Table 1. Distribution of Coleoptera in different habitats of Baghdad Campus, Bahawalpu

Class	Order	Family	Genera	H-1	H-2	H-3	Total
Insecta	Coleoptera	Tenebrionidae	*Blaps*	33	17	4	54
			Eleodes	1	1	0	2
			Pimelia	30	15	4	49
			Gonocephallum	1	0	3	4
			Eusattus	128	65	36	229
		Coccinellidae	*Coccinella*	4	1	60	65
		Carabidae	*Anthia*	1	0	0	1
			Scarites	0	0	1	1
			Calosoma	0	0	30	30
		Elateridae	*Agriotes*	0	0	1	1
		Carculionidae	*Rhynchophorus*	0	0	1	1
		Chrysomelidae	*Altica*	363	154	67	584
		Scarabaeidae	*Thyce*	0	0	10	10
			Heteronychus	1	0	0	1
			Tomarus	0	0	1	1
			Phyllophagaa	0	0	4	4
Shannon's Weiner Diversity Index					1.07	1.00	1.75
Simpson's diversity index					0.45	0.46	0.21
Dominance Index					0.54	0.53	0.78
Number of individuals							1037
Number of genera							16

The field survey was carried out from July 2020 to April 2021. During this period, 1037 samples of Coleoptera fauna were collected from three habitats like H-1, H-2, and H-3. After identification, 1037 samples were comprised of 1 order, 7 families and 16 genera. In total count, maximum diversity of genus *Altica* was found and *Anthia, Scarites, Agriotes, Rhynchophorus, Heteronychus* and *Tomarus* showed minimum but almost equal diversity. Genus Blaps is present

in different habitats of Baghdad Campus, Bahawalpur. From July 2020 to April 2021, 33 individuals from habitat-1, 17 from habitat-2 and 4 from habitat-3 were collected. Total number of collected beetles was 54. Maximum number was seen in habitat-1 and minimum in habitat-3. Genus *Eleodes* samples during collection period were 1 in habitat-1, 1 in habitat-2 and none in habitat-3. Total number of sampled beetles in all habitats was 2. Maximum number was reported in habitat-1 and habitat-2. 30 individuals of genus *Pimelia* were captured from habitat-1, 15 individuals from habitat-2. Its 4 individuals were present in habitat-3. Total samples were 49. Its maximum number was found in habitat-1. Minimum distribution was observed in habitat-3 (Biffi et al. 2021).

Genus *Gonocephallum* belongs to family Tenebrionidae and its collected individual was only 1 in habitat-1. None was found in habitat-2. 3 samples were collected from habitat-3. Total number of reported beetles was 4. Its maximum number was found in habitat-3. Genus *Eusattus* is from the family Tenebrionidae and total number of reported specimens was 129. 128 beetles were present in habitat-1, 65 in habitats-2 and 36 in habitat-3. Maximum number was observed in habitat-1and minimum number was in habitat-3.

It was the second largest genus reported during research conduction. Genus Coccinella belongs to the family Coccinellidae. 4 samples were found in habitat-1, 1 ladybird was seen in habitat-2 and 60 samples were collected from habitat-3. Total 65 ladybirds were collected in selected sites. Maximum number was seen in habitat-3 and minimum number in habitat-2. Genus Anthia is carabid beetle in the family Carabidae. Only one sample was sorted out from habitat-1. No beetle was captured from habitat-2 and habitat-3 during research period. Genus *Scarites* belongs to the family Carabidae. No beetle was found in habitat-1 and habitat-2. Only one beetle was collected from habitat-3. Its reported individuals were minimum during research conduction. Beetles of the genus *Calosoma* were found in only habitat-3. Total numbers of samples reported were 30 from habitat-3. No beetle was seen in habitat-1 and habitat-2. It was in minimum number during the study period.

Genus *Agriotes* is from the family Eleteridae. It was only observed in habitat-3. Only 1 sample was present in habitat-3. No beetle of this genus was found in habitat 1 and habitat-2. Genus *Rhynchophorus* belongs to the family Carculicidae. Only one weevil was sorted out in habitat-3 during research period. It was absent in both habitat-1 and habitat-2. Genus *Altica* belongs to the family Chrysomelidae. 363 individuals were collected from habitat-1 during the field observations. 154 individuals in habitat-2 and 67 individuals in habitat-3 were found. Maximum individuals were reported in habitat-1. Minimum number was reported in habitat-3. It is the largest genus sorted out during research conduction in Baghdad Campus, Bahawalpur. Genus Thyce is placed in the family Scarabaeidae. It was absent in habitat-1 and habitat-2. 10 individuals were reported in habitat-3. Total samples of this genus were 10 during collection period (Biffi et al. 2021; Souza et al. 2021).

Genus *Heteronychus* belongs to family Scarabaeidae. It was found in habitat-1 only. Sample collected from habitat-1 was 1. It was absent in habitat-2 and habitat-3. The family scarabaeidae also includes genus *Tomarus*. Only one beetle was observed in habitat-3. None was found in habitat-1 and habitat-2. Genus *Phyllophaga* is also included in the family Scarabaeidae. It was absent in habitat-1 and habitat-2. Its samples sorted out from habitat-3 were 4.

CONCLUSION

The present research has been conducted for taxonomic understanding of Coleoptera fauna in Baghdad Campus, Bahawalpur. After identification, 1037 samples were comprised of 1 order, 7 families and 16 genera. In total count, maximum diversity of genus *Altica* was found and *Anthia, Scarites, Agriotes, Rhynchophorus, Heteronychus* and *Tomarus* showed minimum but almost equal diversity. All identified genera are affected by type of vegetation, temperature and rainfall.

Conflict of Interest: There is no conflict of interest

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Characterization of Plant Growth-Promoting Activity of Bacteria Isolated from Forest and Coastal Regions of Saurashtra, Gujarat, India

Vivek B. Pattani*, Jinesh P. Kaneriya, Krishna Joshi and Gaurav V. Sanghvi
Department of Microbiology, Marwadi University, Rajkot, Gujarat, India

ABSTRACT

The haphazard application of chemical fertilizers and pesticides causes tremendous damage to ecosystems and all biota. One of the most effective ways to tackle the threat is to use biofertilizer. Plant growth promoting bacteria (PGPB) are an important bacterial source for microbial fertilizers that can boost agricultural yields by encouraging plant growth. Bacterial isolates isolated from Saurashtra region, Gujarat, India were analysed for their capability to solubilize inorganic 'P' from tri calcium phosphate and production of indole acetic acid (IAA) quantitatively by bacterial. Production of ammonia, siderophore and hydrogen cyanide (HCN) by selected bacteria isolates was analysed. Biochemical characterization of selected bacterial isolates was done using Vitek 2 Compact system. Isolate GFS15C2 showed highest amount of phosphate solubilization, followed by isolate GFS07C1 and GFS01C1. Bacterial isolate GFS15C2 produced highest amount of IAA. All bacterial isolates were able produce ammonia. Eight bacteria isolates were be to produce HCN. Siderophore was produced by 14 bacterial isolates. In biochemical characterization all the bacterial isolates were able to use D-glucose. Based on biochemical characters clustering of bacteria isolates was done using Paleontological statistics software package for education and data analysis(PAST). Using cluster analysis by euclidean distance method based on biochemical characterization isolates GFS16C2 & SCS12C3 was found to have distinct characters than other isolates. The present study attempts to characterize PGPB which could be harnessed to improve plant growth. Several phosphate solubilizers and IAA producers also showed production of siderophores and HCN which suggests that these organisms do possess biocontrol ability. These PGPB microbial inoculants can be utilized to improve agricultural systems or as an alternate means of environmentally friendly plant disease biocontrol.

KEY WORDS: BACTERIA, HCN, IAA, PHOSPHATE, VITEK.

INTRODUCTION

Application of haphazard use of chemicals fertilizers and pesticide leads to incredible harm to ecosystems and all biota. Alternative to these chemical applications, biofertilizer are one of the best solutions to combat the hazard. These microbes can be used as both plant growth promoting bacteria and as biocontrol agent for suppression of plant pathogenic microbes (Kour et al. 2019; Sharma et al. 2020). The most commonly known plant growth promoting bacteria are *Bacillus, Rhizobia, Pseudomonas, Azotobacter* etc. PGPB, it has the ability to fix nitrogen fixation to solubilize phosphate, iron chelation by siderophores and phytohormone production whereas, being a biocontrol agent, it has the ability to produce HCN and secondary metabolites to suppress the growth of plant pathogenic microbes (Backer et al. 2018; Swarnalakshmi et al. 2020).

Article Information:*Corresponding Author: vivek.pattani@gmail.com
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Hence, its multifaceted capability makes it appropriate as biofertilizer and biocontrol agent in agriculture field. PGPB compounds can exhibit a number of features that influence plant growth, either directly or indirectly. Auxin, gibberellin, ethylene, and other plant growth regulators are produced, as are siderophores, HCN and antibiotics, as well as phosphate solubilization (Suresh et al. 2020; Rawat et al. 2020). The synthesis of siderophore for iron sequestration and the auxin IAA, which has been linked to plant growth promotion, notably root initiation and elongation, is one of the most significant processes involved in plant promotion (Prasad et al. 2019; Morales-Cedeno et al. 2021).

Microbial inoculants provide a number of benefits over chemical inoculants (Vassilev et al. 2020). They are environmentally friendly, sound sources of renewable nutrients that are necessary for soil health and life (Basu et al. 2021). They also fight abiotic stressors and have antagonistic action against a variety of crop diseases. Based on their capacity to absorb nutrients from the soil, fix atmospheric nitrogen, induce nutrient solubilization, and

function as biocontrol agents, a variety of microbial taxa have been commercially employed as effective biofertilizers (Tabassum et al. 2017; Kenneth et al. 2019; Kumar et al. 2021). Plant growth promoting bacteria were isolated from the Gir forest coastal region of Saurashtra, Gujarat. using the soil samples by screening for nitrogen fixing bacteria and further screening was done for phosphate solubilizing bacteria and production of IAA producing bacteria was also done (Kaneriya et al. 2021).

Table 1. Solubilization of Phosphate by bacterial isolates and change in pH on day 5 & 10 (P Indicates Solubilised phosphate and values indicate mean standard error).

Day		Day 5		Day 10	
Sr.	Isolate	P (mg/L)	pH	P (mg/L)	pH
1	GFS01C1	50.48 ± 0.12	5.11 ± 0.05	106.33 ± 0.68	4.19 ± 0.11
2	GFS03C1	44.18 ± 1.08	5.06 ± 0.01	93.73 ± 2.76	4.14 ± 0.15
3	GFS04C1	42.72 ± 0.69	5.07 ± 0.04	90.80 ± 1.96	4.15 ± 0.14
4	GFS05C2	43.53 ± 0.94	5.10 ± 0.02	92.42 ± 2.18	4.18 ± 0.16
5	GFS07C1	55.45 ± 0.83	5.03 ± 0.06	115.23 ± 1.28	4.12 ± 0.17
6	GFS08C1	41.85 ± 0.71	5.02 ± 0.06	89.06 ± 1.84	4.11 ± 0.17
7	GFS10C1	44.51 ± 1.38	4.97 ± 0.07	94.38 ± 3.37	4.06 ± 0.20
8	GFS11C1	48.43 ± 0.75	5.08 ± 0.04	102.23 ± 2.05	4.17 ± 0.15
9	GFS12C1	42.52 ± 0.86	5.05 ± 0.05	90.41 ± 2.39	4.14 ± 0.15
10	GFS13C1	35.15 ± 0.36	5.41 ± 0.15	56.75 ± 1.01	4.50 ± 0.19
11	GFS15C2	57.22 ± 1.60	4.98 ± 0.06	111.30 ± 2.22	4.06 ± 0.14
12	GFS16C1	49.83 ± 0.80	5.10 ± 0.06	105.02 ± 2.26	4.19 ± 0.10
13	GFS16C2	49.20 ± 0.79	5.06 ± 0.04	103.76 ± 2.24	4.15 ± 0.14
14	GFS19C2	45.34 ± 1.15	5.08 ± 0.05	96.04 ± 2.70	4.17 ± 0.11
15	SCS03C1	50.92 ± 0.31	5.05 ± 0.02	93.46 ± 0.88	4.14 ± 0.17
16	SCS04C1	43.53 ± 0.64	5.10 ± 0.04	78.68 ± 1.50	4.19 ± 0.12
17	SCS07C3	41.43 ± 0.84	5.09 ± 0.05	74.49 ± 2.02	4.17 ± 0.11
18	SCS12C1	43.22 ± 0.58	5.17 ± 0.04	78.07 ± 1.48	4.26 ± 0.17
19	SCS12C2	54.41 ± 2.00	5.07 ± 0.06	95.51 ± 1.38	4.15 ± 0.20
20	SCS12C3	42.07 ± 0.75	5.12 ± 0.04	75.76 ± 1.81	4.21 ± 0.12
21	SCS12C4	49.26 ± 0.81	5.05 ± 0.03	90.15 ± 1.97	4.14 ± 0.14
22	SCS12C5	48.43 ± 0.75	5.14 ± 0.05	88.49 ± 1.85	4.22 ± 0.20
23	SCS06S1	42.52 ± 0.86	5.06 ± 0.04	76.67 ± 2.24	4.15 ± 0.16

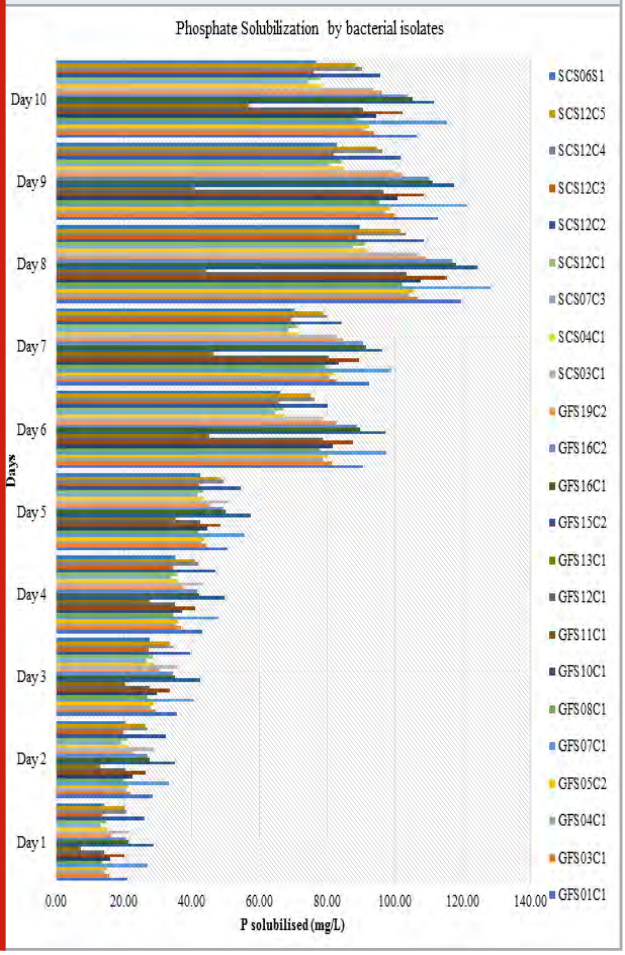
MATERIAL AND METHODS

Bacterial isolates were analysed for their capability to solubilize inorganic 'P' from tri calcium phosphate. The soluble P was determined by using colorimetric chloro-stannous reduced molybdo-phosphoric acid blue method (Dipak and Abhijit 2005). Experiments were set in triplicates and phosphate solubilization and change in the pH of the medium were analysed for ten days. The blue colour produced was measured at 660 nm using UV-1800 UV-VIS Spectrophotometer, Shimadzu. The amount of P2O5 solubilized was expressed in P mg/L of the medium. Isolates which showed multiple properties of nitrogen fixation, phosphate solubilization and IAA production were further analysed for siderophore production, quantitative estimation of phosphate solubilization and IAA.

The production of siderophore with the universal chemical test method for quality detection of siderophore production in fluid crops were tested for bacterial isolates (Schwyn and Neilands 1987). This experiment was carried out using a modified method that was provided by Hu and Xu (Arora and Verma 2017). For inferring the presence of siderophore, the tubes were monitored for colour changes from dark blue to light blue or orange. Uninoculated medium was used as control. Production of IAA was estimated by growing the bacterial isolates in yeast extract-mannitol broth and minimal medium supplemented with 200 µg/ml

L-tryptophan, which acts as a precursor for IAA synthesis at 28 ± 1 ºC. After 48 hours, 5 ml cultures were centrifuged at 10,000 RPM for 15 minutes at 4 ± 1 ºC and the supernatant was collected (Pereira et al. 2019). Experiments were set in triplicates to analyse the production of IAA. Development of pink colour indicates production of IAA and its optical density was recorded at 530nm using UV-1800 UV-VIS Spectrophotometer, Shimadzu. Concentration of IAA produced was estimated against standard curve of IAA (Hi-media) in the range of 10–100 µg /ml. Selected bacterial isolates were analysed for the production of ammonia in peptone water. Cultures were inoculated in 10 ml peptone water and incubated at 28±2 °C for 48 to 72 hours.

Figure 1: Plot for Phosphate Solubilization by bacterial isolates from day 1 to day 10.



Nessler's reagent (0.5 ml) was added in each tube. Ammonia production was indicated by the change in colour from brown to yellow (Cappuccino and Welsh 2017). Selected bacterial isolates were analysed for the production of hydrogen cyanide by the method given by Lorck (1948). Parafilm was used to seal the plates, which were then incubated at 28±2 °C for 4 days. HCN production was indicated by the colour changing from yellow to red (Slama et al. 2019). Biochemical characterization of the selected bacterial isolates was done using Vitek 2 compact, Biomerieux Inc. All the descriptive statistics tests were performed using XLSTAT for Windows (Fahmy 2016). Graphs were created using Microsoft Office Excel 2019. Cluster analysis based on Euclidean distances was done using PAST software by (Hammer et al. 2001).

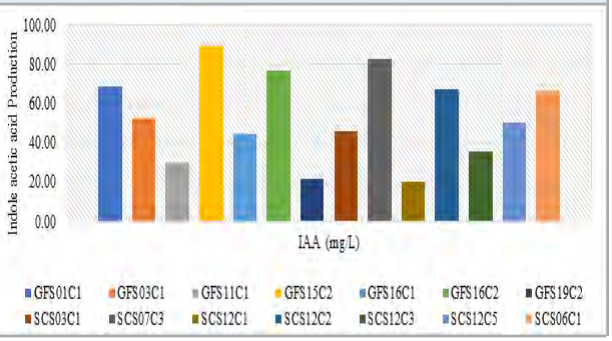
RESULTS AND DISCUSSION

The present study reflects the work done to characterize plant growth promoting parameters isolated from forest and coastal regions of Saurashtra, Gujarat and analyse their biochemical parameters. For soil microorganisms, rhizosphere is appropriate endurance specialty because of rich supplement accessibility (Khatoon et al. 2020). Community of life forms living in a specific specialty is explicit and subject to the actual elements and ecological variables (Mony et al. 2020). Phosphate solubilization is one vital attribute of plant development advancement as microorganisms solubilize insoluble phosphate making it accessible for plants (Joshi et al. 2021). Quantitative analysis of phosphate solubilization: Bacterial isolates GFS01C1, GFS03C1, GFS04C1, GFS05C2, GFS07C1, GFS08C1, GFS10C1, GFS11C1, GFS12C1, GFS13C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS04C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3, SCS12C4, SCS12C5 and SCS07C2 were analysed for the phosphate solubilization (Mony et al. 2020).

Table 2. Production of IAA by bacterial isolates. (Value indicates mean standard error)

Isolate	Indole acetic acid (mg/L)	Isolates	Indole acetic acid (mg/L)
GFS01C1	69.02±0.72	SCS03C1	45.87±0.93
GFS03C1	52.89±1.04	SCS07C3	82.77±1.02
GFS11C1	29.95±0.41	SCS12C1	19.97±0.89
GFS15C2	89.43±1.01	SCS12C2	67.07±0.74
GFS16C1	44.50±0.81	SCS12C3	35.81±0.54
GFS16C2	76.62±0.89	SCS12C5	50.65±1.01
GFS19C2	22.07±0.93	SCS06C1	66.93±0.89

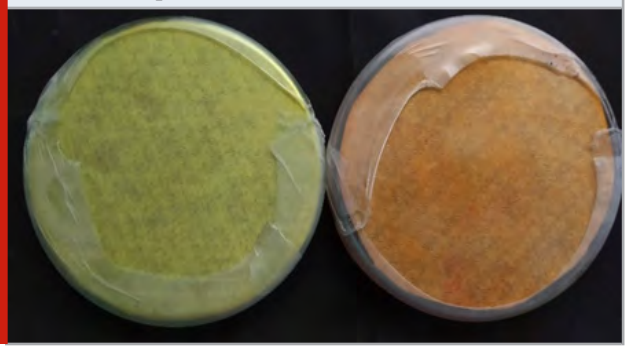
Figure 2: Plot for Production of Indole acetic acid by bacterial isolates.



Solubilization of Phosphate by bacterial isolates and pH reduction of the medium for was analysed for 10 days and of which values of P solubilized on day 5 & 10 are tabulated in the Table 1. Graphical representation for P Solubilization

by bacterial isolates from day 1 to day 10 has been given in figure 1. It was observed that over a period of time, the un-inoculated medium detected no soluble P and no drop in pH as well. In case of inoculated medium solubilization of the phosphate was observed with the decrease in pH. Bacterial isolate GFS15C2 showed highest amount of phosphate solubilization, followed by isolate GFS07C1, GFS01C1, GFS16C1, GFS16C2, GFS11C1, SCS03C1 and SCS12C4. Isolate GFS13C1 showed lowest phosphate solubilization. Marra et al. (2019) reported that all separates which solubilize tri-calcium phosphate (TCP) in fluid and medium could create natural acids causing the pH of the medium acidic. Similarly, all of the isolates that can solubilize TCP in the current investigation demonstrated a decline in the pH of the medium. Similar observation was made Lebrazi et al. (2020). This drop in the pH is supported by secretion of different natural acids by use of sugar (Marra et al. 2019; Lebrazi et al. 2020).

Figure 3: HCN production by bacterial isolates yellow coloured plate (negative control) and orange coloured which indicated the production of HCN.



Production of Siderophore: Selected bacterial isolates were analysed for production of siderophore Bacterial isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3, SCS12C5 and SCS06C1 indicated the production of siderophores. Total 14 bacterial isolates produced siderophores. Bacterial isolates were further screened on the basis of siderophore production and were analysed for their capability to produce IAA quantitatively. Other influential traits of PGPB, which can indirectly enhance plant growth is by producing siderophores, chitinase and HCN, which protects plants against pathogens (Athira and Anith 2020; Legein et al. 2020). Under present study, important traits such as siderophore and HCN production have been assessed *in vitro*.

Indole acetic Acid: Bacterial isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3, SCS12C5 and SCS06C1 which indicated the production of siderophores & higher amount of phosphate solubilization were further analysed for the quantitative assay of indole acetic acid. Production of IAA by bacterial isolates analysed is tabulated in the table 2 and plotted in figure 2. Bacterial isolate GFS15C2 (89.43 mg/L) produced highest amount of IAA followed by SCS07C3, GFS16C2, GFS01C1, SCS12C2 and SCS06C1. Bacterial isolates SCS12C1 lowest. IAA synthesis is an important characteristic of PGPR because this phytohormone allows the plant to form a well-organized root system, which improves nutrient absorption (Kumar et al. 2019). IAA have been reported to stimulate the growth of roots and leaves in plants thereby alleviating plant productivity (Bhat et al. 2020). Among the all the bacterial isolates bacterial isolate GFS15C2 was found produced highest IAA. The IAA produced by bacteria varies between different species and strains. This variation is possibly affected by several factors, such as physiological condition of bacterial isolates, growth stage, and substrate availability (Lebrazi et al. 2020a).

Ammonia Production: Bacterial isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3, SCS12C5 and SCS06C1 were analysed for the production of ammonia. All isolates were able to produce ammonia. HCN production: Bacterial isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3, SCS12C5 and SCS06C1 were analysed for the production of HCN. Week HCN production was indicated by a little change in the colour of Whatman filter paper, with yellow-coloured paper turning pale orange. Bacterial isolates GFS01C1, GFS11C1, GFS15C2, GFS16C2, GFS19C2, SCS03C1, SCS07C3 and SCS12C2 were able to produce HCN.

Total 14 isolates showed siderophore production and 8 showed HCN production. Several of the strains that could inhibit pathogenic *Fusarium oxysporum* growth also had the potential to produce HCN, siderophores, and antibiotics (Karthika et al. 2020, Bandopadhyay et al. 2019). As a result, indirect PGP features are just as significant as direct PGP factors for total plant development (Shastri et al. 2020). Biochemical characterization of bacterial isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3, SCS12C5 and SCS06C1 was done using Vitek 2 compact and data was tabulated in the table 3 & 4. By use of Vitek 2 compact, 47 biochemical characterization tests such as carbohydrate fermentation, lipase, decarboxylases, phosphatases etc can be analysed. All the isolates where able to ferment the D-glucose. Bacterial isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C2, GFS19C2 and SCS12C3 were able to produce phosphatase. Biochemical characterization was carried out to analyse the various biochemical properties of bacterial isolates (Athira and Anith 2020).

Biochemical characterization using Vitex 2 compact was done to analyse biochemical characterization of bacterial isolates. Isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3, SCS12C5 and SCS06C1 were not able to utilise adonitol, D-tagatose and L-arabitol. None of the bacterial isolates were able to produce lipase, ornithine decarboxylase, lysine decarboxylase, Beta-xylosidase, urease, beta-glucuronidase 5-Keto-D-Gluconate and beta-alanine arylamidase. None of

the bacterial isolates were able to assimilate of L- malate. All the bacterial isolates were negative for Ellman reaction and fermentation of glucose. All the bacterial isolate were able to acidify D-glucose which basically leads to drop in pH of the medium.

Table 3. Biochemical characterization of bacterial isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS06C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3 and SCS12C5 using Vitek 2. (Sr. 1 to 23)

Sr	Biochemical tests	GFS 01 C1	GFS 03 C1	GFS 01 C1	GFS 15 C2	GFS 16 C1	GFS 16 C2	GFS 19 C2	SCS 03 C1	SCS 06 C1	SCS 07 C3	SCS 12 C1	SCS 12 C2	SCS 12 C3	SCS 12 C5
1	Ala-Phe-Pro-Arylamidase	-	+	+	-	-	+	-	-	-	-	-	-	+	-
2	Hydrogen Sulfide	(+)	+	+	-	-	-	-	-	-	-	-	+	-	-
3	Beta-Glucosidase	-	-	-	+	-	+	+	+	-	-	-	-	+	+
4	L-Proline Arylamidase	-	-	-	-	-	+	-	-	-	+	-	-	+	-
5	Saccharose/ Sucrose	+	-	+	-	-	-	+	+	-	-	-	-	-	+
6	L-Lactate Alkalinisation	+	-	-	+	+	-	+	-	+	+	+	-	-	+
7	Glycine Arylamidase	-	-	-	-	-	+	-	-	-	+	-	-	+	-
8	O/129 Resistance (Comp.Vibrio.)	+	+	+	+	+	-	+	+	+	+	+	+	-	-
9	Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	Beta-N-Acetyl-Glucosaminidase	+	+	+	+	-	+	-	-	-	-	-	-	+	-
11	D-Maltose	+	+	+	+	-	+	+	+	+	-	-	+	+	+
12	Lipase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	Alpha-Glucosidase	+	+	+	+	-	+	+	+	+	-	-	+	+	+
15	Ornithine Decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	Glu-Gly-Arg-Arylamidase	-	-	-	-	-	+	-	-	-	-	-	-	+	-
17	L-Pyrrolydonyl-Arylamidase	+	+	+	+	-	+	+	+	+	+	-	+	+	+
18	Glutamyl Arylamidase pNA	-	-	-	-	-	+	-	-	-	-	-	-	+	-
19	D-Mannitol	-	-	-	-	-	-	+	+	+	-	-	-	-	+
20	Palatinose	-	-	-	-	-	-	+	+	+	-	-	-	-	+
21	D-Trehalose	+	+	+	+	-	-	+	+	+	-	-	+	-	+
22	Succinate Alkalinization	-	-	-	-	+	-	-	-	-	-	+	-	-	-
23	Lysine Decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-

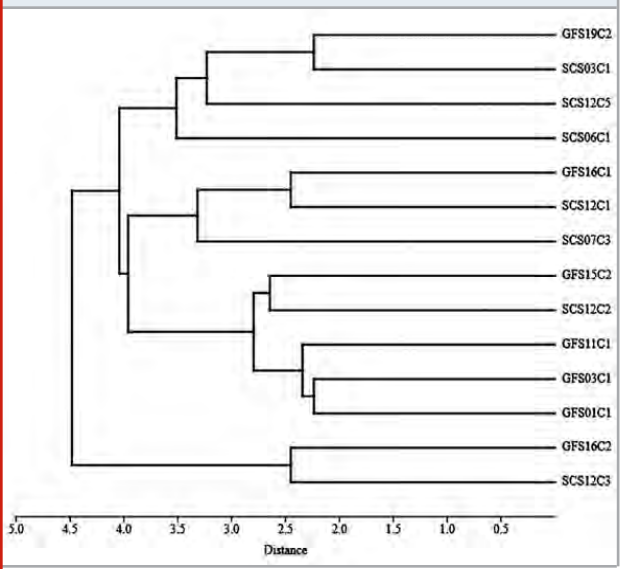
Similar pattern was observed during quantitative analysis of phosphate solubilization. Cluster analysis was performed to study the biochemical relatedness based of biochemical tests among the bacterial isolates, using PAST. ver. 4.07b. (Hammer et al. 2001; Athira and Anith 2020).

Using cluster analysis (figure 4) by euclidean distance method based on biochemical characterization isolates GFS16C2 & SCS12C3 have distinct characters than other isolates. GFS01C1 & GFS03C1 and GFS19C2 & SCS03C1 are more related as compared with other isolates. Individual bacteria cells specialize into unique tasks randomly and independently of one another in some bacteria species by random variations in each cell's metabolic processes. In Bacillus subtilis, for example, whether a cell makes and secretes protease is decided at random (Bettenworth et al. 2019; Li et al. 2021). However, the use of 16S rDNA amplicon sequencing can address this drawback (Liu et al. 2021).

Table 4. Biochemical characterization of bacterial isolates GFS01C1, GFS03C1, GFS11C1, GFS15C2, GFS16C1, GFS16C2, GFS19C2, SCS03C1, SCS06C1, SCS07C3, SCS12C1, SCS12C2, SCS12C3 and SCS12C5 using Vitek 2. (Sr. 24 to 47)

Sr.	Biochemical tests	GFS 1 C1	GFS 3 C1	GFS 11 C1	GFS 15 C2	GFS 16 C1	GFS 16 C2	GFS 19 C2	SCS 3 C1	SCS 6 C1	SCS 7 C3	SCS 12 C1	SCS 12 C2	SCS 12 C3	SCS 12 C5
24	L-Malate Assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27	D-Mannose	+	+	-	-	+	+	+	+	+	+	+	-	+	-
28	Tyrosine Arylamidase	+	-	-	-	+	+	+	+	+	+	+	-	+	+
29	Citrate (Sodium)	-	-	-	-	+	-	+	+	+	+	+	-	-	+
30	Beta-N-Acetyl-	-	-	-	-	-	+	-	-	-	-	-	-	+	-
	Galactosaminidase														
31	L-Histidine Assimilation	-	-	-	-	-	-	-	-	-	-	+	-	-	-
32	Ellman	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	D-Cellobiose	-	-	-	-	+	+	+	+	+	-	+	-	+	+
34	Gamma-Glutamyl-Transferase	-	-	-	-	-	+	+	+	+	-	-	-	+	-
35	Beta-Xylosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	Malonate	-	-	-	-	+	-	-	-	-	-	-	-	-	-
38	Alpha-Galactosidase	-	-	-	-	-	+	(-)	-	+	-	-	-	+	+
39	Courmarate	+	+	+	+	+	-	-	-	+	+	+	+	-	-
40	L-LACTATE Assimilation	-	-	-	-	-	-	-	-	+	-	+	-	-	+
41	Beta-Galactosidase	-	-	-	-	-	(-)	-	-	+	-	-	-	(-)	+
42	Fermentation/ Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	BETA-Alanine Arylamidase Pna	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	+
45	5-Keto-D-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	Phosphatase	+	+	+	+	-	+	+	-	-	-	-	-	+	-
47	Beta-Glucuronidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 4: Clustering of the bacterial isolates using based on Euclidean distance tree constructed using PAST.



CONCLUSION

The findings of the present study indicate the quantitative analysis of phosphate solubilizing capability and IAA synthesis by bacterial isolates. The current study attempted to identify plant growth enhancing microorganisms that may be harnessed to increase plant development. Several phosphate solubilizers and IAA makers also produced siderophores and HCN, suggesting that these organisms are capable of biocontrol. As a result, it can be deduced that some of the screened isolates have a strong capacity to operate as PGPR. Identifying microorganisms and using seed tests can also help us understand dynamics.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Effectiveness of Video-Assisted Teaching on Knowledge and Attitude Regarding Attention Deficit Hyperactivity Disorder among Primary School Teachers in Gurugram, Haryana, India: A Pre-Experimental Study

Nicky Tyagi, Raman Deep* and Poonam Ahlawat
Department of Mental Health Nursing, Faculty of Nursing, Shree Guru Gobind Singh Tricentenary University, Gurugram, Haryana, India

ABSTRACT

The study was conducted in selected schools of Gurugram, Haryana. Sixty (60) primary school teachers were selected from three schools of Gurugram by using total enumeration sampling technique. Self-structured knowledge questionnaire and attitude rating scale was used to assess the knowledge and attitude of primary school teachers regarding ADHD. The findings revealed that in the pre-test, majority 41(68.3%) of teachers had inadequate knowledge and 19(31.7%) had moderate knowledge and 28(46.7%) had unfavourable attitude and 32(53.3%) had Neutral attitude whereas in the post-test, 15(25%) had moderate knowledge and majority 45(75%) had adequate knowledge and 13(21.7%) had neutral attitude and majority 47(78.3%) had favourable attitude regarding ADHD. Paired "t" test was used to observe differences between pre and post-test mean scores and were found statistically significant at 0.05 level. The study concluded that educational material like video assisted teaching had shown significant improvement in knowledge and attitude of primary school teachers.

KEY WORDS: ATTENTION DEFICIT HYPERACTIVITY DISORDER, ATTITUDE, KNOWLEDGE, PRIMARY SCHOOL TEACHERS.

INTRODUCTION

Every kid has the intellectual ability to understand and experience the emotional and physical state of others and have the abilities that might help to reduce discomfort of others (Zahn-Waxler et al. 1984). The well-being, safety. motor and cognitive development of today's children will determine the quality of tomorrow's world, and perhaps even its survival (Sharma et al. 2018). According to global burden of disease, it has been suggested that by the year (2020), mental disorders in the children will increase by more than 50% world- wide and will be among the leading cause of childhood illness and dysfunction. The worldwide occurrence of ADHD is found to be 5.29% in kids, 7.1% in teenagers and 3.4% in adult (Danielson et al. 2018). The global prevalence of any mental disease was expected to be 13.67 percent throughout a lifetime; however, the present rate is 10.56 percent (Alshehri et al. 2020).

Article Information:*Corresponding Author: raman.nursing@sgtuniversity.org
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ADHD is leading issue affecting many children and adults. ADHD is among earliest occurring neurodevelopmental disorders with onset of the condition during formative years and can continue through early life and adulthood. ADHD refers to behavior pattern that is troublesome and is characterized by inattention, hyperactivity or impulsivity that interrupts the growth and functioning and is identified by six or more manifestations from the inattention and hyperactivity & impulsivity group of criteria. Manifestations of ADHD must be exhibited in 2 or more settings (it could be at home, school, or work) and the symptoms must be persistent for minimum 6 months of duration and negatively impacts the social, academic or occupational functioning (American Psychiatric Association. (2013). Although there are various successful management techniques for ADHD, it may lead to academic, vocational and social impairment, if it is left untreated (Cortese 2020).

ADHD is considered to be the result of combination of various factors like biological, psychological and environmental. About 80% of ADHD cases are caused by genetic factors (Biederman et al. 2002). In ADHD, inattention is evident in social and educational context. Manifestations of inattention

includes difficulty in holding attention, not able to finish assignment and not able to complete routine housework and school work. Manifestations of hyperactivity comprises squirming, unable to sit quietly in class, at all the times "on the go," and tend to talk a lot, while a manifestation of impulsive behavior is child cannot wait for their turn (Youssef et al. 2015). Furthermore, the issue is not confined to the childhood years; it has an impact on their mental health and social well-being as adults. (Mpango et al. 2017; Mazaheri et al. 2020).

The Primary school teachers are primary person who notice the signs and symptoms of hyperactivity or inattentive behaviors in the classroom and should be able to manage them and to take a right action (Khademi et al. 2016). Teachers are responsible for teaching the skills to the learners that are part of the curriculum but the teachers also have the responsibility of teaching the learners to function in a way that will help the them to reach educational and societal expectations. The work of the teacher becomes difficult and stressful with the children's having ADHD (Safaan et al. 2017). An instructor has an important role to play in assessing the behavioral and academic issues caused by their extensive interaction with children in different unstructured and structured environments (Mazaheri et al. 2020).

A study conducted in Egypt in (2017) determined that majority 59% of primary school teachers had very little information as compared to only 10.2% adequate information about ADHD. 81.4% of elementary school teachers had never attended any educational program during college regarding ADHD (Safaan et al. 2017; Mazaheri et al. 2020). Another study found that only about one-third of students with ADHD receive classroom management and fewer than two-thirds of students with ADHD receive educational support (i.e., school-based educational support, intervention, or accommodation, such as tutoring, extra help from a teacher, preferential seating, extra time to complete work, or being enrolled in special education) (DuPaul et al. 2018). Traditional ADHD teacher training programs are beneficial in the short term, but initial improvements decrease over time, according to a recent meta-analysis (Mazaheri et al. 2020).

This suggests that more effective long-term solutions are needed (Ward et al. 2020). ADHD negatively impact children's educational achievement because they face problems in preserving concentration, inability to complete given work, being forgetful and engages in non- goal oriented physical activities. There is a social and academic stigma related with ADHD and most of the parents faces difficulties in accepting terms with a diagnosis of ADHD (Moldavsky et al. 2013). Early recognition and management had shown favourable prognosis in several childhood psychiatric disorders. Hence, it is essential to stimulate the teachers by making them realize of their role which is very crucial in early recognition of trouble faced by the children's and make early referral to the health professionals (Arullapan et al. 2019; Ward et al. 2020).

According to a recent population-based study utilising DSM-IV criteria, 15.5 percent of schoolchildren in Grades 1 to 5 suffer from attention deficit hyperactivity disorder. (Rowland et al. 2013). According to the Innovative journal of medical and health research (2016), 3.66 percent of rural Indians suffer from Attention Deficit Hyperactivity Disorder. Based on above literatures the researcher felt that there was need of increasing awareness about ADHD among parents, instructors, and educationists. There are limited researches done on the ADHD in our community. Therefore, it was necessary to conduct a study to assess information and attitude among teachers about ADHD and to sensitize educationists about the need to recognize the manifestations of this disorder at an early stage and refer them to the health-care system (Gupta et al. 2020).

MATERIAL AND METHODS

The Quantitative Research Approach, one group pre-test post-test design was used for this study which was conducted in three selected schools of Gurugram, Haryana. The study population was primary school teachers teaching class from 1st to 5th. The sample size was 60 primary school teachers which were selected using total enumeration sampling technique. Before Data collection, research proposal approval was taken from the research committee of SGT University. The investigator took authorization from the Dean Faculty of Nursing, SGT University and from principal of selected schools of Gurugram, Haryana. The data collection was done in between 5th /04/2021 to 14th/04/2021. Self-introduction and objectives of the study was explained to all the respondents. Written consent was obtained from all participants and confidentiality was maintained by assuring that information provided by them will only be used for study purpose. Data was collected by using self-constructed knowledge questionnaire and attitude likert rating scale.

The instrument contains 3 sections. Section-A included socio-demographic variables such as age, sex, marital status, qualification, teaching grade, teaching experience, previous knowledge regarding ADHD, if yes specify the source of information. Section-B comprises of self-constructed set of questions on knowledge about ADHD which has 30 multiple choice questions (MCQs). Section -C A five-point Likert scale was developed for evaluating attitude of primary school teachers regarding ADHD. This scale contains 20 statements among which 10 statements were positive and 10 statements were negative. After data collection, it was analyzed using statistical package for social science (SPSS). Descriptive statistics like frequency, mean, percentage and standard deviation were used and inferential statistics t-test and chi-square was used. Findings were presented in tables.

RESULTS AND DISCUSSION

The findings of this study revealed that (table-1) depicts the socio-demographic information of primary school teachers according to which 9(15%) teachers were in the 21-25 years category, 17(28.3%) were in the category of 26-30 years, 19(31.7%) were in category 31-35 years and 15(25%) were in category of >35 years. Only 2(3.3%) of the participants

were males and majority 58(96.6%) of the participants were females. Majority 42(70%) of the teachers were married, 15(25%) were unmarried and only 3(5%) were in category of others. Majority 37(61.6%) of the participants were in the category of masters & above and 23(38.3%) of the participants were in the category of bachelor. 10(16.67%) of the participants were teaching the 1st grade, majority 14(23.33%) were teaching the 2nd grade, 12(20%) of the participants were teaching 3rd grade, 13(21.67%) were teaching 4th grade and 11(18.33%) were teaching 5th grade children. 15(25%) had teaching experience less than 3 years, majority 29(48.3%) had experience between 3-8years, 15(25%) had experience between 9- 14 years and only 1(1.6%) had experience of 15 years and above. Only 14(23.3%) were having previous knowledge about ADHD and majority 46(76.6%) were having no information about ADHD. Only 4(6.6%) knew about ADHD through media, 10(16.6%) knew about ADHD through internet, and no one had information through conference, books and other sources.

Table 1. Depicts the socio-demographic information of primary school teacher N=60

S.no	Demographical variables	f	%
1.	Age (in years):		
	21-25	09	15
	26-30	17	28.33
	31-35	19	31.66
	> 35	15	25
2.	Gender:		
	Male	02	3.33
	Female	58	96.66
	Transgender	00	00
3.	Marital status:		
	Married	42	70
	Unmarried	15	25
	Others	03	5
4.	Qualification:		
	Masters and above	37	61.66
	Bachelor	23	38.33
	Teaching grade:		
	1st class	10	16.67
	2nd class	14	23.33
	3rd class	12	20
	4th class	13	21.67
	5th class	11	18.33
5.	Teaching experience:		
	< 3 years	15	25
	3-8 years	29	48.33
	9-14 years	15	25
	15 and above	01	1.67
6.	Previous knowledge regarding ADHD:		
	Yes	14	23.33
	No	46	76.67
	If yes, specify the source of information:		
	Conference	00	0
	Media	04	6.66
	Books	00	0
	Internet	10	16.66
	Other	00	0
7.			
8.			

The table displays in pre-test, mostly 41(68.3%) teachers were in inadequate category and 19(31.7%) were in category of moderate knowledge and in post-test 15(25%) teachers were in category of moderate knowledge and majority 45(75%) teachers were in category of adequate. Similar results were found in study which revealed that during pre-test no school teacher had good knowledge, in experiment group whereas 18(60%) have average knowledge and 12(40%) below average knowledge whereas in post-test test in experiment group 17(56.67%) school teacher had good knowledge, whereas 9(30%) have average knowledge and 4(13.33%) below average knowledge (Kaur et al. 2020).

Table 2. Depicts frequency and percentage distribution of pre & post-test information level about ADHD among the teachers N=60

Information category	Inadequate (0-10)		Moderate(11-20)		Adequate (21-30)	
	f	%	f	%	f	%
Pretest	41	68.3%	19	31.7%	0	0
Posttest	0	0	15	25%	45	75%

Table 3. Depicts frequency and percentage distribution of pre & post-test attitude about ADHD of teachers N=60

Attitude	Unfavourable (20-46)		Neutral (47-73)		Favourable (74-100)	
	No.	%	No.	%	No.	%
Pre-test	28	46.7%	32	53.3%	0	0
Post-test	0	0	13	21.7%	47	78.3%

Table 4. Depicts the comparison of pre-test & post-test information scores about ADHD of teachers. N=60

Knowledge	Mean±s.d	Mean difference	"Paired t value"
Pre test	9.85±2.21	12.55	t=25.936 p=0.00*
Post test	22.40±3.04		

Table 5. Depicts the comparison of pre & post-test attitude score about ADHD of teachersN=60

Attitude	Mean±s.d	Mean difference	"Paired t value"
Pre test	48.97±5.74	30.51	t=23.299
Post test	79.48±7.82		p=0.00*

The table displays in pre-test, 28(46.7%) teachers were in the unfavourable attitude category and 32(53.3%) were in Neutral attitude category, whereas in the post-test 13(21.7%) were in neutral attitude category and majority 47(78.3%) were in favourable attitude category.

Interpretation-Mean difference of 12.55 was found between pre & post-test information score. "Paired t test" was performed to correlate gap between pre & post-test information score and has shown significance. This clearly shows VAT prepared by the researcher had shown great enhancement in information level of teachers in posttest. These findings were congruent with findings in which the pretest mean score of knowledge was 15.66 whereas in the posttest the mean score was 23.98 which clearly shows the significant improvement in the teachers' knowledge (Tungoe et al. 2021). One another study findings were congruent in which experimental group's mean pre-test attention deficit score was 31.03, and group's mean post-test attention deficit score (17.63) was significantly lower than the pre-test score. The influence of behavior therapy is responsible for the difference between the two means. here was a significant link between the mean difference in attention deficit score of schoolchildren with attention deficit disorder (Vanitha 2021).

Interpretation: Mean difference of 30.15 was found between pre & post-test attitude score. "Paired t test" was done to corelate the gap between pre & post-test and has shown significance. This finding was consistent with the findings where it was revealed that the post-test mean score of knowledge was hiigher than the pretest mean score. Hence, it was proved that video-based teaching was beneficial (Bhasin et al. 2020).

The association of post-test information level was not found significant with other selected demographical factors, except teaching experience which had shown significance. Alshehri et al. (2020) findings were congruent with this finding of the study. The association of post-test attitude

was not found significant with selected demographical factors (Alshehri et al. 2020).

CONCLUSION

The findings of the present study revealed that teachers have inadequate knowledge regarding ADHD and also shows that schools should invest in faculty development, and plan workshops to train teachers to deal with the particular requirements of children with ADHD. The educational material in the form of video assisted teaching had shown to be beneficial for the teachers in enhancing their information and attitude about ADHD. The knowledge gained from the educational programme will assist teachers in recognising and managing ADHD pupils in schools and other contexts.

Conflict of interests: There was no conflict of interest among the authors.

Ethical Statement: Ethical approval was given by the Institutional Ethical Committee in a meeting held on 28/11/2020. Ethical Approval Number is FON/SGTU/20/262/15.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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High Performance Thin-Layer Chromatography-Mass Spectrometry Evaluation of Sanguinarine and Dihydrosanguinarine from *Argemone mexicana* Seeds in Edible Mustard Oil

Ramakant Yadav,[1,2] Swati Wavhal,[3] Akshay Charegaonkar,[1] Ranjeet Kaur Bajwa,[2] Prafulchandra Tekale[2] and Vinars Dawane[1,4]

[1]Anchrom Enterprises (I) Pvt Ltd, Mumbai, Maharashtra, 400081, India.

[2]Guru Nanak Institue of Research and Development, G.N. Khalsa College of Arts, Science and Commerce, Mumbai, Maharashtra, 400019 India.

[3]Ismail Yusuf College of Arts, Commerce and Science, Ismail College Campus, Mumbai, Maharashtra, 400060, India.

[4]School of Environment and Sustainable Development, Central University of Gujarat, Gujarat, 382030, India.

ABSTRACT

In this present study, a simple, rapid, cheap, sensitive and reproducible HPTLC-MS Method has been developed for the identification of two important bioactive compounds, Sanguinarine and Dihydrosanguinarine in *Argemone mexicana* Linn seeds. The work further discussed and developed a sensitive HPTLC – MS method to analyse the adulteration and/or contamination of argemone oil in the edible mustard oil by spiking Sanguinarine and Dihydrosanguinarine as biomarkers. The n-hexane: diethyl ether (1:1 v/v) solvent system has been used as an extraction medium to extract the Sanguinarine and Dihydrosanguinarine from the seeds followed by HPLC-MS detection.The CAMAG HPTLC system modules consisted with ATS-4, ADC-2, visualizer-2, TLC scanner-4; Derivatizer and TLC-MS interface-2 have been used for sample application, HPTLC plate development, plate photo documentation, scanning of plate, spraying of derivatization reagent and elution of biomarkers directly from HPTLC plate respectively. The n-hexane: acetone (23:7, v/v) and dragendorff's reagent has been used as mobile phase and derivatization reagent respectively. The UV- spectra and MS data confirmed the detection of selected biomarkers in the samples/spiked samples. Thus, the work highlights the use of HPTLC-MS to develop simple and routine method for the sensitive detection of Sanguinarine and Dihydrosanguinarine in *Argemone mexicana* seeds and able to become bases to these biomarkers' evaluations in other samples such as various edible oils, agro-products, drugs and biomedical products etc.

KEY WORDS: ADULTERATION, ARGEMONE OIL, EDIBLE MUSTARD OIL, HPTLC, SANGUINARINE.

INTRODUCTION

Oils is a product widely used for various preparation of food products such as snacks, sweets, and homemade foods etc., thus Oils have a major role in various edible articles (Mishra and Manchanda 2012; Cárdenas et al. 2021). Food products prepared in good quality standards have been associated with several health benefits while if prepared by contaminated oil may lead to health issues like food poisoning and other long-lasting diseases (Kaur et al. 2019; Almoselhy 2021). Many times, the oils become contaminated or adulterated with other oils and lead to change the quality of pure oil (Li et al. 2021). Several reports have been published about the adulteration of costly edible oils with cheap edible and/or non-edible oils to gain huge profit margin that leads to human poisoning/toxicity or major health issues related to adulterated oils (Salah and Nofal 2021; Xia et al. 2021).

Thus, the proper quality testing of edible oils is the major requirement of today's world. In this regards the adulteration

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of mustard oil with argemone oil is a serious issue and critical analytical problem (Gomber et al. 1994; Thatte and Dahanukar 1999; Singh et al. 2000; Ghosh et al. 2005; Xia et al. 2021). Argemone oil contamination in mustard oil is one of the important issues, which are faced by oil processing industries (Das and Khanna 1997; Babu et al. 2007). The source of this contamination and adulteration is the seeds from the matured *Argemone mexicana* plant (Bui 1974; Babu et al. 2007). *Argemone mexicana* Linn (Mexican poppy, Papaveracea) is an annual weed which grows at the side of agricultural fields and is found mostly in Mexico and Florida of South America as well as in Asian, African and Caribbean countries (Namkeleja et al. 2014). The plant grows under warm temperatures and found commonly on roadsides of Indian fields. Being a medicinal plant, it is reported to have various medicinal properties like antimicrobial, antimalarial, wound healing and cytotoxic effects etc. (Osho and Adetunji 2010; Dash and Murthy 2011; Priya and Rao 2012; Khan and Bhadauria 2019; Xia et al. 2021).

The plant shows the presence of fatty acids, alkaloids, phenolics and other phytoconstituents (Apu et al. 2012). There are two types of alkaloids present namely Sanguinarine and Dihydrosanguinarine that are proven toxic to humans, by simple process of reduction and oxidation both the alkaloids are interconvertible to each other (Sarkar 1948; Vrba et al. 2009; Absolínová et al. 2009). The seeds of *Argemone mexicana* has similar physical appearance as mustard oil seeds, thus after the maturation of the plant, the light weight black colour seeds of *Argemone mexicana* gets contaminated with mustard seeds and during the oil processing, the oil forms argemone seeds have been mixed with oil of mustard seeds. A clinical condition named as Epidemic dropsy is caused due to consumption of mustard oil adulterated with argemone oil; the alkaloid responsible for this is sanguinarine and dihydrosanguinarine from the seeds of *Argemone mexicana* Linn (Sanyal 1950; Sharma et al. 1999; Xia et al. 2021). Various diseases related to the sanguinarine and dihydrosanguinarine such as glaucoma, epidemic dropsy and sometimes leads total blindness were reported in FSSAI oils and global Fat manuals (Mahajan et al. 2014; Srivastava 2015; Xia et al. 2021).

The earlier analytical methods for identification of sanguinarine in argemone oil have been neither reliable nor reproducible because many of the methods contain manual processes and less proper software control associated with instrumental methods. Paper chromatographic earlier reports and/or previous HPLC methods have been facing the lack of LOD detection as well as clean-up alongwith need of high grade of solvents respectively (Hakim et al. 1961; Li et al. 2021). Thus, the present study is an attempt to provide a HPTLC-MS method for this evaluation along with the attributes of method simplicity, quickness, cost effectiveness and solvent saving approach. The developed methods are significantly useful in the evaluation of these two bioactive alkaloids in the *Argemone mexicana* Linn plant and able to target the adulteration issues associated with the respective oils.

MATERIAL AND METHODS

For chemicals and reagents, all the solvent used for analysis was of analytical grade, solvent like methanol, n-hexane, acetone of Merck (Brand). For the derivatization process Dragendorff's reagent is prepared by using, Bismuth nitrate, Potassium iodide. HPTLC Glass Silica Gel 60 F254, 20x10 cm (Merck Catalogue No. 1.05642.0007). If no. of samples and standards to be applied is less than 10, then use a 10 x 10 cm sized plate. *Argemone mexicana* plant seeds used were directly collected from the fields of Saphale, Palghar (19.577778°N 72.819167°E), edible Mustard oil and seeds is procured from the local market of Mumbai, Maharashtra, India. For standards and sample solutions in the present study, standard sanguinarine and dihydrosanguinarine was not used, instead of standards, argemone seeds were used to isolate sanguinarine and dihydrosanguinarine and to identify them in mustard oils. The two benzylisoquinoline alkaloids isolated and identified from *Argemone mexicana* seeds were used as a standard and their identity and purity was confirmed by MS (Mass Spectrometer).

Working standard stock solution of *Argemone mexicana* seed was prepared by taking 1gram of argemone seeds in 15 ml tarson tube, the seeds were crushed with glass rod and extracted with 10 ml of 1:1 n-hexane: Diethyl ether. The solution was shaken vigorously for 2-3 minutes; Sonicated for 15 minutes, centrifuged at 1500rpm for 5 minutes, after the centrifugation the clear supernatant is used for analysis. This supernatant can be used as working standard. Working standard stock solution was kept in standard volumetric flask rapped with paraffin tape and stored at 8°C throughout the analysis. The working standard was applied in an aliquot of 0.5 µl, 1.0 µl, 2.0 µl, 3.0 µl and 4.0 µl on TLC plate with CAMAG Automatic TLC applicator (ATS 4).

For spiking studies, the spiking of the working standard was done on pure edible mustard oil samples. 2.0 µl of working standard was spiked in each track of pure mustard oil. Spiking on mustard oil was done on plate with CAMAG ATS 4 (Automatic TLC sampler) by over-spotting the working standard on the Mustard oil sample tracks.

For derivatization reagent to detect Alkaloids, derivatization was performed with Dragendorff's reagent. Dragondorff's reagent: Solution 1: Weigh 0.85 g of basic bismuth nitrate in a glass bottle and add 40 ml of water and 10 ml glacial acetic acid. Solution 2: Weigh 8gm of potassium Iodide in a glass bottle and dissolve in 30ml of water. Just before dipping, 20ml of each solution was diluted with 80 ml Acetic acid and 200 ml Water. During chromatography for separation and isolation of the two important alkaloids, CAMAG HPTLC modules from (Muttenz, Switzerland) was used. The HPTLC modules consist of Automatic TLC Applicator (ATS 4) equipped with a 25-µl syringe, CAMAG ADC-2, TLC Scanner 4, operated by CAMAG server client software vision CATS 3.0. HPTLC Silica gel 60 F254 (glass plate), was from Merck.

For sample preparation, two types of mustard oil samples were analysed, mustard oil directly from the market and

other is oil extracted from mustard seeds. From Seeds:1 g of Mustard seeds was taken in a 15 ml Tarson tube, the seeds were crushed with a glass rod, add 10 ml of 1:1 n-Hexane: Diethyl ether. The mixture was shaken vigorously for 2-3minutes, then sonicated for 15 mins. After this,it was centrifuged at 1500rpm for 5 min, and taken for the supernatant to continue analysis. From Oil: 2 ml of mustard oil was taken in 15 ml Tarson tube. Then 10 ml of 1:1 n-Hexane: Diethyl ether was added. The mixture was shaken vigorously for 2-3 min and then sonicated for 15 mins. Finally, the solution was used for analysis.

For the chromatographic conditions at the time of application of the working standard and samples, the HPTLC plates were heated at 105 °C for 5 minutes on CAMAG plate heater III, for activation of plate. For The application of standard and sample CAMAG Automatic TLC Sampler (ATS 4) is used. The working standard solution and sample solution was applied in an aliquot of 0.5 µl, 1.0 µl, 2.0 µl, 3.0 µl and 4.0 µl. The spiking of mustard oils samples was done by over spotting the working standard on edible oil bands on plate. Application was done with dosage speed of methanol (150 nl/s) in the form of 8mm bands. The separation was done in a CAMAG ADC-2 automatic developing chamber. The ADC-2 was pre-saturated for 20 minutes with 20 ml of Mobile Phase [n-hexane: acetone (23:7, v/v)] and relative humidity was controlled at 33 % by using MgCl2. 6H2O (saturated salt solution). The plates were developed up to solvent front of about 70mm from bottom edge. The developed plate was dried automatically by ADC-2. The developed plate was photo documented by CAMAG Visualizer 2 under R White, R 254 nm and R 366 nm.

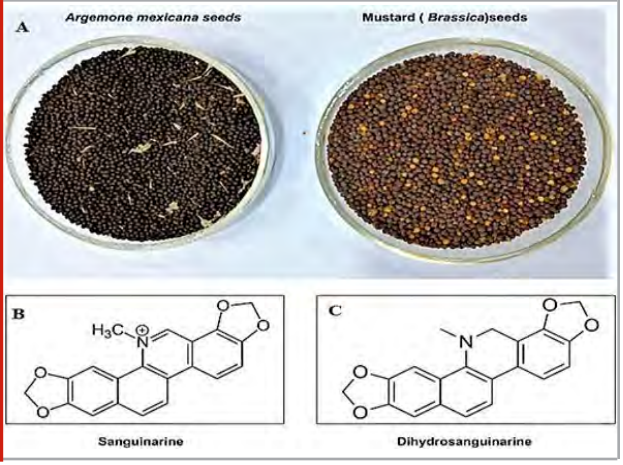
For scanning, CAMAG scanner 4 scanned the developed plate. The plates were scanned at multiple wavelengths (254 nm, 366nm and 480 nm) by using single wavelength and multiple wavelengths in scanner type. Optimization was done for light sensitivity The scanning was done under measurement mode (Absorbance/Fluorescence) mode by using a D2 Lamp for 254 nm Hg lamp for 366 nm and W lamp for 480 nm. The filter used was K 400 and the slit dimensionselected was 6.0 mm x 0.45 mm micro. The Scanning was performed with the scanning speed of 20 mm/s and data resolution of 25µm/step. The scanning of the tracks was done from 4.9mm from bottom of the plate and up to 73.1mm.

For the spectrum analysis, the plates were scanned under scanner type (Spectrum), optimization was done for resolution and the measurement mode selected to absorbance. Scanning was done from 190 to 700 nm to get the UV visible spectra of the two alkaloids at Rf: - 0.20 mm and 0.42 mm. After the spectrum analysis, the well separated bands at Rf:-0.20 mm of sanguinarine and Rf:-0.42 mm of dihydrosanguinarine was marked with pencil under visible light. The marked bands were eluted with the help of TLC-MS interface 2 and analysed by using Acuity QDA 3 mass spectrometer (waters, USA) in chemical ionization mode for TIC /MS Scan/Positive (+) scan.

RESULTS AND DISCUSSION

An HPTLC-MS Method has been developed for simple and rapid isolation of two toxic alkaloids namely sanguinarine and dihydrosanguinarine from argemone seeds. The method can be used to detect the contamination or adulteration of argemone oil in mustard oil.

Figure 1: The seeds of *Argemone mexicana* and Brassica (A) and the structure of two biomarkers; Sanguinarine (B) and Dihydrosanguinarine (C) respectively.



The Mobile phase used was n-hexane: Acetone in the ratio of 23:7 v/v. The mobile phase provides very good separation between sanguinarine and dihydrosanguinarine with reduced matrices from argemone oil and mustard oil extracts. This method was very specific to these two QBA's Sanguinarine and Dihydrosanguinarine; alkaloids other than these two do not interfere and are not detected by this mobile phase. As per FSSAI regulations it is claimed on every pouch or container of mustard oil ''Free from Argemone oil'' therefore the mustard oil should be free from argemone oil. The Method is very simple and fast, as it requires simple steps and less time for the extraction process. The process consists of crushing the seeds, sonicating it for 15 minutes and centrifuge for 5 minutes and collection of the clear supernatant for the analysis. For this simple process, the time required is 30 minutes. Procedures reported in previous research papers include tedious procedures for sample preparation.

The two alkaloids sanguinarine and dihydrosanguinarine at Rf: 0.20 mm and 0.40 mm was active in short and long UV and also in the visible light. The best response of the two alkaloids was in R 366 nm image, which is shown in the Fig-2 (B). The developed plate was scanned at various wavelength such as R 254 nm, R 366 nm and 480 nm and the corresponding 3D Densitogram and the peak data is shown in Fig 3 as (A), (B) and (C) and the alkaloids also show their presence under white (Orange colours bands) when they were treated with dragondorffs reagent. After the photo documentation the UV spectra for both bands at Rf: 0.20 mm and Rf:0.42 mm was recorded. The developed plate was further used for HPTLC-MS analysis, the bands at Rf:0.20 mm and Rf: 0.42 mm were marked with pencil and the elution of both the analytes were done by TLC-MS

interface 2, mass dat is shown in the Fig 4 as (A) and (B) (Almoselhy 2021).

After the extraction of oils from *Argemone mexicana* seeds, the identification of sanguinarine and dihydrosanguinarine from argemone oil has been done by checking the maximum Rf value, spectrum data, λ max values followed by the m/z ratios of both alkaloids by TLC-MS interface 2. After confirming the two alkaloids with the above steps, argemone oil was spiked in mustard oil sample by over spotting with CAMAG ATS 4 applicator. When the developed method was used for the identification of the two alkaloids in the spiked sample, both the alkaloids were identified in the spiked samples without any interference from mustard oil. Thus, this method was suitable for checking the contamination or adulteration of mustard oil by argemone oil. The developed method gives reproducible results (Cárdenas et al. 2021).

Figure 2: Developed plate under various electromagnetic radiation zones. Where, A - Developed plate image in R 254nm, shows the separation of Dihydrosanguinarine (Rf: 0.42) and Sanguinarine (Rf: 0.20) from argemone oil, argemone oil spiked to edible mustard oil. B - Developed plate image in R 366nm, shows the separation of Dihydrosanguinarine (Rf: 0.42) and Sanguinarine (Rf: 0.20) from argemone oil, argemone oil spiked to edible mustard oil. In R366 nm, both the alkaloids Sanguinarine and Dihydrosanguinarine can be easily detected with intense yellow colour fluorescence bands, the upper band of higher intensity is of Dihdrosanguinarine and lower band is Sanguinarine. C -. Developed plate image in R White, tracks 1-5; TLC separation of (i)Sanguinarine (Rf:0.20) and (ii) Dihydrosanguinarine (Rf:0.42) from argemone oil. Track 6- 9 Unspiked mustard oil sample. Tracks 10-12 spiked mustard oil sample. D - Derivatized plate image in R White after derivatization with dragondorff's reagent. Both the alkaloids give reddish orange colour bands after derivatizing it with dragondorff's reagent. Sanguinarine at (Rf: 0.20) and Dihydrosanguinarine (Rf:0.42).

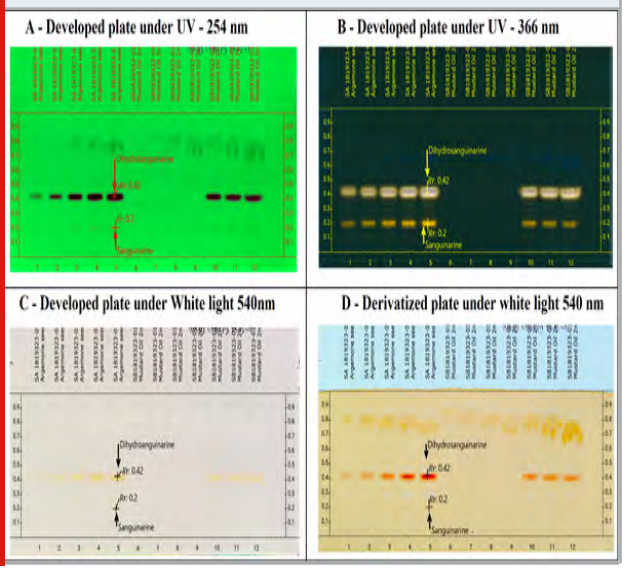


Figure 3: 3D – Densitogram profiles and their respective data table images including no. of peaks, Rf max, Height and area of the peaks and their relative percentage profiles. Here, tracks 1-5; TLC separation of (i) Sanguinarine (Rf:0.20) and (ii) DihydroSanguinarine (Rf:0.42) from argemone oil. Track 6- 9 Unspiked mustard oil sample. Tracks 10-12 spiked mustard oil sample. Where, A – Densitogram profile at UV – 254 nm, B – Densitogram profile at 430 nm, C – Densitogram profile at UV – 366 nm.

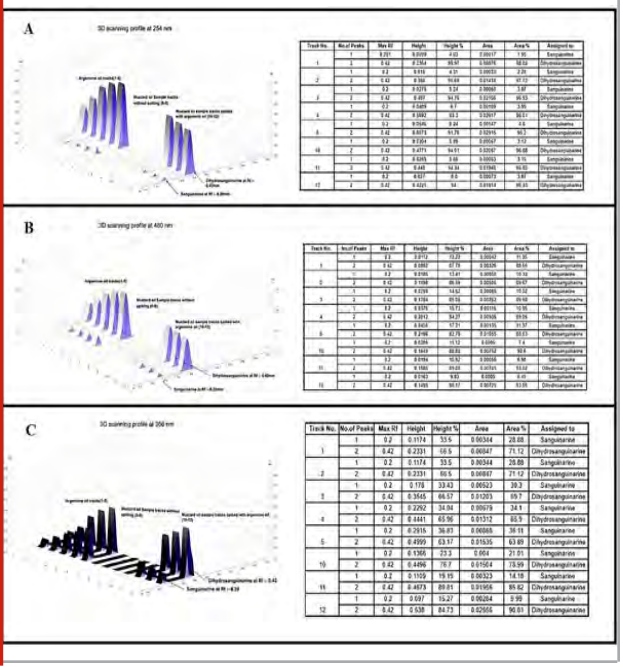
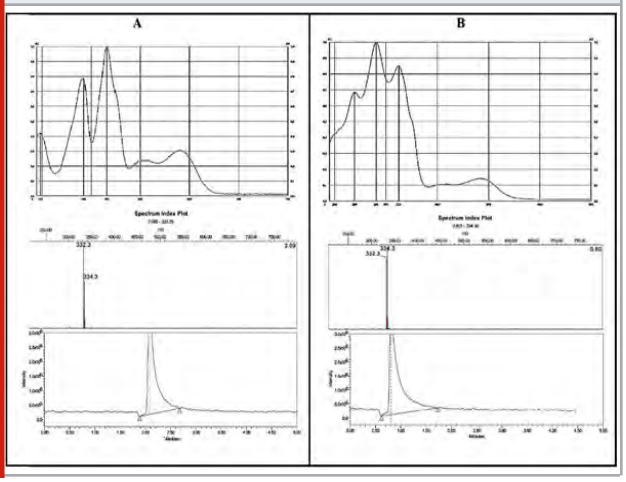


Figure. 4: UV- spectra and correspondent MS- spectra of Sanguinarine (A) and Dihydrosanguinarine (B) respectively. A - In situ UV spectra of Sanguinarine in argemone oil showing the λ max at 194 nm, 281nm and 332 nm along with the peak at 332 m/z in mass spectra. B - In situ UV spectra of Dihydrosanguinarine in argemone oil after UV irradiation (366 nm, for 15 min). Showing the λ max at 240 nm, 281 nm and 325 nm alongwith the peak at 334 m/z in mass spectra.



The identification of Sanguinarine in mustard oil samples was further confirmed by comparing the in-situ UV spectra of Sanguinarine from argemone oil and spectra of Sanguinarine in spiked mustard oil. The Mass spectral analysis of bands corresponding to Sanguinarine (Rf: 0.20) and Dihydrosanguinarine (Rf: 0.42) after exposure to UV-radiation was done with the help of TLC-MS interface 2. The bands were marked with pencil under UV 254nm light and the analytes are eluted from the plate to Mass Spectrophotometer. The base peak (m/z 332 and m/z 334) shown for Sanguinarine and Dihydrosanguinarine (Cárdenas et al. 2021).

CONCLUSION

The findings of the present study suggest that the HPTLC-MS method has been developed to isolate and identify the alkaloids namely Sanguinarine and Dihydrosanguinarine from *Argemone mexicana* seeds. By using this method, Sanguinarine and Dihydrosanguinarine, which is well separated without any interference from oil matrices, can be used as specific markers to detect the adulteration or contamination of mustard oil with argemone seed oil. This method found to be cheap, very simple to use and less time consuming, when compared with earlier reported methods that are having complex sample preparation and reproducibility issues. This method found to be useful to screen multiple numbers of mustard oil samples simultaneously thus significantly potent in industries in the screening of samples on large scale and industries dealing with mustard oil related products as well as applicable in forensic labs for argemone oil poisoning case investigations.

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Conflict of Interests: Authors declare no conflict of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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The Effects of *Costus speciosus* Root Extract on Cultured Human Lung Cancer Cells, A549

Abdulkader Shaikh Omar,[1,2] Rawah Adnan Nasraldeen, Raed Albiheyri,[1] Roqayah H. Kadi[3] and Salah E. M. Abo-Aba[1,2]

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University 21589, Jeddah, Saudi Arabia

[2]Najla Bint Saud Al Saud Center for Distinguished Research In Biotechnology, Jeddah, Saudi Arabia

[3]Department of Biology, Faculty of Science, University Of Jeddah, Jeddah, Saudi Arabia

ABSTRACT

Cancer has become one of the most common clinical reasons of death all over the world, with more than nine million deaths each year. Throughout the history, many traditional plants have been used to treat various types of cancers. The scientifically researched anticancer plants, with over 3000 species are novel candidates for anticancer drugs, because more than 80% of society believes in traditional medicine as its cure. The aim of this study was to determine the effects of *Costus speciosus* root extract on human lung cancer (LC) cells (A549). Treatment of (A549) cells with ethyl acetate *Costus* root extract significantly lowered the cell viability. IC50 was calculated, after 24, 48 and72 h, with concentration of 25, 40, 70 and 100 µg/100µl. The MTT assay was used to test the viability of the cells. The effect of (EACR) on cell adhesion and migration was demonstrated in a wound healing assay, mitochondrial membrane potential was detected in a JC-1 assay, and DNA damage and repair were demonstrated in a comet assay. There was a noticeable reduction in (LC) development. Experiments underlying the anti-cancer activity of (EACR) on (A549) cells may be regarded as one of the most promising targets for novel cancer treatment strategies (LC).

KEY WORDS: A549, ANTI-CANCER ACTIVITY, COSTUS SPECIOSUS, ETHYL ACETATE, CELL VIABILITY.

INTRODUCTION

Dietary risks in addition to bad lifestyle and tobacco are factors that help cancer development and are the main risk factors which at end lead to lung cancer (LC) and accounting for 22% of cancer deaths According to the World Health Organization (WHO). Lung cancer (1.76 million deaths), colorectal cancer (862 000 deaths), and stomach cancer (783 000 deaths) are the most common causes of cancer death., Liver (782 000 deaths), Breast (627 000 deaths). In lung cancer, similar histo-type between patients is a biological prove for different treatment response for each patient, with different levels of molecular heterogeneity have been recognized. In the top 10 cancers affecting males and females in Saudi Arabia, the most affected group by LC is males (Marisa et al, 2019, Alqahtani, and Alghamdi 2020).

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The main types and pattern of cancer treatments include surgery according to NCI, 2015, chemotherapy, radiation therapy according to American Society of Clinical Oncology 2016, immunotherapy and hormone therapy. Traditional medicinal herbs are used worldwide to treat cancer through the ages. With more than 3000 medicinal plants, the scientifically anticancer plants are novel for anticancer drugs, specially that up to 80% of society believe in traditional medicine as it was presented from (WHO), with more than $5 billion/year spent for herbal use in US alone (Andrew-Vickers et al., 2001; Wachtel-Galor, 2011; Lin et al., 2015; Thomas et al., 2015; Sharma et al., 2017; Chen et al., 2018; Hong-lian et al., 2019).

Furthermore, the pharmacologic screening for the treatments from herbs is under congesting advancing. The aerobic glycolysis manufacture of cancer cells is major amount of lactic acid whilst taking oxygen through mitochondrial oxidative phosphorylation. Due to a decrease in the number of the receptors in the cell surface according to Warburg

observation. One of the functions of mitochondria is the regulation of apoptosis. By knowing the importance of the relation between cancer cells metabolism, mitochondria and apoptosis, it is vital to study relationship of the extracts of medicinal herbs and proliferation of cancer cell lines (Andrew-Vickers, et al., 2001; Rivlin et al., 2011; Guaman-Ortizet al., 2017; Mirza et al., 2018).

Costus speciosus grows on the moist, slopes environments, originally from South East Asia although it has been naturalized in some tropical areas such as Hawaii as well. But currently it is more found In India, Sri Lanka, Indonesia and Malaysia. Largely found in south India. This plant is cultivated for its roots which are used in perfume industry, incense and medicine. It has an acrid, sweet and bitter taste. *Costus grandis* and *Costus speciosus* is the most important used two plants in this family, Many scientific studies have been conducted or are now being conducted on these two plants in order to commercialize their positive properties and for pharmacological effects as well, although many of these studies are still in vitro phases, and not progressed onto human trials (Pandey et al., 2009; Vishalakshi et al., 2010; Pawar and Pawar, 2014; Waisundara, et al., 2015).

Some researchers discovered that this plant's anticancer action is mediated by overexpression of proapoptotic and downregulation of antiapoptotic molecules, which together reduce cancer cell proliferation and progression. Anti-inflammatory Activity, Rheumatism, bronchitis, asthma, flatulence, constipation, leprosy, skin illness, and anemia are among the conditions for which it is prescribed. The oil extracted from the roots is known as Costus Oil. As a result of over-exploitation for various medical and commercial purposes, the supply of this vital plant in the wild is dwindling. As a result, the Indian government has banned the export of 29 medicinal and aromatic species. (Pandey et al, 2009; Pawar, and Pawar, 2014; El-Faret al., 2017; Selim and Jaouni 2017; Majiet al, 2020). Therefore, the current study was carried out to investigate the effect of ethyl acetate extract of *Costus cpeciosus* roots on cultured human lung cancer cells (A549).

MATERIAL AND METHODS

Preparation of Costus roots' extract: Fresh 500 g of *C. speciosus* roots was purchased from Mayajan abazeer in Makkah, KSA. The root material was extracted by macerating for 2 days in 75% methanol, filtered and dried. According to (Mohammed, et al., 2019), the single dose contains 0.05% DMSO to give the final desired concentration of methyl acetate of C. speciosus roots (ECR).

Using A549 Cell Line for culture: In a 15 ml tube, the collected cells were washed, centrifuged at 250 rpm for 5 min. and the obtained cell pellet was resuspended in one ml of cold cell medium (30 ml free medium with 1ml DMSO and 3ml FBS) and transferred to cryogenic vials, stored in the -80ºC.

Thawing of cells: The prepared cell suspension was transferred to a 15 ml tube, and mixed gently with 10 ml of complete culture medium. After centrifugation at 250 rpm for 5 min, the cells were collected in fresh culture medium, preserved in a CO_2 incubator at 37ºC and examined each 24 hours and sub cultured if necessary.

Cancer cell lines and Sphere forming culture: Human Lung cancer cells A549 were grown at 37 °C in RPMI medium supplemented with 10 % fetal bovine serum and 1 % penicillin and streptomycin under 5% CO_2 and 95 % air. The cells were collected, washed with saline phosphate-buffered, incubated with 0.05 % trypsin/ EDTA and collected at the exponential phase to carry out all the experiments.

Cell Viability Assay (MTT Assay): The effects of *C. speciosus* roots on the cell viability and proliferation of A549 cells were detected by (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) cell proliferation assay kit (Invitrogen), following manufacturer's instructions.

Clonogenicity Assay: Clonogenic assay test was used to detect the efficacy of cytotoxic agents and other anti-cancer therapeutics on colony forming ability were done using different cell lines. In 6 wells plate, the suspended cells (3×10^3 cells/well) in 3 mL of culture medium, containing different concentration from the tested material (25, 40 and 70µg/100 µl) were incubated at 37°C and 5% CO_2 for 24 hrs. After treatment for growth and colony formation, the medium was removed then replaced with fresh medium and incubated for another 7 days.

Colonies fixation and staining: Media was removed from each well by aspiration and by washing each well with 2ml PBS. colonies were fixed with 1.5 ml 10% formalin-based buffer solution for15-30 minutes. Washing the wells again with 2 ml PBS well done again, 600 µl of 0.25 % triton was added to the cells and washing with PBS to remove the tritone residues. Colonies were stained with 1.5 ml of Giemsa (1x) for 30-60 minutes, then removing the stains by washing with PBS then allowing cells dry. The observation was done using inverted microscope with a magnification lens x10.

Wound Healing Assays: The classic and commonly wound healing assays are a applied to study cell motility and biology according to (Jonkmanet al., 2014). In 6 wells plate, Cells (2×10^5cells/well) were incubated in 3 ml of the culture medium for 24 hrs at 37°C and 5% CO_2. After 24 hrs, the formed cell ~70-80% confluence as a monolayer were scratched across the center of each well, washed by PBS incubated, treated with different concentrations the tested material (25, 40, 70 and 100 µg/500 µl), allowed to migrate and examined under inverted after 0, 24, 48 and 72 hrs post-induction of injury.

The Comet Assay, or Single Cell Gel Electrophoresis Assay (SCGE): DNA damage in individual cells can be detected by the common Comet assay (Product Manual, OxiSelect™ Comet Assay Kit (3- Well Slides). Cells (2×10^5cells/well) were incubated in 3 mL of culture

medium in 6 wells plate for 24 hrs and treated by different concentrations of the tested material (25, 40, 70 and 100 µg/500 µl) for 24 hrs.

DNA fragmentation assay: Agarose gel electrophoresis was used to detect DNA fragmentation and distinguish apoptotic cell using to DNA purification kit (Promegacataloge#A1120).

Isolating Genomic DNA protocol: The DNA isolation methods were done according to Wizard® Genomic DNA Purification Kit, Promega, electrophorized using gel electrophoresis with EtBr staining method and visualized under UV illumination.

Measurement of the Mitochondrial Membrane Potential (ΔΨm) with JC-1: Apoptosis is a cellular process involving a genetically programmed series of events leading to the death of a cell. During this process, several key events occur in mitochondria, including loss of mitochondrial transmembrane potential (ΔΨm). For this reason, ΔΨm is an important parameter of mitochondrial function and has been used as an indicator of healthy cells. The principle of all the following detection methods is done according to (Zhang et al., 2010). The cells were treated at different concentrations (25, 40, 70 and 100 µg/100µl) of plant extract for 24 hrs.100 µl of JC-1reagent was added to each well and incubated for 30 min. The fluorescence was measured by using a microplate reader with excitation/emission = 535/595 nm for red fluorescence (viable cells) and485/535 nm for green fluorescence (apoptotic cells).

Human Interleukin 32 ELISA Assay: The shortest and most abundant antibody is IL-32 alpha. Potential modifications include myristoylation and N-glycosylation. Transfected IL-32 alpha was more likely to be cell-associated as compared to IL-32 beta, suggesting an intracellular function. IL-32 is induced by mitogens in peripheral lymphocytes, by IFN-gamma in epithelial cells, and in turn induces cytokine expression.

Plate Preparation and assay: For coating a 96-well microplate, 100 µL per well of PBS (Capture Antibody working concentration) was used and the sealed plate overnight and incubated at room temperature. Each well was washed with 400 µL by the buffer. After removing the washing buffer, 300 µL of reagent diluent was added to each well and the plates were incubated at room temperature for an hour then 100 µL of reagent dilution was added to each well and incubation was applied to another two hours at room temperature. 100 µL of the detection antibody diluted in reagent diluent were added to each well, incubation 2 h at room temperature was done. 100 µL of the working dilution of streptavidin-HRP added to each well at room temperature in the dark for 20 min. 100 µL of substrate solution was added to each well with gentle tapping. 50 µL of Stop Solution was added to each well with gentle mixing. The microplate reader set used to measure the optical density at 450 nm.

Statistical analysis: Data was collected and mean value ± standard deviation (SD) from two independent experiments with three plicate was calculated. The statistical Package for Social Sciences (SPSS version 20) was used and variables between more than two groups was detected using one way ANOVA test. A probability at $P< 0.05$ was considered significant.

RESULTS AND DISCUSSION

To investigate the effects of ECR on the proliferation of A549 cells were assayed by using MTT Assay test. Different increasing concentrations of treated cells (25, 40, 70 and 100µg/100µl) of ECR for 24, 48, or 72 h. ECR showed a dose- and time-dependent inhibitory effect on the growth of A549 cells. The extract caused 50% growth inhibition (IC50) at around (40 and 70µg/100µl) for 24, 48, and 72h, respectively (Table 1).

Table 1. The Effect of ECR on MTT levels in A549. Data were expressed as mean ± SD and Statistical analyses were performed using One Way ANOVA

Doses (µg/100µl)	0µg/100µl	25µg/100µl	40µg/100µl	70µg/100µl	100µg/100µl	Sig.(2-tailed)
Cost. 24h	100±0.04879	97.075±0.087558	66.685±0.137552	56.407±0.131724	39.292±0.1101	.181
Cost. 48h	100±0.07103	94.704±0.128571	61.541±0.161742	53.593±0.139114	31.394±0.131354	.092
Cost. 72h	100±0.034648	89.136±0.033723	57.121±0.023381	48.161±0.027541	21.123±0.023049	.029

The Effect of ECR on A549 Cells Colony Formation: The anti-proliferative and cytotoxic effects of the ECR on A549 cells were further determined and verified using anchorage-dependent colony formation assay (also referred to as clonogenicity). The ECR suppressed colony formation in a dose-dependent manner demonstrates that (25, 40, 70 and 100µg/100/µl) of ECR reduced colony formation in A549 cells (Fig 1).

To determine whether ECR inhibited cell growth by inducing apoptosis, A549 cells were treated with increasing concentrations for different time intervals and the cells were assessed using wound healing assay, comet assay and DNA degradation methods. The effect of ECR on migratory capacity of A549 cells was determined by wound healing assay. Treatment of A549 cells with ECR, dose-dependently, led to prevention of wound gap closure (Fig. 2a, b and c).

In an apoptotic assay, DNA degradation is an irreversible process. The ECR caused DNA fragmentation by DNA degradation. The comet assay is a reliable method for detecting single DNA strand breakage at the single-cell

level, and it is widely used as an apoptosis biomarker. Treatment of A549 cells with increasing doses of ECR (40 and 70 µg//100µl) for 24 h resulted in significant DNA damage. The images show most DNA migrated out of nuclei indicating that ECR caused severe damage to the nuclear scaffold (Fig 3). These findings suggest that ECR induced DNA damage in A549 cells.

Figure 1: Cell viability was evaluated using MTT assay. The A549 cells were treated with different concentrations of extract for 24, 48 and 72 h. The values are represented as the mean ± SD, P < 0.0microscope; magnification 4x.

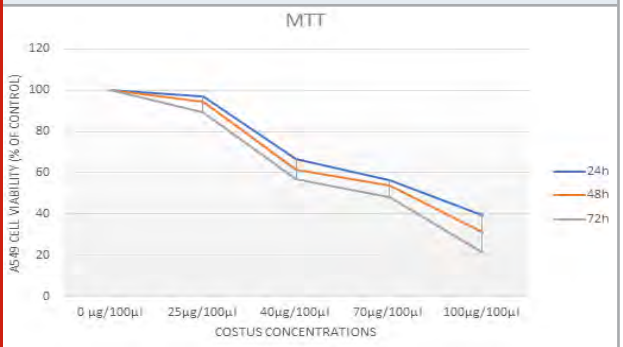
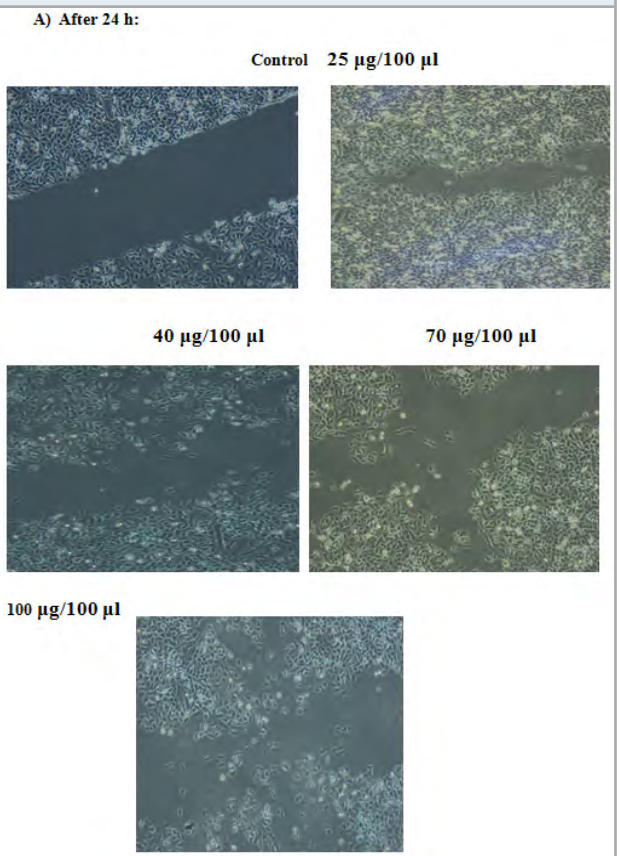
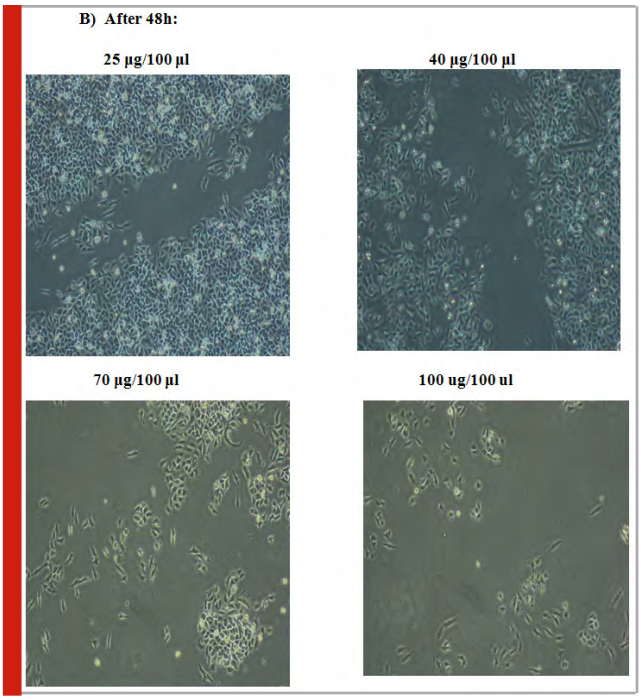
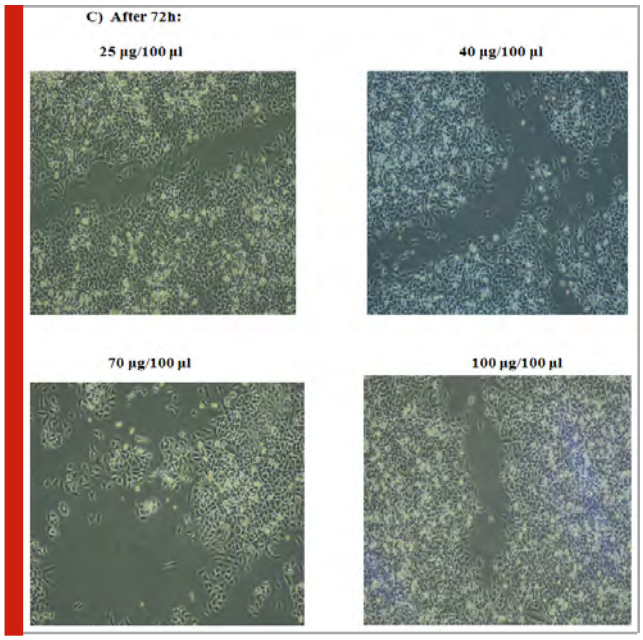


Figure 2: Effects of ECR on cell migration. A549 cell migration was determined by wound healing assay after treatment of A549 cells with increasing concentrations of the extract for A): 24, B): 48 and C0: 72 h; magnification ×10.







To obtain a further proof that ECR induces apoptotic death, cells were treated with increasing concentrations of the extract for 72 h and DNA fragmentation was visualized using agarose gel electrophoresis. The observations in (Figure 4) showed ECR induced inter-nucleosomal DNA cleavage forming discrete bands of 200 base pairs.

One of the first intracellular events after the onset of apoptosis is the disruption of mitochondrial membrane potential. The vital mitochondrial dye JC-1 is a useful tool for investigating mitochondrial function.

To prove that ECR induces loss of mitochondrial membrane potential, the effect of ECR was monitored after labelling cells with the sensitive dye JC-1. Thus, A549 cells were

treated with increasing concentrations of ECR for 24h, then the intensity of JC-1 fluorescence was assessed using microplate reader. Results in (Fig.5) indicated that ECR dose-dependently decreased the levels of JC-1 red/green ratio in A549 cells in a dose- and time-dependent manner ($P<0.05$) as compared to the untreated cells (Table 2).

Figure 3: In commit assay test in A549 cells treated with ECR, shows the creation of a DNA tail and damaged DNA nuclei as shown a bright head and tail, whereas nuclei with undamaged DNA appear round with no tail.

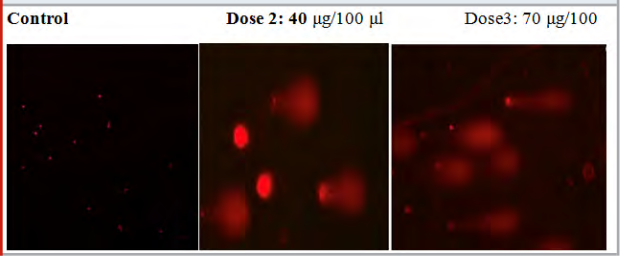
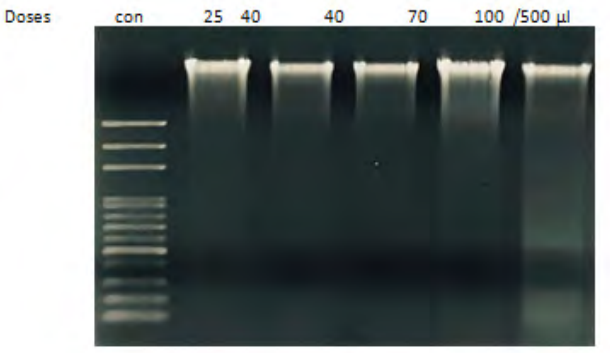
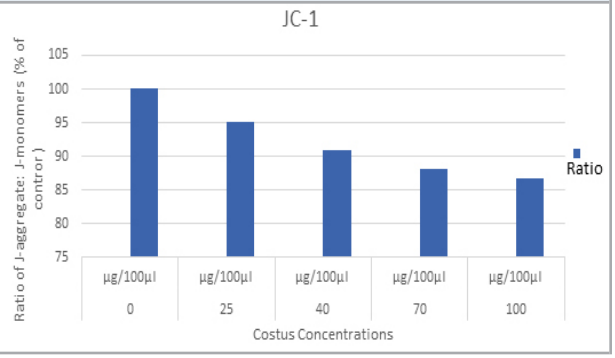


Figure 4: ECR induced oligonucleosomal degradation of the genomic DNA. A549 cells were incubated with the indicated concentrations of ECR for 72 h, then genomic DNA was extracted and electrophoresed.



The metastatic and unrespectable conditions of LC patients have a poor prognosis. The disadvantages and low efficiency

Figure 5: Fluorescence microplate reader analyzed the dissipation of mitochondrial membrane potential in A549 cells with increasing concentrations of ECR for 24 h and stained with JC-1 and the average ratio of red/ green fluorescence intensity, $P<0.05$ significantly different compared with control.



of available treatments of conventional chemotherapy, toxicity and drug resistance in metastatic patients (Qian,et al., 2017). Therefore, there is an urgent need to discover some more plant-based anti-cancerous agents with no or less cytotoxic effects. *C. spesious* is one of the important traditional medicinal herbs, having potent therapeutic capabilities (Vickers and Zollman, 2001; Hanahan and Weinberg 2013; Marinoet al., 2019).

Recently, the discovery of a natural antioxidants from different fruits and vegetables have been recently extensively studied for their antioxidant and/or free radical scavenging activity. A previous study indicated that the intake of fruits rich in antioxidants increases the antioxidant capacity of plasma and reduces the risk of chronic health ailments including cancer (Pandey et al., 2009; Lin et al., 2017; Abuelgasimet al., 2018). The present study was carried out to investigate the potential anti-cancer activity of C. Spesious in the A549 cell line. Primarily, MTT assay was done to evaluate the antiproliferative effect of ECR on A549 cell line in a time- and dose-dependent manner for 24, 48- and 72-hours treatment (Jonkman et al., 2018).

Table 2. The effect of ECR on JC-1 levels in A549. Data were expressed as mean ± SD and the Statistical analysis was performed by using One Way ANOVA

Doses (µg/100µl)	0µg/100µl	25µg/100µl	40µg/100µl	70µg/100µl	100µg/100µl	Sig.(2-tailed)
Costus	100±1.195203	95.16±0.177130	90.85±0.351164	88.20±0.372296	86.64±0.107190	.008

Cancer is often characterized by too little apoptosis and too much proliferation of cells. So, the induction of apoptosis is an advantageous strategy for cancer therapy. Cancer therapies depends on the extent of their ability to induce the death of cancer cells while allowing the survival of healthy cells. The comet assay and gel electrophoresis in our data confirmed that the DNA damage by ECR, which is a characteristic feature of apoptosis. Cells with damaged DNA could undergo apoptosis as damaged DNA is hard to be repaired; therefore, the observation of these assays suggests that ECR treatments triggered events leading to DNA damage and initiation of apoptotic cascade, which leads to programmed cell death (Zhang et al., 2008: Ardekani and Jabbari 2009; Mazina et al., 2015; Pistritt et al., 2016; El-Far et al., 2018).

CONCLUSION

A key regulator of apoptosis is Mitochondria which have shown to be involved in the dissipation of mitochondrial

membrane potential and integrating different pro-apoptotic pathways via the release of cytochrome c into the cytosol. The results of ECR depicted that mitochondrial membrane potential (ΔΨm) was gradually decreased with the increase of the dose, which is an important trigger to activate the intrinsic pathway.

Conflict of Interest: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Differential Effects of Solvents on Extraction, Pharmacognostic Evaluation and Antioxidant Activity of Long Pepper *Piper longum* Fruit Extract

Soma Chauhan and Amita Mittal*
University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra Haryana, India

ABSTRACT

The present study proposed differential effect of solvents on extraction, pharmacognostic evaluation and antioxidant activity of Long pepper (*Piper longum*) fruit extract. The bio-analytes present in *P. longum* fruit were extracted by dissolving powder fruit in water, ethanol, methanol, acetone, and ethyl acetate. Further, the extracts were dried using rotavapor at 55 °C and used to assess the phytochemical analysis where total phenolic compounds (TPCs) were evaluated using Folin-Ciocalteu Assay. Additionally, ascorbic acid content was measured, and Fourier Transform Infrared *Spectroscopy* (FTIR) analysis was performed to predict the presence of various compounds based on their surface functional groups. Further, antioxidant efficiency was determined by considering DPPH radical scavenging activity. The quantitative measurements of the TPC and ascorbic acid reveal the presence of the pharmacognostic compounds in *P. longum*, which is also supported by FTIR analysis. The FC assay test confirmed the highest polyphenols (181.0 ± 8.69 mg GAE/g) in ethyl acetate extract, while methanolic extract showed the maximum ascorbic acid content (1.51 ± 0.067 mg AE/g). Methanolic and ethyl acetate-based extraction process showed great results than other solvents as they showed maximum DPPH radical scavenging activity (% degradation), 81.57 %, followed by ethyl acetate (79.35%). The obtained results announced *P. longum* extract can be used as a raw material in pharmaceutical industries and local levels to improve health conditions.

KEY WORDS: ANTIOXIDANT ACTIVITY, ASCORBIC ACID, EXTRACTION, P. LONGUM, TOTAL PHENOLIC COMPOUNDS.

INTRODUCTION

Tremendous development in the technologies and improved medical facilities result in an increase in the population with a great pace where highly healthy and nutritive food is required. Many plants and their extracts are being used for several applications at the industrial and household level since ancient times, i.e., medicinal plants, spices, herbs, and nutritive plants (Goyal et al. 2018; Giannenas et al. 2020). Along with the aforementioned applications, various medicinal plants have been used to get several benefits, i.e., nutrients, medicine and preservatives (Deekshith et al. 2021; Ahmad et al. 2021). Additionally, spices and medicinal plants have been reported as potential antimicrobial (antibacterial, antifungal) and antioxidant agents, which increased their demand in medical biotechnology, promising many beneficial outcomes against newly emerging harmful microbes and diseases (Cenobio-Galindo et al. 2019; Szymandera-Buszka et al. 2020).

Medicinal plants are a rich and combined source of beneficial compounds, i.e., antioxidants, proteins, vitamins, polyphenols etc. (Khan et al. 2010; Chikatipalli 2021). Various studies have been performed to analyze the nutritive and medicinal potential of many spices, herbs, medicinal plant parts and their extracts. Various analytical techniques can be used to evaluate the presence of the aforementioned compounds in the plant raw (Boukhatem and Setzer 2020; Fitzgerald et al. 2020). Directly or indirectly, medicinal plants are a vast part of animal's life and human being in the whole world. Medicinal plants are highly used in INDIA in the form of food, spices, juice and preservatives daily, which increased the demand for some optimal study to find out the potential of spices and their most delicate use (Olalere et al. 2017; Srikacha and Ratananikom 2020; Beya et al. 2021).

Indian spices are not just limited to taste, colour and aroma, but it also has potential in therapeutic properties, which increased their uses in pharmaceutical industries (Kalra et al. 2021). Now Indian spices are used worldwide for food

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and medicinal purposes. Indian Ayurveda of medicine also enlightened the properties of Indian spices, their varieties, their benefits, and their potential use in treating or preventing various diseases (Chauhan et al. 2015). Piperine, an alkaloid, is a highly used spice in medicine and homemade "nukshas" for health purposes. Piperine, *Piper longum* also known as Indian long pepper or pippali, is highly used to treat various diseases, i.e., indigestion, bronchial disorders, snake venom, and breathing problems. Additionally, it has also shown beneficial results in infertility, menstrual problems and pregnancy (Kumar et al. 2011; Chauhan et al. 2019; Tiwari et al. 2020).

Additionally, a variety of compounds increase the availability of both reducing and capping agents in *P. longum*, which might increase its demand in nanotechnology and material science for the synthesis of nanoparticles for various applications, i.e., wastewater treatment, drug delivery system, antimicrobial activity, implants, and chemical conversion (Jamila et al. 2020; Favre et al. 2021; Schnabel et al. 2021; Cheraghipour et al. 2021).

As mentioned, these spices and other food items are highly rich for small but highly beneficial tracing compounds, but optimum use and combinations had not been evaluated. Hence, optimising the composition of spices and their constituents should be done to get the maximum possible results. To accomplish the present demand, we have successfully extracted the bioanalytes present in the selected *Piper longum* in different solvents, i.e., water, ethanol, methanol, acetone, and ethyl acetate. Further, the dried extracts were characterized using FTIR spectroscopy and the phytochemical analysis studies, i.e., Total phenolic compounds (TPCs), ascorbic acid equivalent and DPPH radical scavenging activity, performed to evaluate the constituents present in the *P. longum* and their potential activity.

MATERIAL AND METHODS

The dehydrated fruits of *Piper longum* were procured from the local shop of Kurukshetra. The purchased fruits were washed under tap water followed by distilled water and kept at 40°C for 72 hrs. Further, washed fruits were precast into powder form by grinding and stored for further analysis. Morphological appearance of *Piper longum* present in literature, and images of seeds used to identify the fruit (Kumar et al. 2011; Murphin Kumar et al. 2020). All the chemicals were purchased from Himedia, INDIA. For extraction, 10g of *P. longum* powder was macerate to 100 ml solvents (methanol, ethanol, acetone, ethyl acetate, and water) at room temperature for 48 hours by frequent mixing.Further, all the extracts were centrifuged at 5000 rpm, and the supernatant was filtered using Whatman no.1 filter paper. Then, the filtrate was dried using a rotary vacuum evaporator (Heidolph, VE-11) and stored at 4°C for further analysis. The schematic representation of the extraction process and phytochemical analysis is shown in Fig. 5 (Du et al. 2012; Cheraghipour et al. 2021).

Polyphenols (Folin ciocalteu) assay used with some modification to Deng et al. (2013) to detect the total phenolic compounds present in each extracted sample. Further, 0.2 ml of prepared solution, 4 ml of sodium bicarbonate solution (2% w/v) and 5.6 ml of DI water were mixed and added with 0.2 ml of 50% (v/v) Folin-Ciocalteu reagent and incubated for 15 minutes at RT after proper mixing. Further, the resultant mixer was analysed for absorbance value at 750 nm in UV-Vis Spectrophotometer and concentration was calculated by using a standard curve (Deng et al. 2013; Golder et al. 2021).

The ascorbic acid concentration was measured using Chelli and Golder (2018) method. 200 µl of extract (10 mg/ml) was mixed with 1800 µl DCPIP followed by measurement of absorbance at 604 nm after 20 minutes. A calibration curve was plotted between various ascorbic acid concentrations and their absorbance after incubation. Further, the linear fitting was performed for the standard curve equation. The obtained equation was used to calculate the AA concentration of unknown samples by using their absorbance value (Chelli and Golder 2018). (Chelli and Golder 2018). The obtained extracts were analyzed by using Fourier-transform infrared spectroscopy (FTIR) under KBr mode (PerkinElmer, spectrum two). FTIR spectrums were compared with the standard library and literature to assign the functional groups and their expected compounds. Additionally, % transmission or absorbance values for a specific peak (peak intensity) can be used to compare the fraction of specific compounds present in two different samples. In this case, high absorbance or low % transmission defines more percentage of a specific compound in solution (Ravi and Pandey 2019).

In-vitro DPPH radical scavenging activity was investigated using DPPH as an indicator of antioxidant activity. For this, 250µM DPPH stock solution was prepared by diluting with 9.9 mg DPPH with 100 ml ethanol (96% v/v). The control solution was prepared by adding DI to the DPPH stock solution maintaining the ratio (1:1). The sample solution was prepared by adding 0.5 ml Bio-extract to 1.5 ml DPPH solution, and blank was prepared by adding ethanol to DI and incubated at 25°C for 30 minutes for homogenization. Further, the absorbance value was measured at 517 nm using a UV-vis spectrometer (Jirankalgikar et al. 2014, More and Makola 2020). The scavenging activity was examined as % DPPH radical activity by using the expression given below:

DPPH radical scavenging activity = (Ac-As)/Ac x 100

Where, As defined the absorbance value of sample (extract) and As defined the absorbance value of control solution.

RESULTS AND DISCUSSION

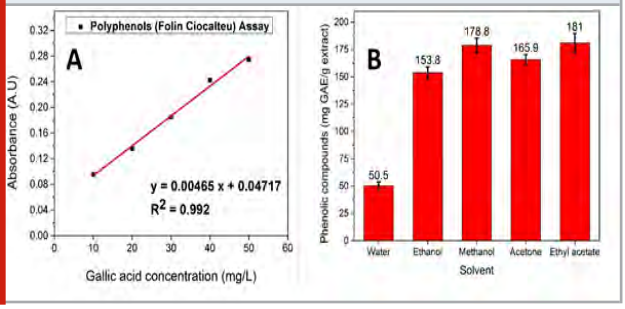
Determination of total phenolic compounds (TPCs): Polyphenols (Folin Ciocalteu) assay was used to detect the TPCs present in each extracted sample. A standard curve was plotted by using various gallic acid concentrations and their absorbance after incubation. Further, the linear fitting was performed for the standard curve equation. The obtained calibration curve and calibration equation is given

in Fig. 1 (A) and eq. (2). The obtained calibration curve equation is as follows:

$$y=0.00465x+0.04717 \quad (2)$$

Where y defines the absorbance value and x defines the concentration of the gallic acid (gallic acid equivalent).

Figure 1: (A) Calibration curve of gallic acid and (B) Total phenolic compounds concentration present in each extract (mg GAE/g extract)



Further, the gallic acid equivalent of extract samples were calculated by using a calibration equation with respect to their absorbance value. The number of phenolic compounds obtained in each sample of bio-extract is stated as mg GAE/g Extract. The obtained gallic equivalent values and absorbance of all the samples are given in Table 1 and Fig. 1 (B). The obtained data confirmed that *P. longum* is a rich source of polyphenols containing up to 181.0 ± 8.7mg polyphenols per g of dry extract. Additionally, ethyl acetate showed the best extraction of the polyphenol with 181.0 ± 8.7 mg GAE/g while water showed the least extraction with 50.5 ± 3.3 mg GAE/g (Alara et al. 2021).

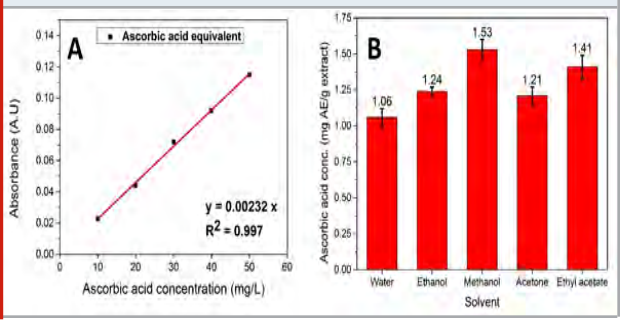
Furthermore, methanol (178.8 ± 6.6 mg/g) showed better extraction of polyphenols than acetone (165.9 ± 4.5 mg/g) and ethanol (153.8 ± 5.4 mg/g). The FTIR pattern also confirms the presence of maximum total phenolic compounds in ethyl acetate-based extract. An increase in the extraction of phenolic compounds in organic solvent than water might be attributed to the high solubility of polyphenols due to the suitable polarity of organic solvents than water (Alara et al. 2021). Methanolic extract of *Knoxia sumatrensis* leaves showed similar with 55.0 ± 1.3 mg GAE/g total phenolic compounds, whereas ethyl acetate extract of X. granatum leaves showed ethyl acetate extracts with 28.36±0.50 mg GAE/g TPC in the aqueous state (Loganathan et al. 2021; Darmadi et. al. 2021).

Determination of ascorbic acid equivalent (AE): The standard curve was plotted between known ascorbic acid concentrations and their absorbance, as shown in Fig. 2 (A). AE content was calculated from its absorbance value by using the standard curve equation as follows:

$$y=0.00232x \quad (3)$$

Where y defines the absorbance value, and x defines the ascorbic acid equivalent (AE) concentration (Darmadi et. al. 2021).

Figure 2: (A) Standard curve of ascorbic acid and (B) Ascorbic acid concentration present in each extract



The amount of ascorbic acid obtained in each bio-extract sample is expressed as mg AE/g dry extract. The obtained ascorbic acid concentration and absorbance of all samples are given in Fig. 2 (B) and Table 1. The obtained data confirm that P. longum also contains ascorbic acid but in very less amount. Additionally, the methanolic extract showed the highest ascorbic acid concentration (1.53 ± 0.07 mg/g), which attributed to the high solubility of the ascorbic acid in methanol than others. Unlikely, the water showed the least extraction of the ascorbic acid (1.01 ± 0.06 mg/g) and ethyl acetate (1.41 ± 0.08 mg/g) showed better extraction than acetone (1.21 ± 0.06 mg/g) and ethanol (1.24 ± 0.03 mg/g). Hence, ascorbic acid has better solubility in methanol than others which might be due to appropriate polarity and partition coefficient. *Pleurotus floridanus* extract showed a higher 17.54 mg/g ascorbic acid content and osage orange's extract showed around 12 mg/g ascorbic acid, which claimed presence of less amount of ascorbic acid (vitamin C) in spices than citric acid fruits (Bains et. al. 2020; Dadayan et. al. 2021).

Analysis of compounds present in extracts by FTIR: All the bioextract samples were analysed by using FTIR spectroscopy to study the corresponding functional groups. The FTIR spectrum for all the bio-extract samples and their corresponding functional groups are given in Fig. 3. The small peak obtained at 3742 and a broad peak at 3325 cm^{-1} (in methanol solvent extraction) correspond to the O-H group of flavonoids/tannins and tannins/phenols/ polyphenols, respectively. Further, a peak around 2922 cm^{-1} and 2853 cm^{-1} represents the O-H stretching vibration of polyphenols and C–H stretching vibration of aromatic compounds, respectively. The peak present at 1720 cm^{-1} is allocated to the C=O stretching vibration of the carboxylic group of hemicellulose. Peaks at 1617, 1442, and 1360 cm^{-1} claimed the C=O group of ketone/aldehyde functional groups, C-H bending mode of lignin/carboxylic acid, and CH_2/CH_3 deformation due to the presence of glycosides/ carbohydrates, respectively. Further, peaks of 1248, 1039, and 990 cm-1 –are linked to C-O bonding of polyphenols/ phenols, OH-CH stretching in sugar and polysaccharides, and β-linkage of the polysaccharide, respectively (Brangule et al. 2020).

Lastly, small peaks at 803 and 712 cm^{-1} correspond to the C-H chain of carbohydrate or fatty acid chain (Purkayastha et al. 2012; Trifunschi et al. 2015; Oliveira et al. 2016;

Monisha and Vimala 2018; Ravi and Pandey 2019). Conclusively, all the extracts showed the same peaks, but the % transmittance of these peaks vary due to the different extraction power of solvents. However, a broad peak is clearly visible in methanol (represented by blue), corresponding to the O-H group of tannins, phenols, and polyphenols, which claimed the high level of phenolic compounds present in methanol solvent-based extract than others. Additionally, % transmittance value of ethyl acetate showed the least transmission than others which confirmed the presence of the high amount of analytes in the extract (Brangule et al. 2020; Manasa et al. 2020).

Figure 3: FTIR spectrum of *P. longum* bio-analytes extracted in water, ethanol, methanol, acetone, and ethyl acetate.

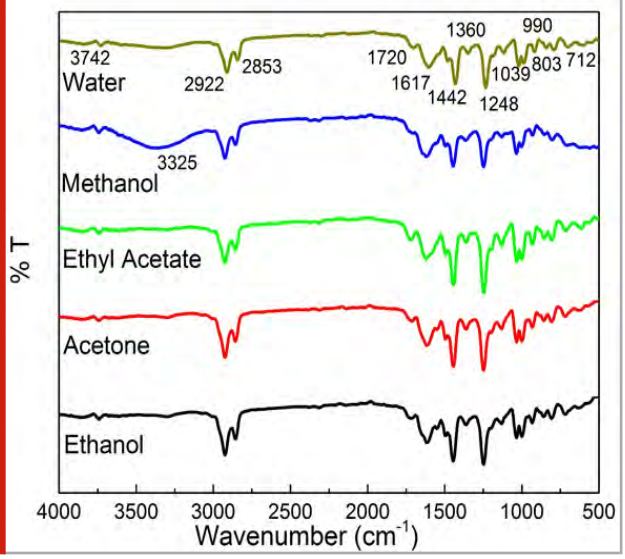
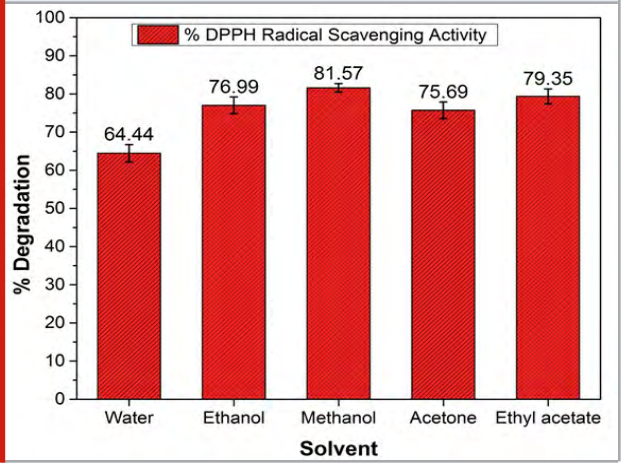


Figure 4: DPPH scavenging activity (% degradation) of each extract



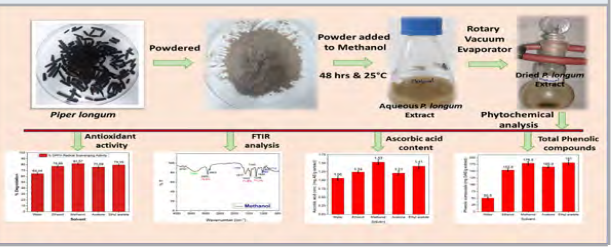
***In vitro* antioxidant activity:** The antioxidant activity of all the extracted samples was examined. The obtained results are shown in Table 1 and Fig. 4. The obtained data were fitted to equation 3 to calculate % DPPH degradation. The obtained data confirmed that the methanolic extract showed maximum DPPH radical activity (% degradation), 81.57 ± 1.1 %, while water extract showed the least DPPH radical activity (64.44 ± 2.3 %) (Espinoza et al. 2020).

Additionally, ethyl acetate (79.35 ±1.9 %) showed better DPPH radical scavenging activity than acetone (75.69 ±2.2 %) and ethanol (76.95± 2.1 %). The obtained result confirmed that methanol and ethyl acetate are the better solvents for extraction of DPPH radical scavengers than other solvents, which might be attributed to the presence of scavenger compounds in methanolic solvent.

Table 1. Total phenolic compounds concentration, ascorbic acid concentration and antioxidant activity of each extract

Solvent	Gallic acid equivalent (mg GAE/g)	Ascorbic Acid Concentration (mg AE/g extract)	% Degradation (Antioxidant activity)
Water	50.53 ± 3.29	1.01 ± 0.063	64.44 ± 2.30
Ethanol	153.76 ± 5.41	1.25 ± 0.028	76.95 ± 2.13
Methanol	178.85 ± 6.57	1.51 ± 0.067	81.57 ± 1.12
Acetone	165.94 ± 4.48	1.21 ±0.058	75.69 ±2.18
Ethyl Acetate	181.0 ± 8.69	1.38 ± 0.076	79.35 ± 1.93

Figure 5: Schematic representation of extraction of the bio-analytes and their analysis.



The obtained results are well supported by total phenolic compound and ascorbic acid content, which might influence enhanced DPPH scavenging activity. Similar results have been reported where ethyl acetate extract of *Mangifera indica* leaves showed 79.73 % DPPH inhibition, and methanolic extract of *Conyza bonariensis* L. leave showed 47.83% inhibition (Ibrahim et al. 2020; Espinoza et al. 2020).

CONCLUSION

The findings of the study claimed the excellent DPPH radical scavenging activity of bio-analytes present in *Piper longum* extracted by using different solvents. Additionally, the obtained results confirmed the presence of polyphenols and ascorbic acid, which can be used for other applications. Methanolic and ethyl acetate-based extraction system showed great results than other solvents as they showed maximum DPPH radical scavenging activity (% degradation), 81.57 %, followed by ethyl acetate (79.35%). Variation in phytochemicals quantity and quality with change in the solvent, rectify the optimization of solvent for extraction to contract maximum benefits. Hence, *Piper longum* and other spices bio-analyte's composition can be stimulated in the future for various health benefits with appropriate practice.

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Conflict of Interests: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Enhancing the Occupational Health Safety among Radiology Nurses Working in the Hospital of Gurugram, Haryana, India

Naorem Chanu Sofia, Raman Deep* and Kavita Pillai
Faculty of Nursing, Shree Guru Gobind Singh Tricentenary University, Gurugram, Haryana, India

ABSTRACT

This pre-experimental study was done on forty nursing personnel posted in the radiology section of a selected hospital of Gurugram National Capital Region using one group pre-test post-test design. The study sample was selected using convenience sampling technique. A structured knowledge questionnaire and observational checklist were used to assess the effectiveness of the educational package regarding occupational health safety. The mean pre-test knowledge score was 16.65 and mean post-test knowledge score was 22.13. Similarly, the mean pre-test practice score was 12.42 and mean post-test practice score came to be 14.70. The calculated 't' value for knowledge score was 37.099 and the practice score was 11.801 at a 0.05 level of significance. The result reported that nurses were aware of radiation safety and protection, even though it would be helpful if their knowledge and practice are apprised regularly.

KEY WORDS: OCCUPATIONAL HEALTH, OCCUPATIONAL HEALTH SAFETY, RADIATION HAZARDS, RADIATION PROTECTION, RADIATION SAFETY.

INTRODUCTION

In Occupational safety we try to manage dangers at the workplace to achieve an admissible level of risk, whereas in workplace safety pertains to safeguard employees' health and safety while on the job, irrespective of their vocation (Aluko et al. 2016). Occupational health is an area of health care emphasized by many disciplines. It has been dedicated to the well-being and safety of workers in the workplace. It has mainly focused on injury prevention, protection, and employee education. Occupational health and safety focus on to create and maintain a safe and healthy working environment (Aluko et al. 2016; Albander 2021). As stated by National Institute of Occupational Safety and Health (NIOSH), every year, nearly one hundred thousand people pass away owing to occupational health problems, while approximately four hundred thousand fresh cases of occupational diseases are identified each year (Sreekumaran and Balachandran 2018; Albander 2021).

Around 20 lac workers lose their life before natural death time each year from occupational illnesses such as occupational poisonings and cancers, with 16 billion workers suffering from occupational diseases, 27 billion from workplace injuries and physical hazards such as radiation. Artificial sources account about 16 percent of occupational hazard radiation, mainly medical exposures (Megeed et al. 2019; Albander 2021). The health of the workers faces many risk factors at the workplace which lead to various health hazards. The workers might have cancers, accidents, musculoskeletal problems, respiratory problems, hearing loss, stress-related disorders, infections, etc. Patients and health care workers in several disciplines, including diagnostic radiology, interventional cardiology, interventional radiology, nuclear medicine, and surgery, are concerned about radiation safety (Saha 2018; Frane and Bitterman 2020; Dabhekar and Naik 2021).

Radiation is a sort of energy that manifests itself as waves or particles and is present in everyday lives. Radiation exposure can be internal or external, and it can occur through a variety of routes. Due to the increasing use of ionizing radiation for diagnosis and treatment, patients and healthcare professionals are facing serious health and safety issues. Minor radiation exposure also creates a threat to healthcare personnel (Ploussi and Efstathopoulos 2016; Khamtuikrua and Suksompong 2020; Frane and Bitterman 2020; Dabhekar and Naik 2021). The majority (80%) of our ionizing radiation exposure comes from natural sources, the most significant of which is radon gas, while the remaining 20% comes from artificial origin, mainly medical X-rays

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(Awosan 2016). Emission from these sources is both unseen and odorless, and because health care personnel are generally preoccupied with performing tasks, they may overlook it. As a result, health-care workers are exposed to significantly more radiation than is necessary. Excessive radiation exposure can cause a variety of health problems, including cataracts, hair loss, congenital abnormalities, and cancer development (Salim et al. 2022).

Exposure to these emissions to would be parents may lead to reduced potency, unsuccessful fertilization or implantation, or fetal malformations. Maternal exposure after conception, might cause the fetal death or anatomical and physiological problems of the newborn. In addition, there may be other ill effects like spontaneous abortion (both early and late), significant or slight congenital anomalies, death around the time of birth, less than average weight at birth, issues related to development or behavior of the newborn, and exposure of carcinogen to fetus by crossing placental barrier (Khamtuikrua and Suksompong 2020; Park and Yang 2021; Salim et al. 2022). Therefore, healthcare professionals need to be fully aware and knowledgeable about the dangers of radiation in order to protect themselves and their patients from adverse effects (Salim et al. 2022).

Table 1. Socio- demographic variables of participants

Demographic variables	Categories	Frequency	Percentage
Age (years)	20 to 29 years	37	92.5%
	30 to 39 years	2	5.0%
	40 to 49 years	1	2.5%
	50 years and above	0	0%
Gender	Male	4	10%
	Female	36	90%
Qualification	GNM	9	22.5%
	PB B. Sc Nursing	5	12.5%
	BSC Nursing	25	62.5%
	Postgraduate	1	2.5%
Designation	Staff nurse	26	65%
	Senior staff nurse	11	27.5%
	Assistant head nurse	2	5%
	Head nurse and above	1	2.5%
Working experience (Years)	Less than 5 yrs	30	75%
	6 to 10 yrs	7	17.5%
	11 to 15 yrs	2	5%
	16 to 20 yrs	1	2.5%
	More than 20 years	0	0%
Have you attended training/ refresher courses on radiation safety?	Yes	40	100%
	No	00	00%
How frequently have you attended training/ refresher courses on radiation safety?	Once	25	62.5%
	2 to 4 times	13	32.5%
	5& more than 5 times	2	5%

Three principles of radiation protection stated by The International Commission on Radiological Protection (ICRP) are: justification, optimization (as low as reasonably achievable (ALARA)), and limitation of dose of radiation. The major purpose of safeguard against radiation is to ensure that everyone who works with radiation is properly protected. Health care providers may experience symptoms such as nausea and vomiting within hours as a result of the high degree of radiation exposure administered over a short period of time, which sometimes can lead to mortality in days or even weeks afterward. This research was done with an objective to give adequate and effective information regarding the importance of using safety measures and protective devices while doing the duty in the radiology department (Ploussi and Efstathopoulos 2016; Frane and Bitterman 2020; Khamtuikrua and Suksompong 2020).

MATERIAL AND METHODS

A pre-experimental, one group pretest posttest design was conducted for this study. This study was approved by the ethical committee of SGT University, Gurugram. Forty nursing professionals posted in Radiology Department were selected using convenient sampling technique. The

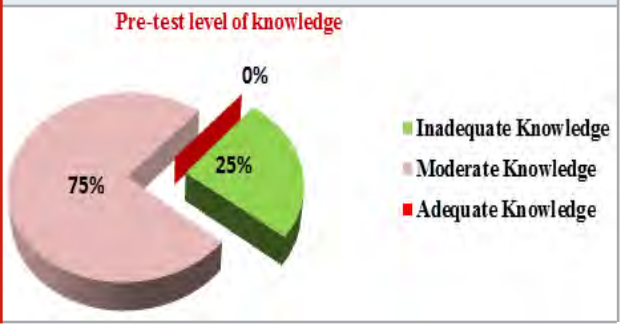
knowledge questionnaire consisted of 25 closed- ended questions that contain selected variables such as age, gender, qualification, designation, year of experience, training/refresher courses attended, and frequency of training undergone, and information regarding the radiation safety measures, protective devices, risks and health effects of radiation exposure. The second questionnaire was an observational checklist, framed to assess the radiation safety and protective measures.

Data collection tools were evaluated by the guide and co-guide and 10 experts from the different fields regarding the content, clarity, and language of the tool. Suggestions were taken into account, and relevant improvements were made to increase the validity of the tools. The willingness to participate in the study was obtained through their consent. The goal of this research project was conveyed to the participants, and they were assured of confidentiality. The study was conducted at Medanta the Medicity, Gurugram in the month of April 2021 after taking formal permission. The data collected in the Microsoft excel sheet was analyzed in SPSS software version 28 and descriptive and inferential analysis was done.

RESULTS AND DISCUSSION

Times to time many studies are done related to the different aspects of occupational health safety among nursing personnel. Present study was conducted to evaluate the efficacy of the educational package in terms of information and practice of nursing personnel related to occupational health safety. The discussion of this study is based on the data gained through statistical analysis and interpretation of data.

Figure 1: Pie chart showing percentage of pre-test level of knowledge (N=40)



Distribution of the selected variables of the participants: Majority of the participants (92.5%) were between the ages of 20- 29 years. Five percent were between the ages of 30-39years, 2.5 percent were between the ages of 40 and 49years, and none of the respondents were between the ages of 50years and above. Ninety percent were female and 10% were male. Most of the samples (62.5%) were with BSc Nursing and 65% were Staff nurses, 75% were with 0-5years of experience. All the nursing personnel had undergone training/ refresher courses and 62.5% had attended these sessions only once, 32.5 % had attended 2- 4 times, and only 5% had attended 5 and more than 5 times.

Percentage Distribution of Staff Nurse's Pre Test and Post Test level of knowledge and Practice regarding Occupational Health Safety.

Fig 1 reveals that during pre-test score of 75 percent of nurses had a moderate knowledge score, 25 percent had an inadequate knowledge score, and 0 percent had adequate knowledge regarding occupational health safety,

Figure 2: Pie chart showing the percentage of the post-test level of knowledge (N=40)

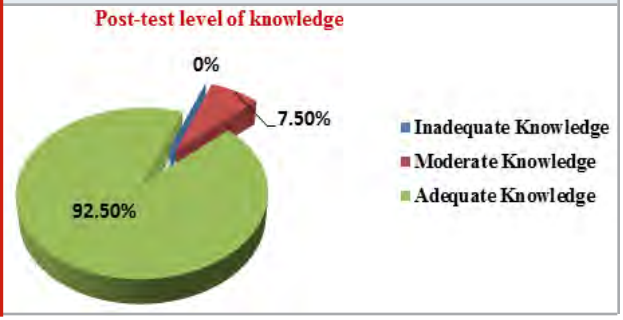


Fig.2 reveals that 92.50 percent of Nurses had adequate knowledge and 7.50 percent had moderate knowledge regarding radiation safety. Thus, most of the respondents had adequate knowledge regarding occupational health safety in the post test. This result agrees with the results of other researchers that the participants had a good understanding of ionizing radiation, knowing their source, benefit, and potential hazard. This is most likely due to a common awareness of radiation and the threats it poses. Even though the nursing personnel are aware of the radiation safety and protection, it would be better to update them regularly. (Luntsi and Ajikolo 2016; Abuzaid et al. 2018; Mohamed et al. 2018). Also, this result goes contra with the findings that all of the nurses did not attend any courses related to radiation protection and most of the nurses were not aware of safeguarding against radiation and their risks. The study concluded that this lack of awareness may lead to very critical effects on both nurses and patients. During and after the formal nursing education, the nurses need some formal training regarding radiation risks and protection courses (Maliro 2011; Muhammad et al. 2015; Partap et al. 2019; Salehi et al. 2020; Salim et al. 2022).

Figure 3: Pie chart showing the percentage of the pre-test level of practice

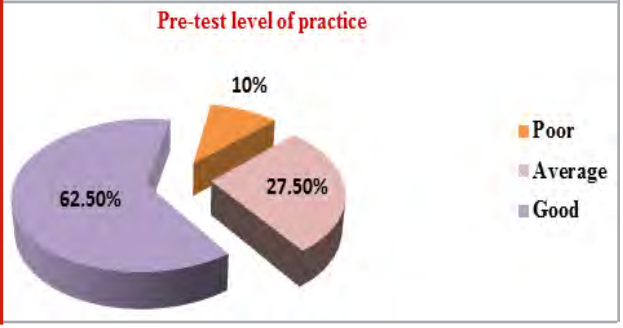


Fig. 3 and Fig. 4 highlight that before administering the educational package, more than half of the respondents had good practice levels (62.50%) regarding radiation safety, although after administering the educational package, their practice levels increased to 95%. Thus, most of the participants had good practice level regarding radiation safety. A similar study was conducted by a researcher on occupational hazards and protective measures among Radiographers in terms of knowledge and practice (Megeed et al. 2019; Salehi et al. 2020).

Figure 4: Pie chart showing the percentage of the post-test level of practice

Table 2. Comparison of the mean pre-test and post-test level of knowledge and practice and standard deviation to assess the effectiveness of educational package.

	Group	Mean ±SD	Mean difference	df	't' value	p-value
Knowledge (n= 25)	Pretest	16.65±1.673	5.48	39	37.099	0.001*
	Post-test	22.13±1.800		39		
Practice (n=15)	Pretest	12.42±1.55	2.28	39	11.801	0.001*
	Post-test	14.70±0.46		39		

*Significant; p- value= statistically highly significant at 0.05 level of significance

The findings revealed that about two-thirds of the participants had satisfactory practice scores regarding radiation hazards and safety measures (Megeed et al. 2019). On contrary, another study revealed that the lack of knowledge and practice made the nurses unable to protect the patients and themselves against ionizing radiation. The study concluded that the potential benefits of Medical Professionals need to be emphasized in universities and hospitals, where the curriculum contents in radiation sciences are insufficient and recommended medical schools or hospitals for additional training (Ibrahim 2018; Partap et al. 2019; Salehi et al. 2020).

The statistics revealed that the mean difference between the pretest and post-test scores for knowledge was 5.48 whereas the mean difference in practice score between pre-test and post-test was 2.28. These data depicted that there is a true difference between the mean score and has not occurred by chance. So as a result, the educational package effectively improved the information and practice regarding occupational health safety among nursing personnel posted in the radiology department. The study concluded that the effectiveness of the educational package was highly effective (Jihad and Khudur 2020).

Chi-square values revealed that no association was found between the post-test data of knowledge and practice regarding occupational health safety among nursing personnel with the selected variables. A similar study conducted revealed that there was no significant relation of post-test knowledge scores with their demographic variables (Ibrahim 2018). This result of the present study disagrees with another research which revealed that there was statistically significant relation of post-test knowledge scores with their demographic variables (Megeed et al. 2019).

CONCLUSION

The findings of the present study conclude that most of the participants had adequate level of knowledge and practice regarding radiation safety and protection after administration of educational package. Even though the nursing personnel had awareness regarding radiation safety and protection, it would be better to update them on regular basis. Periodic practical courses, regular in-service education program and continuing education courses can be provided to improve and maintain their knowledge and practice regarding radiation hazards and protective measures. Regular monitoring of health care workers who are exposed to the radiation is also very essential.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

Ethical Statement: Ethical approval was given by the Institutional Ethical Committee in a meeting held

on 28/11/2020. Ethical Approval Number is FON/SGTU/20/262/06.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Antimicrobial Activities of *Coriandrum sativum, Anethum graveolens* and *Linum usitatissimum* Essential Oil-Nanoemulsions For Use as Alternatives Food Preservative

Alia M. Aldahlawi,[1,2] Ghaida S. Bin Siddik[1] and Magda M. Aly[1,3*]

[1]Department of Biological Science, Faculty of Science, King Abdulaziz University Jeddah, Saudi Arabia

[2]Immunology Unit, King Fahad Medical Research Center, King Abdulaziz University, Jeddah Saudi Arabia,

[3]Botany Department, Faculty of Science, Kafrelsheikh University, Kafr El Sheikh, 33516, Egypt.

ABSTRACT

Bacterial infectious diseases are still one of the main causes of death and severity of bacterial infections, which have markedly gone up mainly due to the emergence of multidrug resistant bacteria. The aim of this study was to prepare nano-emulsions using Coriander and Dill and Flaxseed essential oils and investigate their antibacterial activities. Three nano-emulsions (NEs) were produced by mixing essential oils, surfactants and water with droplet sizes of NEs formulations in the range of 25-62 nm. No toxicity was recorded for Coriander and Dill at 100 µl/ml while Flaxseed NE showed moderate toxicity. Standard local pure cultures of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Candida albicans* were obtained. Dill NEs and Coriander NEs showed moderate activities against both *S. aureus* and *E. coli* with inhibition zone diameter rang ing from 12-14 mm and weak activities against *K, pneumoniae* and *P. aeruginosa*. The three tested Oil Nanoemulsions showed weak inhibition activity with an inhibition zone diameter of 10 mm against *Candida albicans* as a test yeast. The best minimum inhibitory concentrations (MICs) of nanoemulsions was for flaxseed NE against all the tested Gram negative bacteria but the results were higher than that obtained by the control antibiotic that showed excellent activity. In conclusion, the tested NEs showed inhibitory activity against the tested bacteria due to inhibition of vital microbial functions such as cellular transport and/or energy production.

KEY WORDS: PATHOGENIC BACTERIA, NANOEMULSION, ESSENTIAL OILS, DILL, CORIANDER, FLAXSEED.

INTRODUCTION

All over the world, infections with bacteria are considered severe public health problems and almost food-borne diseases are caused by bacteria which cause diarrheal disease, the commonest food borne disease. The Gram-negative bacteria, *Escherichia coli, Shigella* and *Salamonella* in addition to the Gram positive, *Bacillus cereus, Staphylococcus aureus,* methicillin-resistant *S. aureus* (MRSA) were the main causative agents of almost all food-borne diseases. The huge use of antibiotics to treat these dangerous infections led to the emergence of many multidrug resistant bacteria which are the main threats that affect human health (Berglund, 2015; Dadonaite et al., 2018; Awol et al., 2019; Tariq et al., 2019; Al-Nabulsi et al., 2020; Alsayeqh, 2020; Al-Seghayer and Al-Sarraj, 2021).

Mortality and morbidity are increased due to the increase in appearance of multi drug resistant bacteria, thus demand for new antimicrobial agents is increased. There is increasing interest in secondary and aromatic metabolites of medicinal plants due to their well known antimicrobial properties and their success in treatment of several diseases. These plant metabolites can be used especially as natural food preservative instead of chemical ones which were very harmful to human health. Nowadays, several natural materials especially plant essential oils (EOs) have been shown to exhibit broad spectrum inhibitory activities against various Gram positive and Gram-negative pathogenic bacteria and in fact, they can be used to replace synthetic antimicrobial agents. These plant essential oils inhibit undesirable bacterial growth without the appearance of resistant isolates (Dhifi et al., 2016; Tariq, 2019; Long et al., 2019; Raut and Karuppayil, 2014; Long et al., 2019). The antibacterial activities of plants essential oils of oregano, cinnamon, and rosemary, sage thyme, lemon grass, clove, lavender and tea leaves were extensively studied (Najafi-

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Taher et al., 2018; Gago et al., 2019; Yazgan, 2020; Guo et al., 2020; Falleh et al., 2021).

They showed excellent to moderate antimicrobial activities against both Gram positive and negative bacterial pathogens by denaturation of the bacterial cell membrane which cause potassium and ions leakage in addition to other cell components and finally, cell death. These plant essential oils are hydrophobic compounds and usually have quite low solubility in water, which limits their utilization in aqueous-based foods and beverages. This problem could be simply resolved by encapsulating these essential oils within emulsion-based delivery systems to enhance their biological activities. Therefore, several attempts to incorporate essential oils into different nano-delivery systems have been reported (Donsı and Ferrari, 2016; Tariq, 2019; Aly et al., 2019; Al-Otaibi, 2021; Hassan et al., 2021; Upadhyay et al., 2021; Almasi et al., 2021; Mansouri et al., 2021).

After essential oils are encapsulated into suitable emulsion delivery systems (Nanoemulsion), they can then be incorporated into aqueous-based foods (e.g., beverages) and other products by simple mixing. The nano emulsions has a broad spectrum of activity against bacteria, viruses, fungi and some dermatophytes and spores forming bacteria (Alkhatib et al., 2013, Chime et al., 2014; Chang et al., 2015; Alkhatib et al., 2016 a, b). These nano-emulsion solutions were mainly oil dispersed in water with a diameter ranged from 2-200 nm and stabilized by an interfacial film of surfactant and/or co-surfactant. Essential oil nanoemulsions enhance the antimicrobial properties of EOs and prevent the growth of bacteria. Therefore, the aims of this study were to extract and prepare essential oil nano emulsion from *Coriandrum sativum* (Flaxseed), *Anethum graveolens* (Coriander) and *Linum usitatissimum* (Dill) to be used as alternatives food preservative or as antimicrobial agents against some bacterial pathogens.

MATERIAL AND METHODS

Standard local pure cultures of *Escherichia coli* 25922, *Staphylococcus aureus* 29213, *Pseudomonas aeruginosa* 27853, *Klebsiella pneumoniae* 700603 and Candida albicans 10231 were obtained from Microbiological lab., Faculty of Science, King Abdulaziz University. All the tested isolates were checked for purity using different phenotypic and biochemical assays like Gram reaction, API20E, hemolytic activity and Oxidase, peroxidase and gelatinase tests (Ibrahim et al., 2014). Pre-culture of each test organism was prepared using a nutrient broth medium. Under aseptic conditions, Agar well diffusion method and minimum inhibitory concentration were used to determine the antimicrobial activity of NEs.

Plants seeds were oven dried for 24 hrs at 70°C. Then, the dried seeds were broken into fine powder and essential oils were extracted from 600 g of the dried and powdered seeds by Hexane at 65 - 70°C using Soxhlet extractor (Stony Lab 500 ml Soxhlet Type Extraction Apparatus). The resulting essential oils were stored at -20°C in sealed brown glass vials until use. Water in oil NEs were prepared by mixing 1ml from distilled water, 500 µl from previously extracted essential oils and 100µl from each non-ionic surfactant (tween80, span 20), except for *Linum usitatissimum* where 200 µl from tween80 were used (Alkhatib et al., 2013). The mixtures were sealed and placed in boiling water up to 90-100°C in the water bath until slightly opaque nanoemulsion was formed, similar to the color of their extracted essential oils (Al-Sowayigh et al., 2019, Aldahlawi et al., 2020). Different concentrations of nanoemulsions, 35, 50 and 100 ppm were prepared from each plant oil and all the prepared concentrations were preserved at 4◦C until used.

All prepared NEs were diluted with media (DMEM) and centrifuged at 4500 rpm for 15 min. Then, droplet size and zeta potential of nanoemulsions were obtained at 25°C and analyzed using Zetasizer Nano analyzer. *Artemia salina*, commonly known as brine shrimp, was used as test organism to investigate the toxicity of essential oils nanoemulsions using lethality bioassay technique (Aly and Gumgumjee, 2011; McClements and Rao, 2011; Aldahlawi et al., 2020). About 8 ml of solution containing *Artemia salina* (water life De-capsulated brine shrimp eggs) were added to a hatching chamber containing 300 ml of tap water with a small spoon of food salt and a little of baking soda.

The hatching chamber was left under an inflorescent bulb and air pump for 24-48 h for the eggs to hatch into shrimp larvae. Hatching larvae were collected into a beaker with plastic pipette and separated from the eggs. Then, 10 larvae of *Artemia salina* were collected and added to small Petri plate. The final volume of water in each plate was adjusted to 5 ml with seawater, immediately after adding the larvae. Different concentrations of NEs including 25, 50, 75 and 100 µl were then added to plates and left at room temperature for 4 hrs. After 4 hrs the number of larvae that survived in each plate was determined under stereo microscope (OPTIKA). Percentage of cell mortality of the brine shrimp obtained for each concentration of NEs was recorded.

Under aseptic conditions, Petri plates (90 mm×15 mm) were prepared by pouring 15 ml of sterile Mueller Hinton agar medium in each plate and the agar was allowed to solidify. About 100 µl of freshly prepared inoculum suspensions of bacteria (1.5×10^8 CFU/ml) and yeast ($2x10^6$ CFU/ml) was added using micropipette and uniformly swabbed all over the surface of the Mueller Hinton agar plates using sterile cotton swab by rotating the plate 60 degrees after each application to spread the bacteria and yeast on the surface of the agar plate completely. After inoculum absorption by agar, three wells of 7mm diameter were made in the agar with the help of sterile cork-borer and labeled properly (Nolte and Metchock, 1995). Each well was filled with 100 µl of the nanoemulsion using micropipette. Plates were left for 1-2 hrs at room temperature to allow proper diffusion of the nanoemulsion to occur in the medium. The plates were then incubated without inverting at 37°C for 24 hrs. The average diameter of the inhibition zone surrounding the well was measured (Al-Sowayigh et al., 2019).

Antimicrobial activity of prepared nanoemulsions was determined using Broth microdilution method. In sterile plastic, disposable, microtitration plates with 96 flat-bottom wells, 100 µl/well of sterile distilled water was added into

12 wells using micropipette. The tested microorganisms were prepared from overnight nutrient broth culture and adjusted to 0.5 turbidity using McFarland standard. Then, 5-7 drops of phenol red indicator were added to the adjusted suspensions (Phenol red solution 0.5% in DPBS, purchased from Sigma Chemicals Co., St. Louis, MO, USA). After that, 25 µl/well of prepared bacterial inoculum (1.5×108CFU/ml) and yeast (2x106CFU/ml) was added into 12 wells, and then 125 µl of prepared NEs was added into well number 1 for each organism and mixed properly.

Two-fold dilution of NEs was prepared by transferring 125 µl from well 1 to well 2 and so on and keep diluting and mixing until well 11. The last well 12 serves as growth control (negative control). Finally, the plates were incubated for 24 hrs at 37°C with shaking. The procedure was repeated for each a nanoemulation with all tested organisms. The MIC was determined by changing in the color of the medium. Appearance of pink color indicates the MIC which is the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism (Al-Sowayigh et al., 2019). All data were expressed as mean ±standard deviation ($\bar{x}$ ± SD). Statistical analysis was performed with one-way analysis of variance (ANOVA) and pairwise t-test using MegaStat and Microsoft Excel. The statistical significance difference was considered when p-value <0.05.

RESULTS AND DISCUSSION

Zetasizer Nano analyzer was used to identify the Droplet size (Z-average), polydispersity index (PDI), homogeneity or quality of the dispersion and Zeta potential (electrophoretic mobility) of the nanoemulsions formulations. Droplets size was in the range between 25-62 nm. All NEs have negative zeta potential values as shown in Table 1.

Table 1. The Z-average diameters, PDIs and zeta potential measurements of Nanoemulsion formulations expressed as $\bar{x}$ ± SD.

Oil Nanoemulsions		Z-average diameter (nm)	PDI	Zeta potential (mV)
Scientific name	Common name			
Linum usitatissimum	Flaxseed NEs	38.3 ± 8.28	0.216	0.26± 0.048
Coriandrum sativum	Coriander NEs	55.25 ± 6.13	0.111	-2.36 ± 2.74
Anethum graveolens	Dill NEs	28.67 ± 3.13	0.109	-2.17 ± 2.13

Table 2. Toxicity of NEs against *Artemia salina*

Tested NEs	Toxicity (% of mortality)			
	25 µl/ml	50 µl/ml	75 µl/ml	100 µl/ml
Flaxseed NEs	20%	33%	30%	50%*
Coriander NEs	10%	23%	20%	20%
Dill NEs	5%	20%	20%	40%
Control	0%	0%	0%	0%

*Toxic Concentration

Artemia salina was used to test the toxicity of 3 different essential oils nanoemulsions. The mortality percentage was determined under stereo microscope. The NE concentration that kills 50% of the brine shrimps (LC_{50}) considered toxic. No toxicity was found for each of the NEs concentrations even for the highest concentration 100 µl, except for flaxseed NE at 100 µl which showed inhibition of 50% of the brine shrimps as shown in Table 2. LC_{50} of flaxseed NEs was obtained at 100 µl/ml. This is meaning that increase NEs concentration may be lead to more mortality percentage of *Artemia salina* larva.

The activity of NEs was tested against pathogenic bacteria and yeast using agar well diffusion method. The results of Table 3 represent the mean diameters of inhibition zones (mm) exhibited by NEs against the tested bacteria and yeast. NEs showed moderate activity against the tested bacteria and *Candida*. Flaxseed NEs showed excellent activity against Gram negative bacteria with mean diameter of 18 mm while low activity was recorded against Gram positive bacteria with mean diameter of 14 mm. Dill NEs and Coriander NEs showed moderate activities against both S. aureus and *E. coli* with inhibition zone diameter ranged from 12-14 mm and weak activities against *K, pneumoniae* and *P. aeruginosa*.

The three tested Oil Nanoemulsions showed weak inhibition activity with inhibition zone diameter of 10 mm against *Candida albicans* as a test yeast. Also, minimum inhibitory concentrations (MICs) of nanoemulsions were determined for the tested pathogens represented in Table 4. The lowest MIC range (19-25 %) was recorded for Flaxseed NEs while the range was 29.7 to 31.0% for Coriander NEs and was ranged from 24.2-42.1 % for Dill NEs. Cefaclor (control antibacterial agent) had MIC ranged from 2-5 µg/ml while Fluconazole (control antifungal agent) had MIC (5 µg/ml) against *Candida albicans*.

A lot of attention was given to nanoemulsions which were produced for protecting and delivering certain plant essential oils. These nanoemulsions were characterized mainly by measuring the droplet size, viscosity, thermal stability and refractive index. Three nanoemulsions (NEs) of Flaxseed, Coriander and Dill were produced by mixing the tested essential oil, surfactants, and water and heating the competent in a water bath until clearance. The physical properties of the obtained NEs formulations were determined and the results were similar to that obtained by

Ozogula et al. (2020) for thyme essential oil nanoemulsions. The roles of the previous nanoemulsions in controlling Dendritic cells phenotype expression, apoptosis, and cytokine secretion were recorded before by Aldahlawi et al (2020). Non-significant effect on the viability and apoptosis of dendritic cells was noticed by these NEs and they have a tolerogenic effect on these cells. The prepared Flaxseed, Coriander and Dill NEs had droplet sizes in the range of 25-62 nm. Similarly, Neem nanoemulsion was successfully prepared and had means droplet size of 67.85 nm (Ghotbi et al 2014; Donsı and Ferrari, 2016).

Table 3. Antimicrobial activity of NEs using agar well diffusion assay.

Tested NEs	Diameter of inhibition zone (mm) mean ±SD				
	Staphylococcus aureus	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Candida albicans*
Flaxseed NEs	14.7±0.6ac	18.3±1.5d	18.3±2.1d	18.0±1.0d	10.0±1.0e
Coriander NEs	$13.0\pm1.5_{ac}$	$14.0\pm1.5_{a}$	$11.0\pm1.1_{c}$	$11.0\pm1.4_{c}$	$10.5\pm1.5_{e}$
Dill NEs	$13.0\pm1.0_{ac}$	$14.0\pm0.9_{c}$	$10.0\pm0.5_{c}$	$10.0\pm1.6_{c}$	$10.4\pm2.5_{e}$
Cefaclor*	$25.0\pm1.6_{ac}$	$34.6\pm1.5_{b}$	$39.3\pm1.5_{c}$	$30.0\pm1.0_{b}$	NA
Fluconazole*	NA	NA	NA	NA	$22.0\pm1.3_{f}$

*Cefaclor was used as control for bacteria, Fluconazole was used as control for yeast. NA: Not applicable. The data are presented as mean ± SD, n= 3. The same letters indicated non significant results at $p< 0.05$.

Table 4. MICs of NEs against the tested microorganisms.

Tested NEs	MICs of NEs (%, v/v)				
	Staphylococcus aureus	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Candida albicans*
Flaxseed NEs	25.7±5.0#	21.8±2.9#	21.8±2.2#	19.9±1.9#	43.7±2.0#
Coriander NEs	29.7±2.3#	31.0±3.9#	31.0±4.5	31.0±0.9#	31.0±9.9#
Dill NEs	26.7±1.0#	24.3±2.7#	42.1±11.9	31.0±2.1#	34.3±7.9#
Cefaclor* (µg/ml)	5.0±0.12	2.0±0.09	3.9±0.06	3.9±0.11	NA
Fluconazole* (µg/ml)	NA	NA	NA	NA	5.0±0.03

*Cefaclor was used as control for bacteria, Fluconazole was used as control for yeast. NA: Not applicable., The data are presented as mean ± SD, n= 3. The symbol # : indicates significant results at $p< 0.05$ compared to control.

It was reported that using a mixture of surfactants to prepare nanoemulsions from plant essential oils provides better effectiveness than one. The droplet size increases as the amount of water increases and decreases with the amount of surfactant due to the increase in interfacial area and the decrease in the interfacial tension. It was clear that the amounts of components used in the preparation of NEs affects the droplet size of the nanoparticles. Non-ionic surfactants have hydrophilic and lipophilic molecules that balance size and strength of these opposing molecules is called a hydrophilic-lipophilic balance number (Porras et al., 2004; Ibrahim et al., 2015).

Artemia salina, known as brine shrimp, is one of the standard organisms for testing the toxicity of chemicals. It is the most convenient test organism for toxicity tests, because of its easy hatching from dry cysts and its eggs can be stored for years without losing their viability (Sorgeloos et al., 1978, Ruebhart et al., 2008). All the tested NEs had different antimicrobial activities against all the tested microorganisms, Gram positive and negative bacteria and yeast. The best MIC was for flaxseed NE against all the tested microorganisms. Agar diffusion tests are often used as qualitative methods to determine whether a bacterium is resistant, intermediately resistant or susceptible to the tested antimicrobial agent.

Another method to determine the antimicrobial activity of NEs was minimum inhibitory concentrations (MIC) which is a quantitative method and considered as the 'gold standard' for determining the susceptibility of organisms to antimicrobials, therefore it was used to judge the performance of all other methods of susceptibility testing (Andrews, 2001). Although, the Gram negative

bacteria contain a high lipid layer which is less susceptible to antimicrobial agents, the NE prepared using the surfactant can overcome the lipid barriers and increase the susceptibility of the bacterial cells (Gupta et al., 2014). Ziani et al. (2011) studied the activity of thyme oil (TO) nanoemulsions against some yeast isolates such as *Saccharomyces cerevisiae Brettanomyces naardenensis* and *B. bruxellensis* while Meral et al., (2019) recorded the antimicrobial activities some bacteria, *Pseudomonas aeruginosa*, *E. coli* and *Salmonella typhimurium*.

Nanoemulsions based on essential oil of thyme showed inhibitory activity against some food borne pathogens bacteria (*Salmonella paratyphi, Staphylococcus aureus, Enterococcus faecalis* and *Klebsiella pneumoniae* in addition to some spoilage bacteria of fish. The nanoemulsions affect the cell wall morphology in addition to the damage in bacterial cell membranes. Thus, preparation of nanoemulsions from essential plant oil increased antibacterial activity and can be used as food preservative agent during processing or packaged fish or food products.

CONCLUSION

As a conclusion from experiments that performed on different pathogens, all nanoemulsions had no toxic effect (LC50) at different concentrations when tested against brine shrimp, except for flaxseed NEs that showed the highest inhibition zones against all the tested microorganisms, and the best activity was against Gram negative bacteria, *E. coli, K. pneumoniae, P. aeruginosa*. Dill NEs and Coriander NEs showed the highest inhibition against *E. coli*. The best (lowest) MIC was recorded for flaxseed NE against the three tested Gram negative bacterial while higher MICs (lower activities were recorded for coriander NE and Dill NEs. So, all NEs have antimicrobial activity against multidrug resistant pathogens, thus they can be used in medicine and food industries.

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Conflict of Interest: The authors declare no conflict of interests.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Morin Mitigates Unpredictable Chronic Mild Stress-Induced Depression By The Regulation of Endoplasmic Reticulum Stress and Brain-Derived Neurotrophic Factor-Mediated Apoptosis

Ramaraj Kiruthika,[1] Asokan Prema,[2] Selvaraj Aruna Devi,[1] Thamilarasan Manivasagam[1] and Arokiasamy Justin Thenmozhi[1,3]*

[1]Department of Biochemistry and Biotechnology, Annamalai University, Annamalainagar, Tamil Nadu, India.

[2]Department of Chemistry, Government College of Arts and Science, Tittagudi, Tamil Nadu, India

[3]Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India.

ABSTRACT

According to the report of World Health Organization, it is expected that depression will be one of the major causes for disability and disease in the world, by 2030. Currently used drugs will not be effective to the depressive patients, due to their lower efficiency with side effects. Hence, there is a need to concentrate on natural products to overcome therapeutic hassles. Morin, a bioflavonoid found in fruits, vegetables, some herbs and wine is reported to have an antidepressant-like effect against acute stress conditions. It is reported to possess various pharmacological properties such as antioxidant, anti-diabetic, hepatoprotective, anti-cancer and anti-inflammatory activities. To understand the antidepressant effect of morin against unpredicted chronic mild stress (UCMS), rats were divided into normal, UCMS alone, UCMS and morin (30 & 60 mg/kg) and morin alone (60 mg/kg) groups. Serum corticosterone levels, expression of brain derived neurotrophic factor (BDNF), apoptotic and endoplasmic reticulum (ER) stress related indices were compared among the groups. UCMS alone exposed rats showed less crossings with diminished activities in open field test (OFT), increased serum corticosterone levels and enhanced expression of BDNF signaling and ER stress related markers associated with apoptosis as compared to control. In contrast, morin (60 mg/kg b.w.) cotreatment attenuated UCMS induced abnormalities as compared to UCMS alone exposed animals. The present results indicated the antidepressant-like actions of morin against UCMS in rats were partially due to its anti-apoptotic effects by regulating BDNF/Trk-B and ER stress related markers. Moreover the results of the present study indicated that the morin may act as therapeutic agent for the management of depression alone or with other currently used antidepressants.

KEY WORDS: BRAIN DERIVED NEUROTROPHIC FACTOR, CHRONIC MILD STRESS, CORTICOSTERONE, ENDOPLASMIC RETICULUM STRESS, MORIN.

INTRODUCTION

Stress is the unavoidable condition affecting the quality of human life in which the system can acclimatize or fail to acclimatize day-today hassles, abuse or diseased state (Sakr et al. 2015). The failure of adaptation or exposure to prolonged stress leads to development of depression, ultimately leads to various diseases including Alzheimer's, Parkinson's and cardiovascular diseases, diabetes mellitus and rheumatoid arthritis due to the disturbance in brain functions and body physiology (Chakravarty et al. 2013). The World Health Organization predicted that depression will be the second most contributing source of disability and disease in the world by (2030) (Albert and Fiori 2014; Sheng et al. 2021).

Chronic stress exposure induces numerous reactions inside the body via activating HPA axis, thereby stimulating hypothalamic-derived corticotropin-releasing factor (CRF) secretion. CRF enhances the adrenocorticotropic hormone secretion from the pituitary gland, which in turn activates adrenal cortex to release cortisol (Charney 2003; Sheng et al. 2021). Protein folding and secretion is one of the

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main functions of endoplasmic reticulum. Under various pathological state such as oxidative stress, a high fat diet, hypoxia, hypoglycemia, calcium depletion and stress could impair the protein folding and results in their accumulation, a condition called as ER stress (Hetz and Papa 2018). It is shown that both the chronic social defeat stress and UCMS triggers ER stress and depressive behaviors in rodents (Zhao et al. 2013; Tan et al. 2015). ER stress and apoptosis are the key features underlying in the pathology of depression, as the functions of ER are vigorously impaired under stress conditions, apoptotic events are stimulated (Hetz et al. 2015). BDNF is the most studied neurotrophin in depressed conditions, reported to maintain the homeostasis of neuronal proliferation and death that acts via the activation of two receptors, (i) tyrosine kinase receptor-B (Trk-B, and (ii) p75 (Peng et al. 2020).

Unpredictable chronic mild stress (UCMS) exposure to rats mimics the similar behavioural and neuroendocrine changes as that of depressive patients (Lee et al. 2015) and hence used to analyze stress pathology. Hippocampus, an important brain region, reported to play a key role in cognitive function that is mainly affected by depression and anxiety (Biala and Kruk 2009). Incomplete knowledge about the etiology and progression of depression leads to unsuccessful treatment. Currently used drug could not used successfully to the depressive patients, due to their lower efficiency with side effects (Bschor et al. 2012). Hence, there is a need to focus on natural products to overcome therapeutic hassles (Munir et al. 2020).

Morin, a ubiquitous bioflavonoid possesses antioxidant, anti-diabetic, cardioprotective, nephroprotective, hepatoprotective, anti-cancer and anti-inflammatory properties (Rajput et al. 2021). Further, morin exhibited its neuroprotective effect against amyloid, acrylamide, haloperidol, glutamate and 1-methyl-4-phenylpyridinium ion induced toxicity in rodents (Khamchai et al. 2020; Singaravelu et al., 2021). Previous experiment from our lab demonstrated that the oral administration of morin attenuated the UCMS induced behavioural impairments and oxidative stress by its potent antioxidant activity (Kiruthika et al. 2021). However to elucidate further molecular mechanism and confirm its neuroprotective effect, the antiapoptotic role of morin via ER stress and BDNF/TrkB mediated pathways were evaluated in this study.

MATERIAL AND METHODS

Male *Albino wistar* rats (200–225 g) were obtained and kept in standard conditions at Central Animal House, Rajah Muthiah Medical College & Hospital, Annamalai University with food and water *ad libitum*. The Institutional Animal Ethics Committee (Reg. No. 160/1999/CPCSEA, Ethical Clearance No. AU-IAEC/1212/4/18) approved the protocols of the work. Morin and other chemicals of analytical grade used in this study were procured from Sigma-Aldrich, Bangalore, India. Primary antibodies against glucose-regulated protein 78 (GRP78), spliced X-box-binding protein-1 (XBP-1), CCAAT/enhancer binding protein homologous protein (CHOP), cytochome c, cleaved caspases 3and 12, bax, BDNF, TrkB, p-TrkB, p75, β-actin and secondary antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). After the acclimatization phase of one week, thirty rats were randomized into five groups (n = 6). Group I animals were kept without disturbance for 42 days under standard condition. Group II rats were subjected to UCMS for 6 weeks (Lucca et al. 2009; Yang et al. 2017).

Group III and IV rats were exposed to UCMS and oral administration of morin (30 and 60 mg/kg) (Ola et al. 2014) for 42 days. Group V rats were administered with morin (60 mg/kg) alone as group IV for 42 days. After performing the open field test (Rajasankar et al. 2009) animals were decapitated and blood was collected and centrifuged for serum separation. It was stored in frozen condition until biochemical assay was carried out. Circulatory corticosterone level was quantified by enzyme immunoassay using a commercial kit procured from Assay Designs, Inc., Ann Arbor, USA. Hippocampus was procured, ground in an ice-cold RIPA buffer and centrifuged to collect the supernatant. Levels of protein were quantified by Lowry et al. (1951) method. Cellular proteins (50 µg) were separated using SDS–PAGE. It was blotted to PVDF membrane, which were developed with blocking buffer and incubated with primary immunoglobulins of cyto c, caspases 3 and 12, bax, BDNF, TrkB, p-TrkB, GRP78, XBP-1, CHOP and p75 with shaking overnight.

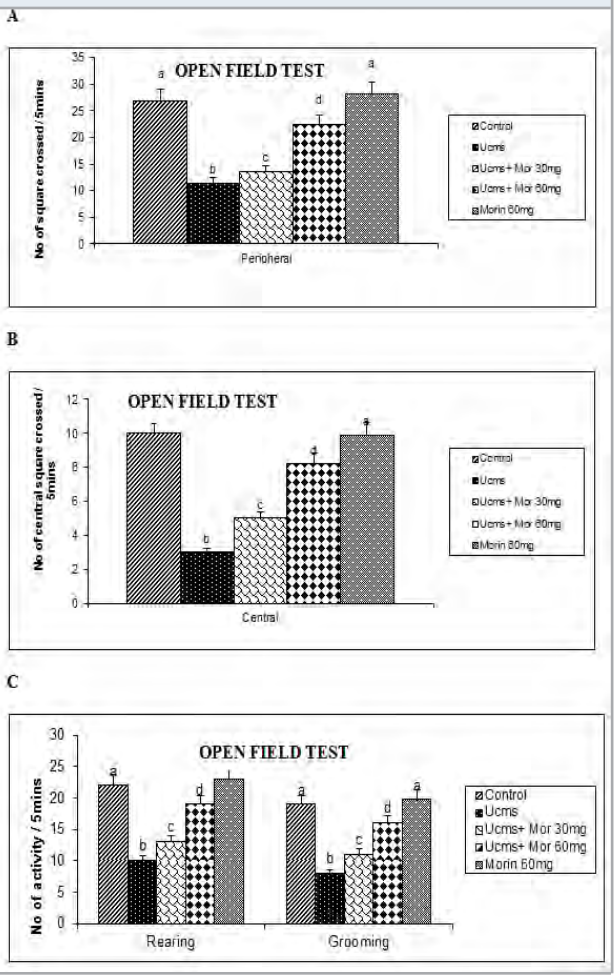
The membranes were then incubated with secondary antibodies for 2 h at 37˚C and washed. Protein bands were quantified by using chemiluminescence's method and obtained bands were scanned and estimated by gel image analysis program. Data were expressed as mean ± Standard Error (SEM) of six rats for behavioural and biochemical studies and of three rats for western blot analysis. The statistical significance was calculated by one-way analysis of variance (ANOVA) using SPSS version 15.0 using Duncan's Multiple Range Test (DMRT). A value of $p< 0.0.5$ was considered as a significant difference between groups and the values not sharing common alphabet differ significantly with each other.

RESULTS AND DISCUSSION

The protective effect of morin on UCMS induced behavioral despair was assessed by the OFT (Figure 1). In OFT, there was a significant reduction in the number of peripheral and central lines crossed, grooming and rearing actions in the rats subjected to UCMS as compared to control animals. However, more crossings with enhanced grooming and rearing activities were exhibited by UCMS and morin (30 and 60 mg/kg) co-treated groups as compared to UCMS alone rats. Liu et al. (2009) indicated that the rats exposed to UCMS for 42 days showed depressive behavioral indices like reduced locomotion and activity. The open field test is used to measure the deleterious effect of UCMS. The enhanced score in OFT represents the increased movement, grooming and rearing activities and lowered state of anxiety (Duan et al. 2008). In our study, diminished locomotion and activities were found in the UCMS exposed and morin

co-administrated rats as compared to UCMS alone exposed animals, which is corroborated with previous studies (Ben-Azu et al. 2019; Hassan et al. 2020).

Figure 1: Effect of morin on movements (A, B) and activities (C) of rats exposed to UCMS.



The protective effect of morin on UCMS persuaded dysfunctions in feedback mechanism regulating endocrine secretion was indicated by estimating serum corticosterone levels (Figure 2). UCMS rats showed an increase in the serum corticosterone levels than the non-stressed control rats. Morin (30 and 60 mg/kg) administration to UCMS rats significantly depleted the levels of serum corticosterone as compared to UCMS exposed rats. Hyperactivation of the HPA axis causes more secretion of corticosteroids from the adrenal cortex. Hence corticosterone is considered as an important marker of the HPA axis hyperactivation that plays a vital function in the determination of therapeutic efficacy of antidepressant drugs (Dean and Keshavan 2017; Nandam et al.2020).

The UCMS induced enhancement of corticosterone levels were observed in this study is consistent with previous results having deleterious effect on health as suggested by Teague et al. (2007). Pariante and Miller (2001) shown that an increased levels of glucocorticoid is found in cerebrospinal fluid, plasma and urine of depressive patients. Checkley (1996) suggested that the enhanced level of glucocorticoid is one of the main causes of depression in animal models. Prolonged glucocorticoid administration induced alterations in both the morphology and functions in the brain regions thereby inducing depressive like behaviors (Gregus et al. 2005). Moreover neuronal atrophy stimulated by glucocorticoids resembles the same as that of depressed patients (Sapolsky 2000). Successful antidepressant therapies are connected with the normalization of HPA axis dysfunction by the reducing serum cortisol levels and enhancing monoamine levels in UCMS rats (Parker et al. 2003; Nandam et al.2020).

Figure 2: Effect of morin on the levels of circulatory corticosterone in rats exposed to UCMS.

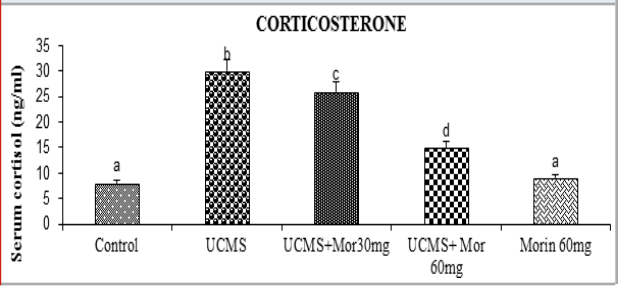
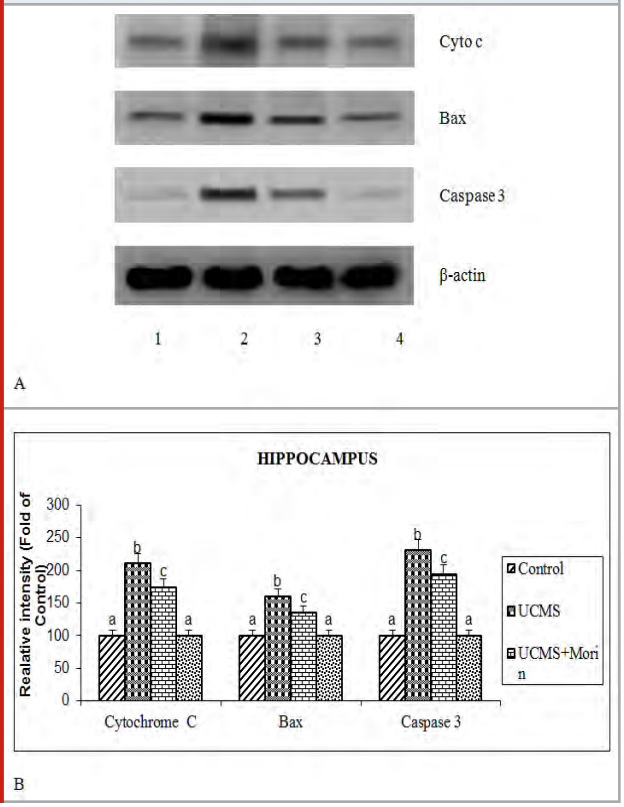


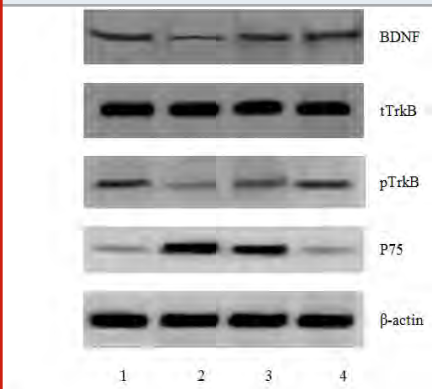
Figure 3: A. Effect of morin on the expression of apoptotic markers in rats exposed to UCMS. B. Immunoblot data are quantified by using β-actin as an internal.



To investigate the antiapoptotic effect of morin on UCMS exposed rats, the expression of apoptotic (bax, caspase 3, cyto c), neurotrophin (BDNF, TrkB, p-TrkB and p75)

and ER stress (GRP78, spliced XBP-1, CHOP, cleaved caspase 12) related markers were studied. The protein expression studies indicated that the elevated expression of bax, cleaved caspases 3 and 12, cyto c, GRP78, spliced XBP-1, CHOP and p75, diminished expression of BDNF and p-TrkB with un-altered expression of total TrkB were found in the UCMS-exposed rats ($p < 0.05$). Administration of morin attenuated UCMS induced alterations in the expression of apoptotic, neurotrophin and ER stress related markers (Figure 3, 4, 5).

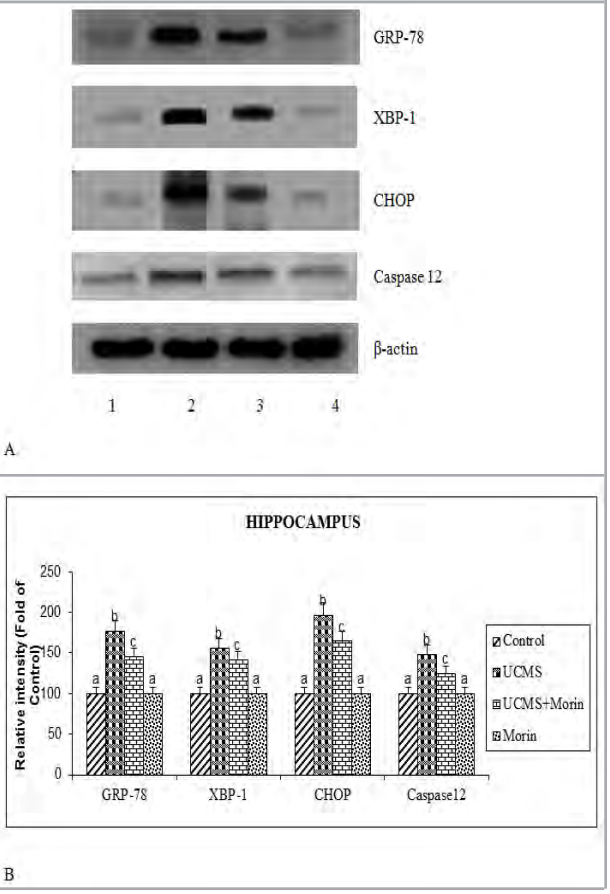
Figure 4: A. Effect of morin on the expression of BDNF, pTrkB and P75 in rats exposed to UCMS. B. Immunoblot data are quantified by using β-actin as an internal.



Levels of BDNF in brain were found to be diminished in experimental models of depression and postmortem samples of patients with depression, while the administration of antidepressants enhanced BDNF expression in hippocampus and cortex (Song et al. 2020). Kimpton (2012) indicated that the reduced expression of BDNF in stressed animals results in diminished neurogenesis and development of depressive and impaired cognitive symptoms, which is corroborated with our present results. BDNF exert pivotal effect through activation of TrkB receptor (facilitating viability, differentiation and synaptogenesis of neurons) and p75 (leading to apoptosis) receptors. The diminished expression of TrkB receptors and enhanced expression of p75 found in UCMS rats thus favored apoptosis, whereas oral treatment of morin ameliorated these alterations by its neuroprotective function (Celik et al. 2020).

Previous studies indicated that neurological disorders like depression, Parkinson's and Alzheimer's disease occurred due to the progression of excessive apoptosis. We

Figure 5: A. Effect of morin on the expression of ER stress related markers in rats exposed to UCMS. B. Immunoblot data are quantified by using β-actin as an internal.



found that the increased bax, cytosolic cytochrome c and caspase 3 expression indicating the enhanced apoptosis of hippocampal neurons in CUMS rats. Activation/inactivation of apoptosis is primarily regulated by the Bcl-2 family proteins (Elmore 2007). The activation of proapoptotic factor Bax, enhances mitochondrial membrane permeability after their entry into mitochondria from cytosol, thereby resulted in mitochondrial cytochrome c release and activation of caspases 3. This eventually leads to apoptotic cell death (Eskes et al. 1998; Lidsky and Schneider 2003). Previous studies demonstrated that morin has been showed to offer the neuroprotective effects against several metabolic disorders and neurodegenerative diseases through its anti-apoptotic effect (Komirishetty et al. 2016; Sharma et al. 2020).

During UCMS exposure, increased expression of GRP78 is reported and considered as the hall mark of ER stress because of its translocation from the membrane receptors and stimulate the unfolded protein response (UPR) (defensive process) in neurons (Zhang and Zhang 2010). The UPR induced apoptosis occurred by the activation of CHOP and caspase-12. CHOP is classified in the C/EBP family which maintains the homeostasis of pro-apoptotic BCl2 proteins to induce apoptosis (McCullough et al. 2001). Similar to GRP78, the upregulated CHOP expression is a

key symbol of ER stress-induced apoptosis and its inhibition is considered as the restoration of ER function (Nishitoh 2012). The caspase-12, a sole member of caspase family found in ER membrane, and its down regulation was shown to prevent ER stress-induced cell death (Ferri and Kroemer 2001). Lee et al. (2003) indicated that the genes of spliced XBP-1, play a key role in the restoration of ER function by regulating the transcription factors involved in protein folding and degradation. Morin attenuated various toxins induced ER stress related indices in several in vivo and in vitro studies, which is corroborated with our results (Sharma et al. 2020).

CONCLUSION

The findings of the present experiment indicated that morin offered neuroprotective effects on UCMS-induced apoptosis in rat model of depression. The above said antiapoptotic role of morin is seems due to the regulation of BDNF pathway and ER stress. The obtained molecular results were supported by the biochemical and behavioral studies. Hence, morin may act as therapeutic agent for the management of depression with other currently used antidepressants. However further studies are needed for exploring its clinical efficacy and safety.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Use of Color Channels to Extract Heart Beat Rate Remotely from Videos

R. A. Sinhal[1],* K. R. Singh,[2] M. M. Raghuwanshi[3] and K.O. Gupta[4]

[1]Datta Meghe Institute of Engineering, Technology and Research, Wardha, India;

[2]Yeshwantrao Chavan College of Engineering, Nagpur, India;

[3]Symbiosis Institute of Technology, Nagpur, Constituent of Symbiosis International (Deemed University), Pune, India;

[4]St. Vincent Pallotti College of Engineering & Technology, Nagpur, India

ABSTRACT

Since last decades, photoplythesmography (PPG) signals that are extracted from the optical absorption in the tissues are increasingly being used for health diagnosis. Despite a good literature, there are different claims about the use of color channels as red, green and blue for extraction of PPG signal, i.e., pulse rate from the videos captured through high resolution cameras. In this article, we present the technique for extracting the heart beat rate (pulse rate) from the videos captured through the mobile cameras for all three color channels and thier analysis. Experiments were performed on a DMIMS database comprising 720 videos, out of which we used 25 videos for the analysis. The pulse rate estimated from the blue channel, was in good agreement with reference data extracted using an MP20 monitor, used as the gold standard. The findings of the present study demonstrated the non-invasive color intensity method for detection of pulse rate from the pre-recorded video of 30 seconds. The algorithm is tested on the DMIMS dataset which we have captured in uncontrolled setting. The green channel is proven to be statistically significant for the video recorded followed by red and then blue channel. The accuracy of the pulse extracted is still low because of low signal to noise ratio.We therefore conclude that the presented technique is best for pulse rate extraction through a blue channel followed by red and green channels respectively.

KEY WORDS: COLOR CHANNELS; PULSE RATE; REMOTE PHOTOPLYTHESMOGRAPHY; RGB COLORSPACE; VITAL SIGNS.

INTRODUCTION

Photoplethysmography (PPG) is an optical technology that detects changes in blood volume under the skin bed of microvascular tissue. This non-invasive technology was commonly used in wrist, finger or ear-based pulse oximetries to measure vital signs as pulse-rate and peripheral oxygen saturation (SpO2) (Verkruysse et al. 2008; Sinhal et al. 2017). The PPG theory is based on the optical absorption of arterial blood by some light wavelengths into other areas of biological tissue (Zhang et al. 2014). A light source lights up a pulsatile blood-containing piece of human skin and a camera catch this. There are two steps in rPPG checking; first region of interest detection, and second signal generation to detect a pulse (Finžgar et al. 2021).

The oxygenated blood circulation induces changes in the amount of hemoglobin molecules and proteins as the pulsatile blood circulates in the human cardiovascular system, creating differences in optical absorption and scattering around the light spectrum. We can get a PPG signal that reflects changes in blood volume by emitting light through the skin layers and measuring the amount of light spreading through the tissue. Physiological variables (e.g., pulse-rate/heart-rate, pulse-rate variability/heart-rate variability, respiratory rate, SpO2, blood pressure, etc.) may be further determined by the PPG waveform and cardiovascular states assessed (e.g., arterial diseases, stiffness, aging, etc.) (Wang et al. 2017). The traditional use of a PPG calculation (i.e., pulse oximetry) involves a light source that emits light into the skin (i.e., a Light-Emitting Diode (LED)) and a light receiving photodetector that has spread through the skin. Depending on their geometric location around the skin, the light source and photodetector have two distinct operating modes: transmissive and reflective (Sinhal et al. 2017; Finžgar et al. 2021).

rPPG camera based vital sign monitoring can be categorized in two trends detection of signals through the variations in skin and detecting head motions induced by changes in heart cycle. rPPG method mainly focus on measuring pulse rate, blood rate and heart rate using videos acquired from

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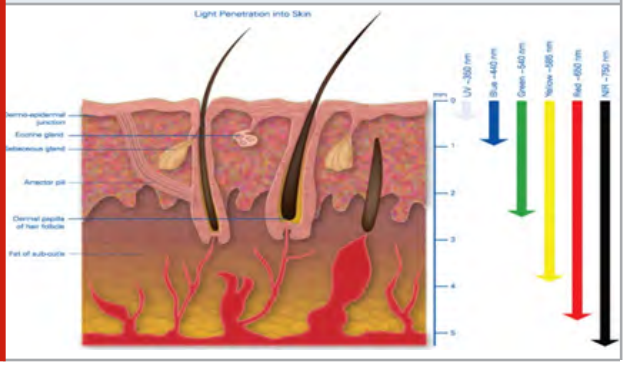
camera. The authors in (Sinhal et al. 2017) used DSLR cameras to capture three color channels in most of their studies. In our research, we focused on capturing videos with a 5-megapixel lens and a video resolution of 1280×720 pixels via mobile phone HTC. The camera sensors capture each pixel corresponding to one color detecting light in red, green and blue bands. Therefore, specific frequency information was extracted from the pixels. Verkruysee's previous work has shown that green channel works best for BPV signals in the RGB colorband (Verkruysse et al. 2008). The ICA algorithm was developed by Poh et al., to extract the BPV signal from one independent source (Zhang et al. 2014; Zhang et al. 2021).

For extracting signals from the video, selection of region of interest (ROI) is also important. Many researchers adapted different methods and chosen different region of interest. Wang et al. (2017) proposed a technique which automatically chose the region of interest by detecting live skin. Mannapperuma et al. (2014) chose forehead for extracting pulse rate, as forehead is less influenced from the motion but has highest signal to noise ratio. Bobbia et al. (2016) developed a model that represented living skin tissue and has region of interest favoured to the region where pulse are more prominent. Po et al. (2018) developed and adaptive ROI technique which improved signal to noise ratio and the quality of extracted rPPG signal. They also developed a new frame adaptive ROI system to divert color saturation or cut-off distortion in the process of capturing fingertip video to improve efficiency due to variance and limited dynamic range of camera sensors indifferent smart phone models (Po et al. 2015; Zhang et al. 2021).

Figure 1: Light penetration into skin illustrating the depth to which wavelengths penetrate human skin



The contributions of this paper are: 1) to show that mobile phone cameras can also work for extracting rPPG signals from videos 2) to show the difference in signals extracted from the traditional DSLR camera and mobile phone camera sensors 3) to present the result of signals extracted from channels compared with the gold standard recorded through pulse-oximeter. From the literature it is evident that progressive work has been done in field of estimating heart rate from the photoplethysmography signals obtained remotely. The researchers have used the good quality sensors for the video acquisition of subjects and therefore the algorithms were able to perform better. In our work we have created database of videos for pregnant women using HTC one mobile phone camera sensor. Also, in Verkruysse et al. (2008) have stated that green channel performs better as compared to blue and red channel which contradicts our finding in the result. Clement et al. (2005) demonstrated the penetration of the different light spectrum in human body for using iPulse technology as treatment. In the finding it has been observed the blue light is extinguished at 4.4mm below the skin surface whereas green extinguishes below 5.4mm. The reference figure for the light penetration into human skin is show in figure 1 below (Zhang et al. 2021).

MATERIAL AND METHODS

In this section, we present the technique to extract pulse rate from remotely recorded videos for three different colours viz; red, green and blue. The videos are recorded in uncontrolled environment with movement. Additionally, there was no filtration or correction done for improvement in videos. Initially, vector frames for recorded video are created. The first frame of the video is displayed to identify the region of interest (ROI) for finding the pulse rate. Here, ROI has been identified manually. For the proposed technique, forehead from the facial portion has been chosen as the ROI. Whenever heart pumps blood, it flushes the blood in entire body and even in the face portion. The changes brought in the body or face due to blood pumping cannot be visualized through naked eyes.

Figure 2: First frame for subject id #5025

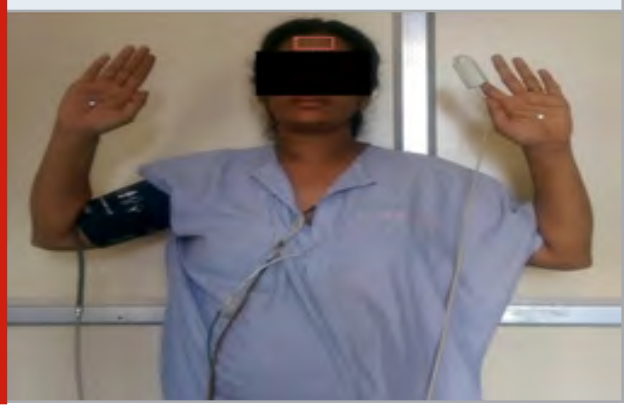
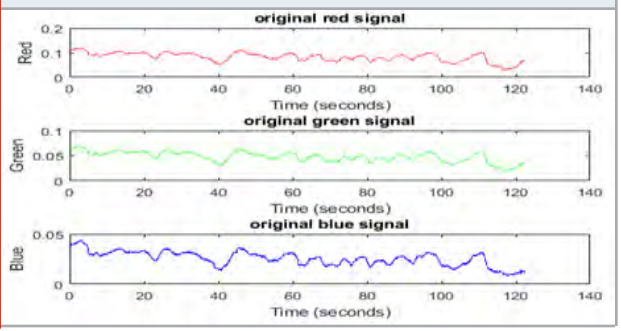


Figure 3: Intensity matrix Original RGB color channels for subject id#5025



However, these changes can be very well monitored by changes in color channels. Moreover, the forehead region of the face is more in area that is exposed to the

light. Therefore, forehead has been selected as the ROI to extract signals with more 'signal to noise' ratio. The other regions such as eyes, cheeks, neck, and hand region were not considered for extraction of pulse signals from the video. For example, a sample frame of video for subject with 'id#5025' is shown in figure 2 depicting ROI with a rectangular box. This selected ROI is kept constant for the frame vector to extract the intensity vector Ir, Ig and Ib different color channels from the video. The intensity vector is then plotted against time for subject id#5025. The plot of red, green and blue signal is shown in the figure 3 below. The average of all color channels (red, green and blue) pixel values within resulting ROI were calculated from each frame (f1, f2, f3 …fn) from raw signal extracted. The average pixel matrix gave the intensity of each color channels extracted from the pixel (Ir, Ig and Ib). The intensity matrix gave the raw traces of the signals extracted from the ROI as shown in figure 3.

In figure 4b, we have plotted the obtained pulse count for id number after filtering them using Butterworth band pass filter and elliptic IIR filters. These values where compared with the real time MP20 recorded values for all 20 patients. We found that butterworth filter outperformed as compared to elliptic filter. Therefore, the original signal is distended filtered with Butterworth bandpass filter to filter out non-physiological frequencies within range of 40 bpm to 180 bpm. Also, it was evident from the signal plotted in figure 4a after applying the elliptic IIR filter the signal was not getting filtered.

The filtered signal as shown in figure 5 is stored in the vector and plotted as a graph. The following figure 5 shows the filtered signal for all three channels for subject id#5025 FSr, FSg, and FSb for red, green and blue channel respectively. The filtered signals were given as input to peak counting algorithm. The peak counting algorithm finds the number of peaks in the filtered signal and count was represented as pulse count for the signal as Pr, Pb, and Pg. This count was compared with the pulse count we had collected for patient during recording of video using ixTrend software.

RESULTS AND DISCUSSION

Experimental Setup: We have recorded the videos for remote-PPG in visible light conditions, using the following video recording setup including HTC one mobile camera as shown in figure 6(a) and figure 6(b). Different components of experimental setup will be explained in the following subsection. Recording Environment: The recording environment consists of a computer system with windows operating system of 32 bit. The system was having the software ixtrend installed to record the heart rate, respiratory rate and SpO2 of the subjects. Mp20 monitor is attached to the system for storing the data for patients as shown in figure 6 (a). Camera: HTC one m9 mobile phone was used to capture the videos of 720 healthy pregnant women in AVBRH hospital having period of gestation from 45 to 300 days.

The database has been created at Datta Meghe Institute of Medical Sciences research laboratory in association with HSPH, Boston, funded by USAID which is described in section 3.2. The subjects were in standing position and the video was recorded with mobile phone mounted on tripod. There was no special illumination on the subject face or the area being captured except the daylight in the room from the window. The video is recorded with 1080p 13Mp camera sensor having 30fps frame rate. Figure 6(b) is showing the mobile phone and subject on screen (Finžgar et al. 2021; Zhang et al. 2021).

Figure 4. a): Original red signal obtained from video and filtered using bandpass filter and elliptic filter design for subject id#5025, b) Plot of HR obtained after applying Bandpass filter and Elliptic filter (Between 40 and 180 bpm) in relation with the recorded MP20 value

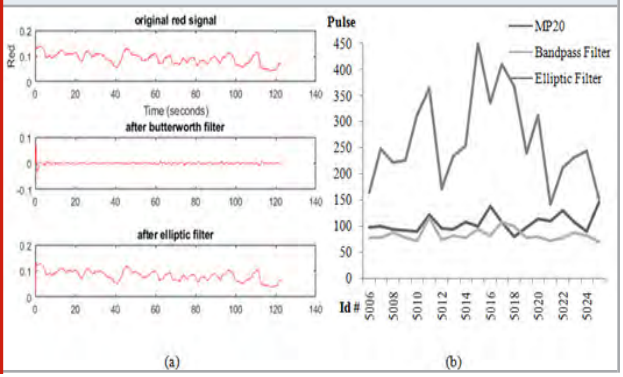


Figure 5: Extracted pulse and filtered pulse for subject id#5025

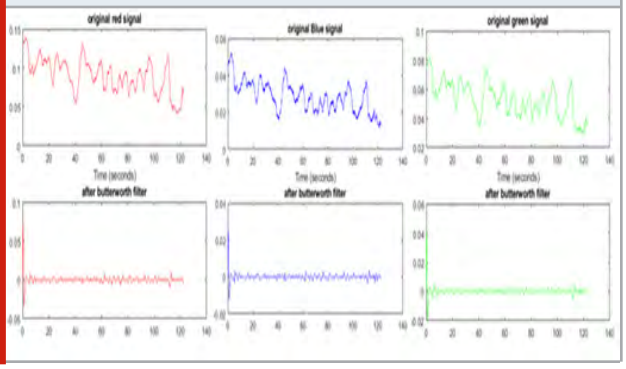


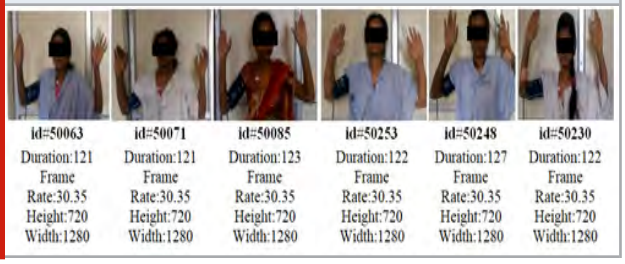
Figure 6. a): Recording environment b) HTC Desire phone and tripod used for recording videos

Database Description: The database consists of 720 videos of different individuals or subjects. Each individual or subject is a pregnant woman of Indian origin with varying skin tone categorized from type-I to type-VI according to

Fitzpatrick scale (Sachdeva 2009) between 19 to 38 years of age (see Figure 7). The video of each subject was recorded using HTC one M9 mobile phone shown in figure 6(b). The time length of each video is approximately greater than 120 seconds. The reason for keeping the video short is to avoid the physiological changes that occur in longer term. Also, we have recorded the physiological signs thorough iXtrend software which recorded the signs up to one minute. The parameters like heart rate, respiratory rate and SPO2 of each subject were also recorded on the computer digitally with the help of MP20 monitor connected to the system using iXtrend software as shown in figure 6(a). A sample frame of video for subject with 'id#5025' is shown in figure 2 depicting ROI with a rectangular box (Finžgar et al. 2021; Zhang et al. 2021).

Figure 7: Samples images from DMIMS database with variation in skin tone

Figure 8: Thumbnails from DMIMS database with its video properties



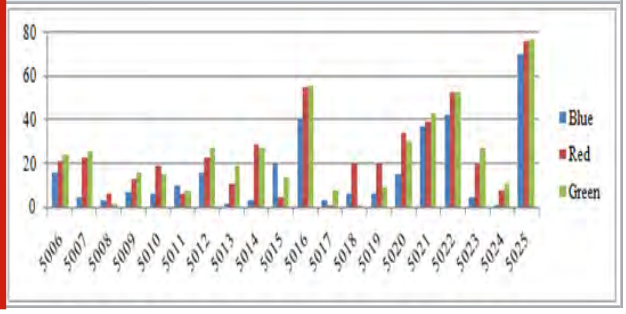
The proposed technique was experimented using MATLAB R2017b with 32GB RAM, 3.60 GHz Intel Core i7 64-bit processor an 1TB hard disk. As discussed about DMIMS database in section 4.2, a subset of videos of 20 different subjects were randomly selected from database for experimentation. One sample frame from each video of 20 different individuals is shown in figure 8. Each video of one subject is represented as (id #number) as shown in figure 8. First four digits '5001' represent the series. The last digit is verhoff coding to distinguish each subject from another subject. The different properties namely; duration, frame rate, height and width of each video are also shown along with each frame for better understanding of the reader. For instance, id#50063 represents the frame for patient id 50063 having duration of video 121 seconds with frame rate of 30.35fps and resolution of 720 × 1280 pixels (Finžgar et al. 2021; Zhang et al. 2021).

This section, presents the experimentation results of proposed technique for detail performance analysis with DMIMS database mentioned earlier. For estimating the pulse rate from the recorded video, the performance of the proposed technique is found for three colour bands. For each color band, intensity signal is extracted which is further filtered using Butterworth bandpass filter as shown in figure 5. The number of peaks in the filtered signal is counted separately for each channel. The number of peaks gives the pulse count for channel which is shown in table 1.

The plotted signal for the intensity values derived for each video frame is the first signal. Using the Butterworth band pass filter, the second signal is extracted and filtered. The derived and filtered pulse plotted for id # 5014 is figure 4. Table 1 demonstrates the accuracy of the pulse derived from the video. The algorithm was tested with three red, green and blue colour channels. More consistency is found for the blue channel, i.e. 89% without any corrections in the video. The accuracy of the red channel is 79.22%, and the accuracy of the green channel is 76.82%. The blue channel, followed by the red channel and then the green channel, offers the highest accuracy respectively.

Figure 9 displays the plot of the discrepancy between the pulse rate obtained from the three video colour channels and the original pulse rate used as the gold standard. The minimum difference is observed in the signal of the blue colour channel filtered by the Butterworth filter followed by a red colour channel and a green colour channel. The blue colour channel was therefore good for extracting the pulse rate from the video database that was captured in the research by adding only the Butterworth filter to the ROI signals (Finžgar et al. 2021; Zhang et al. 2021).

Figure 9: Difference between the gold standard and color channel pulse rate extracted



A paired t-test was run on a sample size of 20 middle aged (25.3 + 4.49) pregnant women to determine whether there was statistically significant mean difference between the pulse count of the subjects acquired from the MP20 monitor (106.35 + 16.99) and the color intensity peak count algorithm applied on video for red (84.25 + 12.14), green (81.7 + 10.99) and blue (94.75 + 14.03) channel. A statically significant increase of 22.1 (95% CI, 11.98 to 32.21) bpm, $t(19)=4.57, p<0.0005$ has been found for red

channel. Similarly, statically significant increase of 24.65 (95% CI, 15.49 to 33.81) bpm, t(19)=5.63,p<0.0000 has been found for green channel. And statically increase of 11.6 (95% CI, 1.7 to 21.5) bpm, t(19)=2.45,p<0.024 is found for blue channel (Zang et al. 2021). The intensity matrix gave the raw traces of the signals extracted from the ROI as shown in figure 3.

Table 1. Accuracy of proposed methodology with three different channels using Butterworth filter

ID	IxTrend Pulses	Pulse Count		
		Red	Green	Blue
50063	99	78	75	83
50071	101	78	75	96
50085	94	88	92	97
50092	92	79	76	85
50102	91	72	76	85
50118	122	116	114	132
50125	97	74	70	81
50139	94	83	75	96
50141	108	79	81	105
50156	100	95	86	120
50160	138	83	82	97
50173	109	108	101	106
50187	81	101	80	87
50194	99	79	90	93
50207	114	80	84	99
50217	111	72	68	74
50224	131	78	78	89
50230	108	88	81	103
50248	91	83	80	90
50253	147	71	70	77
Accuracy		79.22%	76.82%	89.09%

Table 2. Table of analysis between the rPPG signals and gold standard

rPPG Signal	Mean (+StdDev)	t-value	p-value	Std Error	Conf. Interval
Red	22.1(+21.61)	4.57	0.0002	4.83	11.98-32.21
Green	24.65(+ 19.58)	5.63	0.0000	4.378	15.49-33.81
Blue	11.6(+ 4.73)	2.45	0.024	4.73	1.7-21.5

CONCLUSION

The findings of the present study demonstrated the non-invasive color intensity method for detection of pulse rate from the pre-recorded video of 30 seconds. The algorithm is tested on the DMIMS dataset which we have captured in uncontrolled setting. The detected pulse rate is filtered with the three different IIR filters out of which Butterworth filter demonstrated best results. The results obtained where compared with the gold standards recorded form MP20 monitor and was compared for three color channels. The green channel is proven to be statistically significant for the video recorded followed by red and then blue channel. The accuracy of the pulse extracted is still low because of low signal to noise ratio.

Conflict of interest: The authors declare that they have no conflict of interest.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

Financial Interests: The authors declare they have no financial interests.

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An Accurate Embelin Extraction Method for Limited Biomass of *Embelia* Species

Shraddha S. Dandekar and Indu Anna George*

Department of Life Sciences, University of Mumbai, Vidyanagari,
Kalina, Santacruz East, Mumbai, Maharashtra, 400 098 India.

ABSTRACT

The natural benzoquinone, embelin, from the *Embelia* species has therapeutic benefits in a wide range of diseases. Although several extraction methods and solvents have been explored, consensus on the economic use of material and time was ambiguous. The purpose of this study was to devise a protocol for the rapid estimation of embelin. Chloroform, ethyl acetate and acetone extracts were prepared using soxhlet, microwave, sonication and cold extraction methods. The bioactivity of the chloroform extracts was assayed using the DPPH radical scavenging and the Reducing Power Assays. The embelin content in chloroform and ethyl acetate extracts were better in some extraction methods. The chloroform extracts exhibited antioxidant activity which remained unaffected regardless of the extraction technique. The microwave extraction technique yielded quick and accurate results. This technique could be adopted for rapid screening of samples with limited availability of biomass.

KEY WORDS: ANTIOXIDANT ACTIVITY, *EMBELIA* SP., EMBELIN, EXTRACTION METHODS, MICROWAVE EXTRACTION.

INTRODUCTION

Embelia basaal is a large shrub and *Embelia ribes* is a rare woody climber or shrub of the family Primulaceae. *E. ribes* is located up to an altitude of 1750 meters in the western and eastern ghats of India, Sri Lanka, Singapore, China and Malaysia (Annapurna et al. 2013; Patwardhan et al. 2014). The brittle pericarp of the dried berries of *E. ribes* encloses a reddish, single seed with spots of embelin on its surface and is covered by a thin membrane (Sudhakaran 2016). The dried berries of *E. basaal* appear reddish-black with vertical striations on its surface and can be easily differentiated from the grey to black, wrinkled berries of *E. ribes* (Fig. 1) (Nayak et al. 2009). Traditionally *Embelia* has been used extensively for impaired digestion, colon diseases, ulcers, skin diseases, as an anthelmintic, for the treatment of abdominal pain, mental disorders, jaundice, heart diseases and bronchitis (Bhishagratna 1911; Atal et al. 1984; Choudhary et al. 2021).

Embelin has clinically proven its value as an antimicrobial, antioxidant, anti-tumour, analgesic, anti-inflammatory, anti-androgenic, anti-hyperglycaemic, anthelmintic agent and in healing wounds (Radhakrishnan and Gnanamani 2014).

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Different parts of the plant were used in the form of paste, decoction, oil and powder for treating various illnesses. *Embelia ribes* (Vidanga) has been used in the preparation of about 75 ayurvedic formulations (Bhishagratna 1911; Patwardhan et al. 2014). Pharmacological applications aside, it has also been used as a dyeing agent for cotton, nylon, wool and silk (Radhakrishnan et al. 2011a). A recent study of embelin indicated an antiviral property against Covid-19 (Caruso et al. 2020b). The medicinal value of *E. basaal* and *E. ribes* can be ascribed to its active component embelin a yellow-orange crystalline compound (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) that constitutes about 1 to 5% of the berries (Lu et al. 2016; Prabhu et al. 2018; Ferreria and Laddha 2013) (Pandey and Ojha 2011; Nagamani et al. 2013 Ferreria and Laddha 2013; Lu et al. 2016; Prabhu et al. 2018). Bioprospecting or evaluation of the best medium composition for desired results would demand rugged and accurate protocols with an economical investment of biomass and time. The plant biomass has been extracted using various solvents and extraction techniques and the determination of the biochemical activities of phytochemicals in the crude extracts have been regularly explored for better alternatives to the existing options (Altemimi et al. 2017; Tlili et al. 2019; Kamble et al. 2020; Vijayan and Raghu 2021).

Extraction of embelin has been reported using several extraction methods and solvents. However, these studies did not actively explore their efficacy in embelin extraction

from small samples. The present study aims to optimize the embelin extraction from small samples of the berries of two species of *Embelia* using different solvents and extraction methods while retaining their bioactivity.

MATERIAL AND METHODS

The analytical grade solvents were procured from SD Fine-Chem (India) and Merck. The other reagents used for analysis were sourced from Sigma-Aldrich, HiMedia (India), SRL (India) and SD Fine-Chem (India). *Embelia basaal* berries were collected from Supegaon village, Raigad District, Maharashtra. A specimen was deposited in Blatter Herbarium, St. Xaviers College, Mumbai and was identified as *Embelia basaal* (Roem & Schult) A. DC. The berries of *Embelia ribes* were collected from Shimoga District, Karnataka. A specimen was submitted to 'Botanical Survey of India', Pune and was identified as *Embelia ribes* Burm f. The berries of both species were air-dried and stored at room temperature for further analysis. The berries of *E. basaal* and *E. ribes* were crushed, using a mortar and pestle, enough to separate the pericarp from the seeds and expose sufficient seed surface for extraction of the entire embelin content (Ferreria and Laddha 2013). The pericarp and the seeds were subjected to extraction using the solvents chloroform, ethyl acetate, acetone and four extraction methods. The sample to solvent ratio was maintained at 2% (w/v) in each method. The extractions were carried out in triplicate and the embelin content was expressed as mean ± standard error of the mean (SEM).

For Soxhlet Extraction (SE), Coarse ground berries (1 g) of *Embelia* species were refluxed in 50 ml of extraction solvent in a soxhlet apparatus (J-SIL company, India) for 1 h by which time the extract in the siphon arm of the apparatus was colourless. Microwave extraction (ME): The sample (0.2 g) was extracted with 5 ml of solvent for 3 min in a microwave (LG, India) set at 180 W. The sample was heated for 50 s, three times, in a cycle. A resting period of 10 s was given between each spurt of 50 s heating. The solvent was then filtered through Whatman filter paper no. 1 and collected in a beaker. Fresh solvent (5 ml) was added to the sample residue and a second extraction was carried out for 50 s twice, with a resting period of 10 s as described above, followed by filtration. The filtrates obtained were pooled together. Ultrasonication extraction (UE): The sample (0.2 g) in 5 ml of solvent was subjected to sonication using a water bath sonicator (Pure Enterprises, India) for 10 min, followed by filtration. Fresh solvent (5 ml) was added to the sample which was sonicated for another 5 min and filtered. The filtrates obtained were pooled together. For cold extraction (CE), the sample (0.2 g) with 10 ml of solvent was placed on an orbital shaker (Remi RS 12 PLUS) at 200 rpm for 17 h. The samples were then filtered. The extracts obtained by the four extraction methods using various solvents were oven-dried at 40°C and the residues were weighed. The dried extracts were dissolved in chloroform for quantification by UV-VIS spectrophotometer (Shimadzu Scientific Instruments Inc.).

Standard embelin was procured from Sigma-Aldrich and a stock solution (1 mg/ml) was prepared in chloroform. Dilutions were prepared such that the final concentrations ranged between 4 to 24 µg/ml. The UV absorbance at 290 nm of each solution was recorded using a UV-VIS spectrophotometer. A standard graph of absorbance vs concentration of embelin was plotted and the resultant equation was used for calculating the concentration of embelin in the samples. The embelin from samples was also quantified by High Performance Liquid Chromatography (HPLC) for the bioactivity experiments.

Four concentrations of standard embelin in methanol (10, 20, 30 and 40 µg/ml) were run through an HPLC column (Shim-pack GIST C18, 5µm, 20 x 250 of the Shimadzu HPLC system). The mobile phase, 0.1% formic acid in acetonitrile: deionized water (90:10; v/v), was degassed for 30 min. The flow rate was maintained at 0.5 ml/min in isocratic elution mode and the chromatogram was run for 20 min. The volume of sample injected each time was 20 µl. The area under the peak was considered to generate a standard graph for the estimation of embelin in samples. The dried chloroform extracts of the *Embelia* berries, obtained by the four extraction methods were reconstituted in methanol (1 mg/ml) by sonication for 5 min and filtered through a 0.22 µm nylon filter. These extracts would be further referred to as reconstituted extracts. The embelin content in these samples was determined by the HPLC analysis described above and the antioxidant activity was confirmed by the following DPPH and RPA assays.

The DPPH assay method described by Brand-Williams et al. (1995) was adopted with some modifications. The assays included ascorbic acid as a reference standard, a methanol control and the test samples. The DPPH reagent (100 µM) was prepared in chilled methanol and the initial U.V. absorbance of the reagent was approximately 1 (1.0542). Two-fold dilutions of the test samples dissolved in methanol were prepared with final concentrations that ranged from 800 µg/ml to 25 µg/ml. Similarly, standard ascorbic acid solutions were prepared in the range between 40 µg/ml to 1.25 µg/ml. Aliquots (500 µl of standard/ sample) were added to 2.5 ml of DPPH solution and incubated in dark for 30 min before noting the U.V. absorbance at 517 nm. The percentage of radical scavenging activity was calculated by the formula:

$$\text{Radical Scavenging activity (\%)} = \frac{Ao - At}{Ao} \times 100$$

Where Ao = absorbance of the control and At = absorbance of the test sample.

EC_{50} values (50% effective concentration value - the value obtained by plotting percentage radical scavenging activity vs concentration of extract) were calculated for each sample and standard using GraphPad Prism 6.0 software. The reducing power assay (RPA) method described by Muruhan et al. (2013) was adopted with some modifications. A range of two-fold dilutions of standard solution (80 µg/ml to 1.25 µg/ml) and sample (800 µg/ml to 25 µg/ml) was prepared.

Each concentration of the standard or sample was reacted individually with 1.25 ml of sodium phosphate buffer (pH 6.6) and 1.25 ml potassium ferricyanide. This mixture was incubated at 50°C for 20 min. The reaction was stopped with the addition of 10 % trichloroacetic acid (TCA). The solutions were then centrifuged at 3000 rpm for 10 min. The resultant supernatants (2.5 ml) were mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride reagent. The U.V. absorbance of these reaction mixtures was recorded at 700 nm and its reducing potential was expressed as ascorbic acid equivalents. All data obtained were subjected to appropriate statistical treatment and analysis. Two-way analysis of variance (ANOVA) followed by Tukey's test was performed using GraphPad Prism 6.0 software wherever required.

Figure 1: The dried berries of *E. basaal* (A) and *E. ribes* (B); The brittle testa removed to expose the seeds with spots of embelin of *E. basaal* (C) and *E. ribes* (D).

RESULTS AND DISCUSSION

Yield of Extract: The residue obtained after the evaporation of solvent from the extract (yield) varied across the different extraction methods and the solvent used. This ranged from 5% to 7.8% (w/w) for *E. basaal* and 3.5% to 6.2% (w/w) for *E. ribes*. Two-way ANOVA followed by Tukey's test revealed that the yield of the ME - chloroform extract was significantly higher than that of the other methods (*E. basaal*) whereas, for *E. ribes*, the CE - chloroform extract showed maximum yield. The yields obtained by different extraction methods tested in this study were variable but inconsistent with their embelin content (Table 1). Similarly, the Supercritical carbon dioxide extraction resulted in a lower yield than the SE but had a higher flavonoid content (Bimakr et al. 2011).

Legend: The letters and symbols across the cells for each species indicates the statistical significance at $p \leq 0.05$. CH: chloroform; EA: ethyl acetate; AC: acetone; The equation and the regression coefficient (squared) of embelin standard graph is as follows: y = 0.063x - 0.059 and r2 = 0.9963.

Table 1. The yield, embelin content and time required for extraction from berries of *E. basaal* and *E. ribes*

	E. basaal				*E. ribes*		
	Yield (mg/g)	**Embelin content (mg/g)**	**Time (min)**		**Yield (mg/g)**	**Embelin content (mg/g)**	**Time (min)**
	SE					SE	
CH	$71.37 \pm 3.76^{\phi}$	54.69 ± 0.96^{A}	60	CH	$39.50 \pm 1.50^{\alpha\beta}$	18.17 ± 0.25^{ab}	60
EA	$59.90 \pm 2.47^{\phi}$	57.96 ± 1.52^{A}	60	EA	$41.13 \pm 1.86^{\alpha\beta}$	19.34 ± 0.69^{abcd}	60
AC	$64.37 \pm 0.67^{\phi}$	44.94 ± 1.84^{ABC}	60	AC	$40.13 \pm 0.68^{\alpha\beta}$	17.05 ± 1.03^{a}	60
	ME					ME	
CH	$78.83 \pm 3.90^{\Psi}$	54.22 ± 2.65^{A}	5	CH	$53 \pm 0.0.29^{\gamma}$	21.28 ± 0.57^{bcd}	5
EA	$55.33 \pm 5.18^{\phi}$	53.06 ± 5.30^{AB}	5	EA	$40 \pm 1.32^{\alpha\beta}$	21.94 ± 0.14^{cd}	5
AC	$65.83 \pm 5.73^{\phi}$	40.30 ± 2.53^{ABC}	5	AC	$43.33 \pm 1.67^{\beta}$	18.78 ± 0.18^{abc}	5
	UE					UE	
CH	$63.33 \pm 3.03^{\phi}$	53.30 ± 2.08^{AB}	15	CH	$55 \pm 0.76^{\gamma}$	21.18 ± 0.76^{bcd}	15
EA	$50.67 \pm 2.89^{\phi}$	49.45 ± 2.46^{AB}	15	EA	$37.50 \pm 2.25^{\alpha\beta}$	21.25 ± 0.57^{bcd}	15
AC	$54.50 \pm 7.01^{\phi}$	35.32 ± 7.31^{BC}	15	AC	$35.50 \pm 1.00^{\alpha}$	18.63 ± 0.48^{ab}	15
	CE					CE	
CH	$75.67 \pm 7.86^{\phi}$	47.20 ± 1.34^{AB}	1020	CH	$62.50 \pm 0.50^{\delta}$	22.06 ± 0.60^{d}	1020
EA	$63.50 \pm 5.20^{\phi}$	49.41 ± 6.02^{AB}	1020	EA	$42.67 \pm 0.88^{\beta}$	22.51 ± 0.89^{d}	1020
AC	$63 \pm 7.25^{\phi}$	28.88 ± 1.67^{C}	1020	AC	$44 \pm 1.32^{\beta}$	19.81 ± 0.64^{abcd}	1020

Estimation of embelin content by spectrophotometry: Variations in the embelin content in berries could be attributed to its geographical location (1.2 to 4.9%) and/or stage of maturation (1 to 5.2%) (Pandey and Ojha 2011; Nagamani et al. 2013). The embelin content of extracts would also depend on the extraction method and the solvent which was significant in this study of both *Embelia* species. The embelin content in *Embelia ribes* extracts, reported using different extraction methods, ranged from 1.9% to 3.8% (SE), 5% (ME), 0.84% to 23.71 % (UE) and 1.77% (CE) (Madhavan et al. 2011; Radhakrishnan et al. 2011b; Alam et al. 2015; Sathe and Dixit 2015; Kamble et al. 2020). The embelin content of the various extracts in the current study of *E. ribes* was determined as 1.7% to 1.93% (SE), 1.87% to 2.19% (ME), 1.86% to 2.12% (UE) and 1.98% to 2.25% (CE) with different solvents. These values for *E. basaal* ranged from 4.49% to 5.79% (SE), 4.03% to 5.42% (ME), 3.53% to 5.33% (UE) and 2.88% to 4.94% (CE). The highest extracted embelin content in this study was 5.79% from *E. basaal* berries. *E. ribes* was reported to be the best source of embelin when compared to the species tested in a previous study (Vijayan and Raghu 2021).

Figure 2: The HPLC chromatogram of standard embelin (A), λ_{max} of the standard peak from the chromatogram (B), HPLC chromatogram of Sample (*E. basaal* - UE) (C), and λ_{max} of the sample peak obtained in the chromatogram (D).

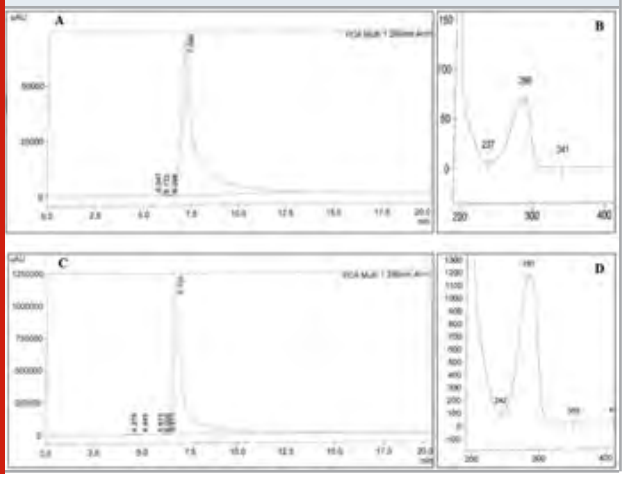


Table 2. Embelin content of reconstituted extracts by HPLC

	Embelin content by HPLC (µg/ml)			
	SE	ME	UE	CE
E. basaal	807.12^{a}	621.58^{ab}	617.07^{ab}	601.55^{ab}
E. ribes	508.77^{ab}	450.58^{ab}	374.3^{ab}	268.96^{b}

A selection of conventional (SE and CE) and modern (ME and UE) methods of extraction were compared in this study. The SE and ME embelin contents were better in *E. basaal* extracts whereas the SE of *E. ribes* was comparatively inefficient than the other extraction methods tested. Solvents used for extraction of secondary metabolites may include a pure organic solvent, a mixture of solvents, water or a percentage of a solvent in water (Gupta et al. 2012).

Figure 3: Percentage of DPPH radical scavenging activity of *E. basaal* (A) and *E. ribes* (B) extracts at different concentrations (µg/ml) obtained using selected extraction techniques. SE: Soxhlet extraction; ME: Microwave extraction; UE: Ultrasonication extraction; CE: Cold extraction.

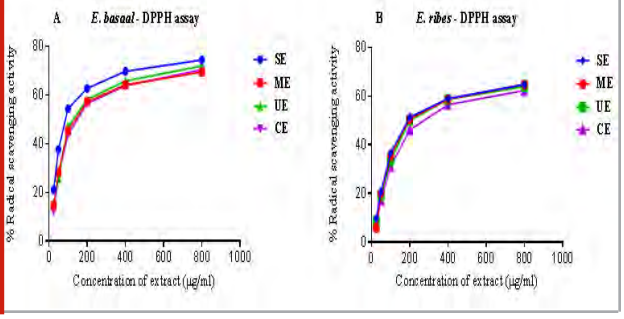
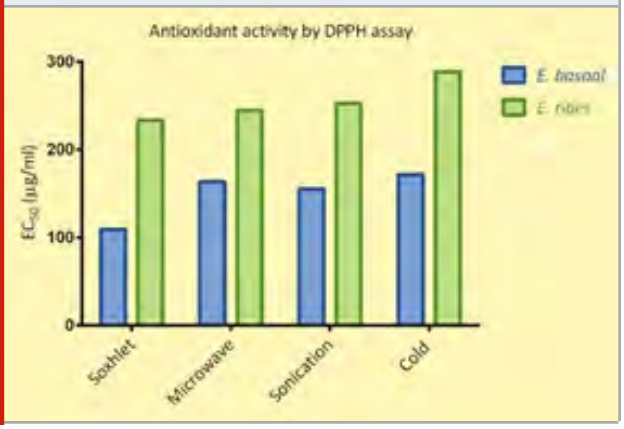


Figure 4: The EC_{50} values of the reconstituted extracts of *E. basaal* and *E. ribes*, using various extraction methods.



Embelin has a polar benzoquinone ring and a nonpolar alkyl saturated chain. The isolated and purified embelin from *E. ribes* is insoluble in water, freely soluble in organic solvents such as DMSO, slightly soluble in methanol and ethanol (Kaur et al. 2015; Caruso et al. 2020a). Previous studies preferred hexane, diethyl ether, chloroform, ethyl acetate, acetone and methanol for extraction of embelin (Madhavan et al. 2011; Alam et al. 2015; Pundarikakshudu et al. 2016), (Vijayan and Raghu 2021). Even so, the results of these experiments failed to reach a consensus on the best solvent for embelin extraction.

Chloroform extraction by maceration, ethyl acetate extraction by sonication and acetone extraction by microwave-assisted extraction method of *E. ribes* yielded

maximum embelin (Latha 2007; Alam et al. 2015; Pundarikakshudu et al. 2016). The most promising solvents were investigated for the development of the rapid analysis protocol. The statistical analysis of this study however indicated that the solvents chloroform and ethyl acetate were found to be marginally more efficient in the extraction of embelin (Table 1). Chloroform was also found to be suitable for the extraction of benzoquinone in *Ficus foveolata* (Meerungrueang and Panichayupakaranant 2015). Methanol was discontinued after the initial extraction process as the residue obtained was sticky, which was also observed by (Pundarikakshudu et al. 2016). Another factor that should be considered is the efficacy of the bioactive principle(s) following an extraction method. The chloroform extracts using all four extraction methods from both species were further analysed for their bioactivity.

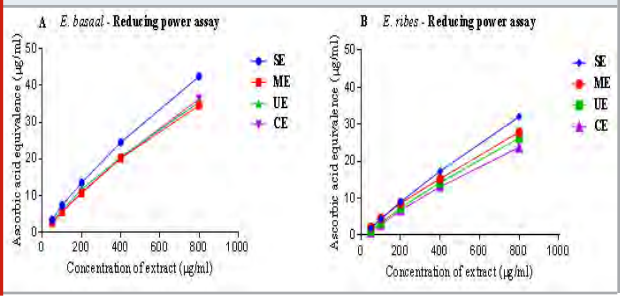
Estimation of embelin in reconstituted extracts using HPLC: The current solvent system was inspired by the existing literature and modified for better resolution (Ferreria and Laddha 2013). The standard embelin chromatogram exhibited one sharp peak at a retention time that ranged from 7.082 to 7.16. The spectrophotometric analysis of this peak showed a λ_{max} at 286 nm. Hence the chromatograms of the samples were monitored at 286 nm and all samples showed one peak with retention times between 6.66 and 7.17 with the λ_{max} that ranged between 277 to 283 nm (Fig. 2). The embelin content of the reconstituted *E. basaal* and *E. ribes* extracts ranged between 602 to 807 µg/ml and 269 to 509 µg/ml, respectively. A significant difference was noticed between the embelin content of *E. basaal* and *E. ribes* extracts. However, all extraction methods were comparatively efficient (Table 2). Contrary to the current observation, a study described that the embelin content varied with different extraction methods (Kamble et al. 2020). The differences in embelin content among species of *Embelia* have been reported in previous studies (Vijayan and Raghu 2021).

Legend: SE: Soxhlet extraction; ME: Microwave extraction; UE: Ultrasonication extraction; CE: Cold extraction. The equation determined for the embelin estimation by the HPLC method was y = 69697x –38593 with the r2 value 0.9972.

Antioxidant Assays; Antioxidants are chemical substances that halt the chain reaction of free radical species generation and protect the cells from cellular damage. The antioxidant activity of plants is generally attributed to the flavonoid, phenolic and tannin content in the extract (Mosquera et al. 2009). Earlier reports of the antioxidant activity of extracts from *E. basaal* and *E. ribes* prompted testing for their antioxidant activity (Ansari et al. 2008; Kamble et al. 2011).

DPPH assay; Antioxidant activity of the chemically synthesized embelin, a benzoquinone, was studied by Joshi et al. (2007) and its activity determined by the DPPH assay was found to be 2.55×10^{-4} mol dm^{-3}. Purified embelin extracts displayed an IC_{50} of 27.92 µg/ml and an EC_{50} of 27 µg/ml (Mahendran et al. 2011; Mohapatra and Basak 2017). Ascorbic acid (standard) was reported to have an IC_{50} that ranged from 3.028 to 4.92 µg/ml (Kamble et al. 2011; Mahendran et al. 2011). The IC_{50} of crude embelin extracts from various *Embelia* species ranged from 9.87 to 50 µg/ml whereas the EC_{50} reported from *E. tsjeriam cottam* was 11 µg/ml (Kamble et al. 2011; Barbade and Datar 2015; Mohapatra and Basak 2017). The radical scavenging activity (the EC_{50}) of the ascorbic acid in the present study was 12.15 µg/ml and that of the samples were 109 to 171 µg/ml (*E. basaal*) and 233 to 289 µg/ml (*E. ribes*). Statistical evaluation of the calculated EC_{50} values indicated that the antioxidant activity of *E. basaal* extracts were significantly higher than those of *E. ribes* and this was more pronounced in the SE and CE extracts of the species. The variation between the extraction methods was insignificant (Fig. 3 and 4).

Figure 5: The Reducing Power Assay using the reconstituted extracts of *E. basaal* (A) and *E. ribes* (B), at different concentrations (µg/ml) obtained using different extraction techniques and expressed as the equivalence of antioxidant activity of ascorbic acid. SE: Soxhlet extraction; ME: Microwave extraction; UE: Ultrasonication extraction; CE: Cold extraction. The equation obtained for the reducing power assay using ascorbic acid as standard was Y = 0.0174 X + 0.0204 (r2= 0.9975) and was used to calculate the ascorbic acid equivalence antioxidant activity of samples.



Reducing power assay: RPA is another standard assay to confirm the antioxidant activity of extracts. High values of UV absorbance indicate high antioxidant capacity. The results of the RPA have been shown in Fig. 5. The average antioxidant activity of the reconstituted extracts (1 mg/ml) expressed as the equivalence of ascorbic acid ranged from 64.33 to 50.52 µg/ml and 41.85 to 27.84 µg/ml for *E. basaal* and *E. ribes* respectively. The statistical analysis of this data (normalized to 1 mg/ml) revealed that there is a significant difference between the reducing power of the *E. basaal* and *E. ribes* extracts. The reducing power of extracts obtained using different extraction methods was insignificant within each species. This is the first report as yet that compared the antioxidant activities of different *Embelia* species. Genotypes of the plants collected from different localities

and solvents have influenced variations in the antioxidant activities of *E. ribes* (Kamble et al. 2020).

CONCLUSION

The findings of the present study indicated that the preferred solvents for extraction of embelin would be chloroform or ethyl acetate. The embelin content of ME extracts was comparable to conventional methods. Antioxidant studies with the reconstituted chloroform extracts confirmed that the bioactivity was retained regardless of the extraction method. Thus, ME has the added advantage of the speed and economic use of biomass. This protocol would be valuable for the screening and selection of plants or callus with high embelin content. This protocol could be adopted for the screening of other important phytoconstituents as well.

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Conflict of Interests: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Role of Senna, *Cassia angustifolia* and Fennel, *Foeniculum vulgare* in Ameliorating Nephropathy in Diabetic Rats

Nadia Nour Osman,[1,2] Abrar Mohammad Al-Ahmadi,[1] Aishah Hussain Ghazwani,[1]* Hanna Mohsen Alhoraibi,[1] Khawla Hassan Alanbari[1] and Wadiah Saleh Backer[1]

[1]Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia,

[2]Food Irradiation Research Department, National Centre for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt.

ABSTRACT

Diabetes mellitus is one of the most challenging metabolic pandemics that affect essential biochemical pathways in the body. The cost of Diabetes mellitus treatment and its side effects may call for an investigation on plant products as sources of treatment. Whereas, traditional medicine has proven that treatment with plant extracts is affordable, effective, and may have fewer negative effects than modern medicines. The current study aimed to investigate the renoprotective effects of senna (*Cassia angustifolia*) and fennel (*Feoniculum vulgar*) against streptozotocin-induced diabetes in albino rats. Male albino rats were used as animal models which divided into five groups, normal group (control), diabetic group (single i.p. 60 mg/kg of streptozotocin, (STZ), and 3 diabetic groups that were treated with aqueous extract of (senna (150mg/kg/day), or fennel (150mg/kg/day) and their combination) by gastric intubation for 4 weeks. Diabetic rats exhibited a highly significant increase in the levels of blood glucose, renal enzymes and renal weight. Also, a significant increase in nitric oxide (NO), thiobarbituricacid reactive substances (TBARS) and xanthine oxidase (XO) accompanied by a significant decrease in vitamin C, catalase (CAT) and reduced glutathione (GSH) in renal tissues as compared to control group. In conclusion oral administration of senna and/or fennel extract reduces oxidative stress in renal tissue by lowering blood glucose levels, increasing plasma insulin, and restoring weight loss and levels of renal enzymes in diabetic rats. The present investigation suggested that the treatment with mix of (senna and fennel) exhibited antidiabetic activity, and had renoprotective effects in streptozotocin-induced diabetes rats.

KEY WORDS: ANTIOXIDANT, DIABETES, SENNA, FENNEL, RENOPROTECTIVE.

INTRODUCTION

Diabetes Mellitus (DM) is one of the world's fastest-growing health issues, which in some countries is now reaching epidemic proportions. One of the most severe and daunting health issues in the 21st century is the increasingly growing prevalence of DM worldwide. It is largely attributed to the lack of exercise, unhealthy diet, obesity and overweight as a result of life style. Over the last few decades, changing lifestyles in KSA have led significantly to the growing prevalence of DM and other chronic diseases. In many diabetes populations, the combination of rising diabetes prevalence and increasing lifespans will lead to an evolving range of types of morbidity associated with diabetes. To date, among the diseases that must be given more and more attention is diabetes, where the statistics and predictions show worrying data. Saeedi et al. (2019) reported that the prevalence of diabetes is estimated at 9.3 % (463 million individuals) at the global level, rising to 10.2 % (578 million) by 2030 and 10.9 % (700 million) by 2045. Besides, about 1.6 million deaths are directly attributed to diabetes each year (Wong et al., 2013; Alnuaim, 2014; Naeem, 2015; WHO, 2020).

One of the features of diabetes is hyperglycemia. Chronic hyperglycemia damages nearly all types of cells in the body. A relationship has been identified between hyperglycemia, oxidative stress and various pathways that can lead to organ and tissue damage. Moreover, complications associated with hyperglycemia include stroke, nerve damage, poor vision, heart attack and renal disease. According to the Center for Control Disease and Prevention (CDC) over a third (37%) of adults diagnosed with diabetes had chronic renal disease; and fewer than (25%) with moderate to severe chronic renal disease. The renal disease of diabetes is referred to as diabetic nephropathy. It is results when diabetes damages

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blood vessels and other cells in the renal tissues. Over time, severe damage to these blood vessels can lead to renal disfunction and kidney failure (Piero et al., 2015; Jangid et al., 2017; NIDDK, 2017; Giri et al., 2018; Clinic, 2019; WHO, 2020; CDC, 2020).

There are a variety of oral hypoglycemic agents are available on the market, but they can lead to a high risk of secondary failure in the long term. Therefore, safe treatment is a demand to prevent diabetes complications with minimal side effects. For several years, plant-based drugs or herbal medicines have been prescribed by conventional medical practitioners to treat different disease conditions as it is low cost and has fewer side effects. In the treatment and control of diabetes, numerous herbal medicines have proved their potential. Sweet potatoes, basil, fenugreek, turmeric, okra and bitter gourd are classified as herbal plants used in diabetes management. Moreover, senna (*Cassia angustifolia*) and fennel (*Foeniculum vulgare*) are reported to possess hypoglycemic, antioxidant and anti-inflammatory effects. Therefore, both plants have the capacity to alleviate diabetes and its related effects (Jamshidi-Kia et al., 2018; Sudhakar et al., 2020; Jani and Goswami, 2020; Rohman and Putra, 2020; Farid et al., 2020; Patle et al., 2020). A study into the ameliorative potential of these plants on diabetes patients' nephropathy could lead to design a safer and more effective drug. So, the current study was planned to evaluate the nephro-protective potential of both senna and fennel water extracts in streptozotocin-induced diabetes in albino rats.

MATERIAL AND METHODS

Chemicals and preparation of the herbal extracts: Streptozotocin (STZ) was obtained from Saint Louis, in Missouri, USA.The leaves of senna (*Cassia angustifolia*) and seeds fennel (*Foeniculum vulgare*) were purchased from the local traditional market in Jeddah, Saudi Arabia. The senna leaves and fennel seeds were ground with the help of a grinder and 100g of this powder was dissolved by bringing to a boil for 30 min with 400 ml of distilled water. The mixture was filtered and centrifuged at 3500 rpm for 20 min. The supernatant was stored at 4ºC prior to its drying, till usage.

Induction of Diabetes in Experimental Animals: Male albino rats (n=50) weighing about (150 to 200 g) were obtained from the Animal Experimental Unit of King Fahd Centre for Medical Research, King Abdul-Aziz University. The animals were kept in plastic cages and were fed with tap water and rat chow ad libitum in a stable environment (temperature 28 ± 2ºC, humidity 60 ± 5%) with 12-hour light and dark cycle. Animal procedures were carried out according to the instruction of the Ethics Committee, King Fahad Medical Research Centre which were in compliance with the international guidelines for proper use and care of laboratory animals.

A single intraperitoneal injection of 60 mg/kg BW of streptozotocin (STZ) was used to cause diabetes in rats(Akbarzadeh et al., 2007). STZ was freshly prepared by dissolving it in citrate buffer (0.5M, pH 4.5). Three days after STZ injection, fasting blood glucose (FBG) level was measured by using *OneTouch* Select Analyzer (Life Scan, Inc., UK) and rats having FGB level less than 200 mg/dL were discarded from the study.

Experimental Design: Fifty animals were randomly divided into five groups of ten rats each as details in Table (1).

Table 1. Test Animal Treatment Groups

Groups	Treatment
Group 1 (Control)	Citrate buffer (0.01M, pH 4.5)
Group 2 Diabetes (Dia)	STZ (60 mg/kg Body Weight (BW), Intraperitoneal (i.p)).
Group 3 (Dia + Senna)	STZ (60 mg/kg BW i.p) + Senna aqueous extract (150 mg/kg BW/ day) (Shanmugasundaram et al., 2011).
Group 4 (Dia + Fennel)	STZ (60 mg/kg BW i.p) + Fennel aqueous extract (150 mg/kg BW/ day) (Sadrefozalayi and Farokhi, 2014).
Group 5 (Dia +Mix (Senna+Fennel))	STZ (60 mg/kg BW i.p) + aqueous extract of mixture from Senna (150 mg/kgBW/ day) and Fennel (150 mg/kgBW/day).
Each aqueous extract was given by oral gavage for a period of 30 days.	

At the end of the experimental period (4 weeks), rats fasted overnight before scarification. Blood samples were taken from the retro-orbital plexus of each anesthetized rat, then centrifuged at 3000 rpm for 10 minutes to separate serum. Immediately after taking a blood sample, the animals were sacrificed; the kidneys of each animal were removed, then

homogenized with 0.1 M cold phosphate buffer (pH 7.4) and centrifuged at 10,000 g for 15 minutes. The supernatant was used for biochemical evaluation.

Biochemical analysis: Blood glucose levels were measured by enzymatic kits according to the protocol of Kunst (1984). Serum insulin levels were determined by solid phase enzyme-linked immune-sorbent assay using Immunospec Insulin Quantitative Test Kit (model E29-88). Determination of the creatinine and blood urea nitrogen (BUN)were measured as described by Burtis and Ashwood (2001). Uric acid was measured according to the methods of Kalekar (1947). The total protein(TP)was measured as described by Henry et al. (1957). Thiobarbituric acid reactive substances (TBARS) was used to measure lipid peroxidation and was determined according to the method of Ohkawa et al. (1979). Xanthine oxidase (XO) activity was determined using the method of Bergmeyer et al. (1974). Nitrite assay was measured as an end product for knowing nitric oxide (NO) concentration according to the method of Miranda et al. (2001). The activity of catalase(CAT) was determined using the method of Aebi (1984). The content of reduced glutathione (GSH) was measured by following the protocol of Beutler et al. (1963). Ascorbic acid (vitamin C) was determined by using commercial kits (CAT NO. AS2516).

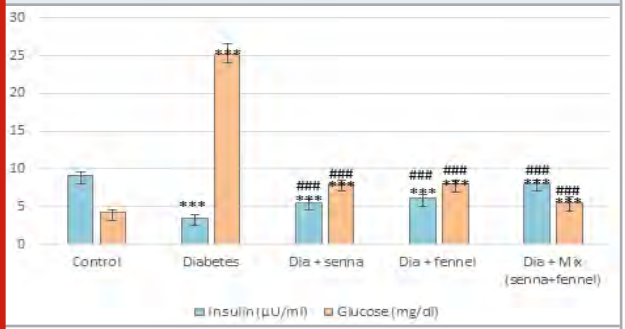
Statistical analysis: Analysis of data was done by Statistics Package for Social Sciences (SPSS) version 20. The data was expressed as arithmetic mean and standard deviation of the mean (SD). One-way analysis of variance (ANOVA), least significant difference (LSD) equation for parametric parameters, was used to analyze the differences between groups. A p-value less than or equal 0.05 was considered significant.

Table 2. Effect of aqueous extracts of senna or/ and Fennel on body and kidneys weight in diabetic rats.

	Initial weight (g)	Final weight(g)	Left kidney (g)	Right kidney (g)
Control	206.4± 6.96	275.2± 4.44	0.546± 0.093	0.556± 0.055
Diabetes	204.0± 6.18	196.1± 5.09***	0.957± 0.129***	0.996± 0.145***
Dia + Senna	200.1± 5.97	231.7± 4.64*** ###	0.764± 0.061***##	0.786± 0.077***##
Dia + Fennel	205.4± 7.66	232.9± 4.28*** ###	0.757± 0.062***###	0.772± 0.057***##
Dia + Mix (Senna+Fennel)	201.2± 9.41	257.4± 2.41*** ###	0.639± 0.060*###	0.695± 0.041***###

Each value represents the mean of 10 rats ± SD.
Significantly different from control value at P<0.05*, 0.001***
Significantly different from the untreated diabetic group at P<0.01## ,0.001###

Figure 1: Effect of aqueous extracts of Senna or/ and Fennel on glucose and insulin in diabetic rats.



Each value represents the mean of 10 rats ± SD, significantly different from control value at P<0.001***, Significantly different from the untreated diabetic group at P<0.001###

RESULTS AND DISCUSSION

The mean bodyweight and kidneys weight of all groups were shown in Table (2). The comparisons between groups indicated that at the beginning of the experiment (zero-day), the mean values of body weight for all groups were matched to the mean value in the normal group. The data of the present study showed a significant decrease (P < 0.00) in the bodyweight of diabetic rats during the experimental period when compared to the control group. While diabetic rats treated with either senna leaves extract (150mg/kg BW), fennel seed extract (150 mg/kg BW), or their mixture gained significant weight (P < 0.00) compared to diabetic untreated rats, the effect of the mixture of both senna and fennel was more efficient than each of them only.

The percentage change in the body in control, diabetic, senna and/or fennel weights were 33%, -4%, 14%, 12%and 22%, respectively. In kidneys weight, the statistical analysis indicated significant variations in the mean values of the right kidney and left kidney between groups. Treatment of diabetic rats with either senna and /or fennel exhibits a significant improvement of the organ weight, although this improvement did not reach the mean values in the control group. The levels of blood glucose and insulin of normal and experimental rats are shown in Figure (1). There was a significant elevation (p=0.000) in blood glucose accompanied by a significant decreased (p=0.000) in plasma insulin in diabetic rats compared with normal rats.

Administration of senna or fennel tends to bring the parameters significantly towards the normal. The effect of the mixture of both senna and fennel was more efficient than each of them only. The Post Hoc analysis using the LSD test exhibits no significant change (p=0.912 and 0.10) between the diabetic group treated with senna and the diabetic group treated with fennel in levels of blood glucose and plasma insulin, respectively.

The results illustrated in Figures (2-5) revealed that STZ resulted in a significant (p<0.00) raise in the level of creatinine, uric acid and BUN accompanied by significant (p< 0.00) decline in the level of total protein in diabetic rats in comparison with normal control. The prolonged administration of diabetic rats with aqueous extract of senna and/or fennel for 30 consecutive days showed a significant (p< 0.00) decreased in the creatinine, uric acid, and BUN and significant (p<0.00) increased in TP when compared with untreated diabetic rats. The statistical analysis exhibits no significant change (p=.218 and 0.105) between the diabetic group treated with senna and the diabetic group treated with fennel in levels of BUN, TP respectively. The changes in the levels of nitric oxide and TBARS, and the activity of xanthine oxidase in renal tissues in control and experimental rats are presented in Figures (6-8).

Figure 2: The levels of Creatinine in diabetic rats treated with aqueous extracts of Senna or/ and Fennel.

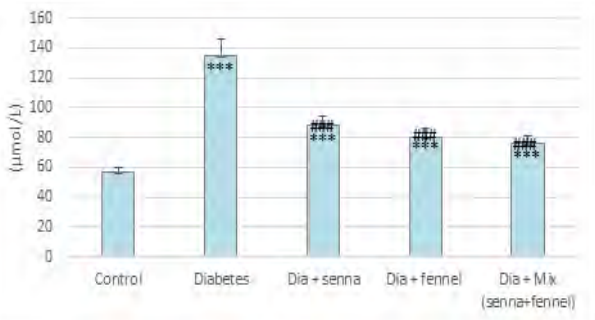
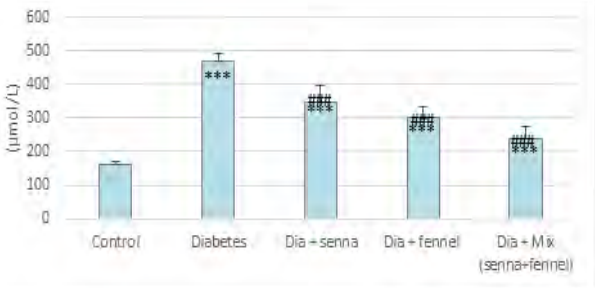


Figure 3:The level of UricAcid in diabetic rats treated with aqueous extracts of Senna or/ and Fennel.



There was a significant elevation (p=.000) in TBARS, nitric oxide and xanthine oxidase in renal tissues in diabetic rats when compared to the control group. The aqueous extracts of senna and fennel offered significant protection against alteration in the oxidative biomarkers of diabetic rats. However, the administration of aqueous extracts of the mixture of senna and fennel was more effective than senna or fennel only. No significant changes in renal TBRAS, nitric oxide and xanthine oxidase between the diabetic groups treated with either senna or fennel.

Figure 4:The levels of Blood Urea Nitrogen (BUN) in diabetic rats treated with aqueous extracts of Senna or/ and Fennel.

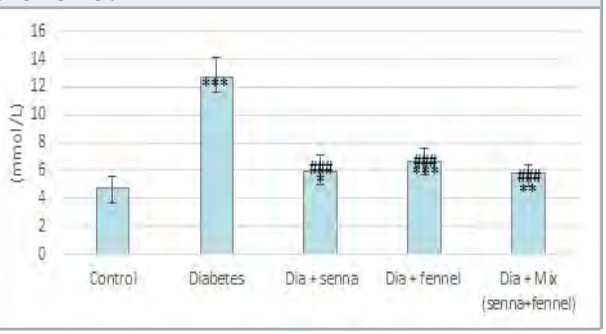
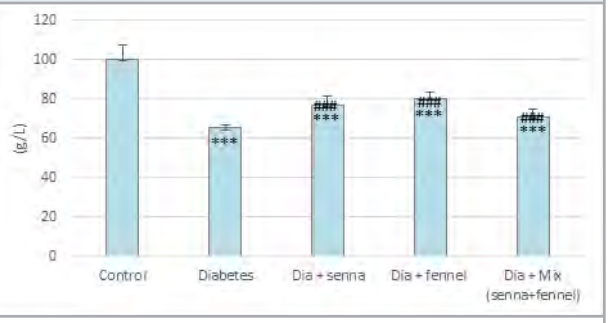
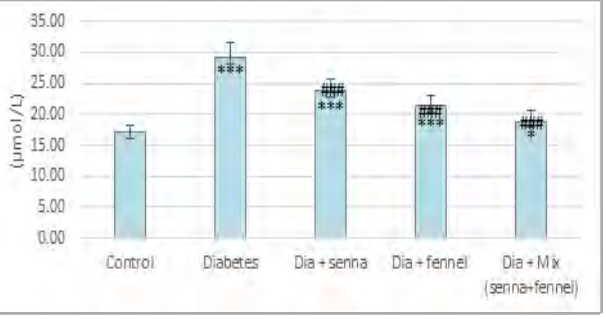


Figure 5: The levels of Total protein (TP) in diabetic rats treated with aqueous extracts of Senna or/ and Fennel.



Each value represents the mean of 10 rats ± SD, Significantly different from control value at P<0.05* , 0.001***, Significantly different from the untreated diabetic group at P<0.001###

Figure 6: The effect of aqueous extracts of Senna or/ and Fennel on renal tissues Nitric Oxide in diabetic rats



Figures 9-11 clearly illustrate the effect of senna and fennel on the antioxidant enzymes. A marked reduction was noted in the level of non-enzymatic antioxidants such as reduced glutathione, vitamin C, and the activity of enzymatic antioxidant (catalase) in the renal tissue of STZ induced diabetic rats when compared with normal rats. Administration senna and fennel or their mixture for the 30 days to STZ induced diabetic rats increased significantly (p < 0.000) the renal vitamin C, GSH levels and the activity of

CATas compared with untreated diabetic rats. Moreover, the results exhibit no significant differences in renal (catalase, glutathione) between the diabetic group treated with senna and the diabetic group treated with fennel.

Figure 7: The effect of aqueous extracts of Senna or/ and Fennel on renal tissues TBRAS in diabetic rats

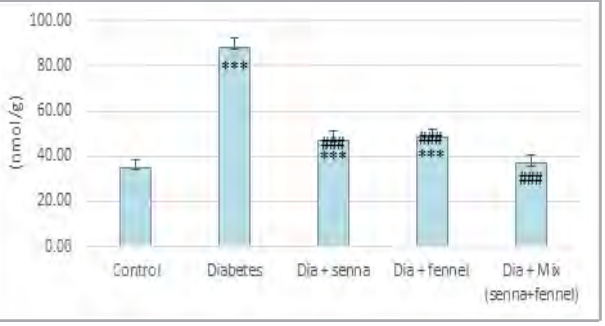
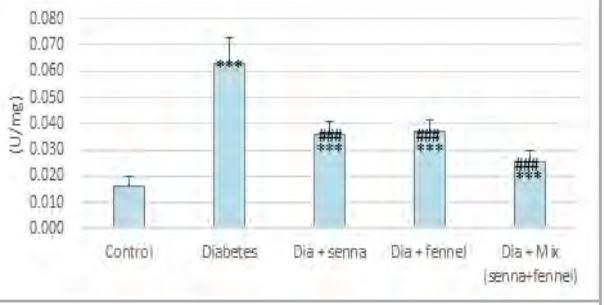
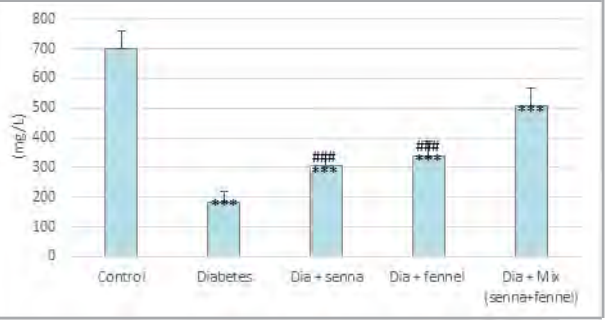


Figure 8: The effect of aqueous extract of Senna or/ and Fennel on renal tissues Xanthine Oxidase in diabetic rats



Each value represents the mean of 10 rats ± SD, Significantly different from control value at P<0.05* , 0.001***, Significantly different from the untreated diabetic group at P<0.001###

Figure 9: The effect of aqueous extracts of Senna or/ and Fennel onVitamin C in renal tissues of diabetic rats.



Despite the abundance of the market with antidiabetic drugs, the ambition still to reach natural products that can use as treatment with the least potential for side effects is an important matter. This has led to an attempt to apply the research on herbs that may be used to produce natural medicine which reduce suffering and potential fears. Therefore, an examination was made to evaluate the antidiabetic effects of the extracts of senna and fennel in diabetic rats and also study their renal protective effects. The data of the present study, showed a significantly decreased in the body weight of diabetic rats during the experimental period when compared to the control group.

Figure 10: The effect of aqueous extracts of Senna or/ and Fennel on Catalase in renal tissues of diabetic rats.

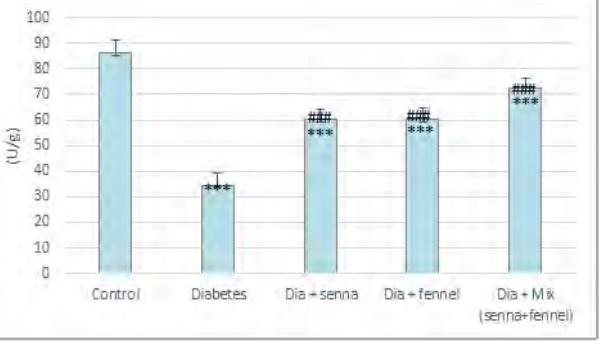
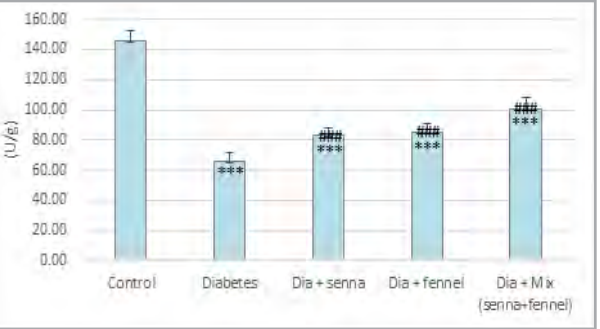


Figure 11: The effect of aqueous extracts of Senna or/ and Fennel on Glutathione in renal tissues of diabetic rats.



Each value represents the mean of 10 rats ± SD, significantly different from control value at P<0.05*, 0.001***, Significantly different from the untreated diabetic group at P<0.001###

The current results are consistent with the findings of Sureka et al. (2021) and Kodidela et al. (2020). This may be due to increased use of protein and fat for energy production, as diabetic rat cells may not be able to use glucose for energy production due to decreased insulin action or secretion. In addition, increased protein catabolism to provide amino acids for gluconeogenesis leads to muscle waste and weight loss(Srinivasan et al., 2014).The diabetic model rats of this study showed ameliorative in body weights after being treated with senna and/or fennel compared to the respective diabetic rat's untreated group. This result might point out the effectiveness of the senna and fennel by their ability to enhance insulin action (Osman et al., 2017).

Moreover, the data of the present study, showed an increase in the weight of the kidney (hypertrophy) in STZ-treated animals when compared with the normal group, which might be due to autophagy failure in the kidney, where Ma et al. (2020) found that autophagy deficiency in kidney tubules led to exaggerated renal hypertrophy in STZ-induced diabetic mice. In this study, diabetes was confirmed by the disparity levels of glucose and insulin, where STZ-diabetic rats showed high levels of glucose and low levels

of insulin. These levels were similar to the findings of Zhang et al. (2020) and Poitout and Robertson (2002), where they demonstrated that STZ causes pancreatic cell defects and reduces the cells' sensitivity to insulin-triggered glucose uptake, resulting in elevated blood glucose levels. Treating diabetic rats with senna or/and fennel extract significantly reduced blood glucose levels.

Mukhtar et al. (2020), reported that diabetic rats treated with 250 mg/Kg BW of aqueous leaf extract of senna for five days showed a significant reduction in blood glucose level. Likewise, El-Ouady et al. (2020) reported that the leaves aqueous extract of fennel at a dose of 10 mg/kg BW reduced blood glucose levels in (STZ)-induced diabetic rats. This decrease in blood glucose levels is attributed to the chemical contents in senna and fennel, which contain compounds that have an antidiabetic activity such as flavonoids. Flavonoids are reported to be able to regenerate damaged pancreatic β cells so that insulin deficiency can be overcome (Chotimah et al., 2008; Alqethami and Aldhebiani, 2021; Lindawati et al., 2021).

Consequently, blood glucose levels will be kept within a safe range as long as the pancreas produces enough insulin (NIDDK, 2018). Diabetes is one of the biggest factors that increases risk for kidney disease and is the number one cause of kidney failure (NIDDK, 2017). Renal damage is evident with a decrease in total protein and an increase in creatinine, uric acid and BUN. Similar structural changes were found in the present study. These results were consistent with Dabdoub et al. (2020).The kidneys remove metabolic wastes such as urea, uric acid, creatinine, and ions in order to maintain the optimum chemical composition of the body fluids (Kishore et al., 2017; Liu et al., 2018; Elkomy et al., 2020). In the case of renal tissue's diseases, the accumulation of those metabolites in the blood may be due to a decrease in their filtering or clearance by kidneys.

In this study, the renal damage may be due to that elevated blood pressure, where Ajayi et al., (2021) proved that there is a positive relationship between STZ-induced diabetes and high blood pressure. High blood pressure reduces the blood supply to the renal tissue through constriction and narrowing of blood vessels, which eventually damages and weakens them. If kidneys' blood vessels are damaged, they may no longer work properly. As a result, the kidneys may stop removing wastes and extra fluid from the blood (NIDDK, 2020).

The daily administration of aqueous extract of senna and fennel for 30 days caused a significant reduction in creatinine, uric acid and BUN, as well as a significant elevation in serum total protein levels in diabetic rats when compared to the diabetic untreated group. This improvement may be attributed to the components of these herbs, where senna and fennel rich in potassium, calcium and magnesium, which are known as key minerals to help control blood pressure (Basak and Reddy, 2017; HHP, 2019; Mehra et al., 2021b).The results of the current study also showed that diabetic rats resulted in an increase in oxidative stress (NO, TBARS and XO) and lowered levels of antioxidants (GSH, vitamin C, and CAT) in tissues of kidneys.

These results have coincided with the results of Samadi et al., (2021)who found that STZ disrupted the oxidative balance in the renal tissue that was proved by the increasing marker for oxidative stress and decreasing the total antioxidant status which was an assessment by Total antioxidant capacity content. Oxidative stress results from the overproduction of reactive oxygen species (ROS) plays a critical role in the pathogenesis of diabetic complications including renal diseases. Under normal circumstances, the body's antioxidant system can swiftly eliminate ROS, but in pathological conditions like diabetes, the excess ROS produced in the body exceeds the antioxidant system's scavenging ability (Pal et al., 2020; Charlton et al., 2021).

Indeed, diabetes triggers oxidative stress through the production of free radicals via glucose auto-oxidation, protein glycosylation and polyol pathways (Obrosova et al., 2002). Moreover, the increase in oxidative stress due to glucose auto-oxidation may possibly inactivate or weakens the activities of enzymatic and non-enzymatic antioxidants (Giugliano et al., 1996, Saddala et al., 2013), and consequently reduces ROS clearance. Many medicinal plants are reported to possess antioxidant properties and protects from complications of diabetes. In congruence with this statement, our study showed that both the aqueous extract of senna and fennel marked significant protection by increasing the antioxidants and reducing oxidative stress.

These results have coincided with Osman et al. (2017), who found that treatment streptozotocin-induced diabetic rats with senna and/or fennel extracts lead to lowering oxidative stress evidenced by the restoration of the enzymatic antioxidative of defense system (SOD, CAT and GSH). The antioxidant scavenging activity result suggests the presence of phytochemicals with antioxidant properties in the extracts of senna and fennel. Previous studies revealed the presence of various bioactive phyto- constituents in senna and fennel plants. Both senna and fennel contain polyphenols like phenolic acids, flavonoids and tannins which are familiar to be liable for the free radical-scavenging and antioxidant activities. These polyphenols' biological actions are thought to be owing to their redox characteristics, which can aid in the absorption and neutralization of free radicals, the quenching of singlet and triplet oxygen, and the decomposition of peroxides (Guarize et al., 2012; Daskum et al., 2020, Farag et al., 2020; Mehra et al., 2021a).

CONCLUSION

Our results suggested that the treatment with extracts of leaves of senna and the seeds of fennel exhibited significant antidiabetic and antioxidant activities in STZ-induced diabetes in albino rats. Both are attenuators of diabetes-induced alterations in body weight, blood glucose and insulin levels. Moreover, these extracts have protective effects in ameliorating diabetic nephropathy through lowering levels of abnormal renal enzymes and promoting renal antioxidants to overcome complications of the

oxidative stress in them. In addition, this study supports the idea that antioxidants from several sources together could provide synergistic benefits.

Conflict of Interest: There is no conflict of interest

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Isolation, Characterization and Quantitative Enumeration of Lactic Acid Bacteria from Human Faeces

Shama Parveen Siddique and *Amar P. Garg

Shobhit Institute of Engineering & Life Sciences, Meerut, Uttar Pradesh, India

ABSTRACT

Human Faeces (HF) is a solid waste material that is secreted as left amount after digestion of food inside the small intestine of the body. It contains large number of viruses, bacteria, fungi, actinomycetes, archaebacteria etc of which Lactic Acid Bacteria (LAB) are very common and play an important role in digestion and immunity. Hence, LAB were isolated and enumerated from HFusing standard protocol in the present study to find out the qualitative and quantitative distribution of LAB in gut microbiome. LAB were isolated using MRS agar medium under anaerobic conditions and was found that *Lactobacillus lactis, L. Acidophilus, L. fermentum* and *Enterococcus faecium* were the dominant species and the populations varied from 3.5 x 106 to 4.5 x 1010CFU/mL. It shows that good populations of LAB in gut microbiome survive under anaerobic conditions. LAB have great efficiency to resist against antibiotics. Such species of LAB should be commercialized and marketed at a global stage so that problems related to imbalance in gut microbiome can be solved.

KEY WORDS: ANAEROBIC, GUT MICROBIOME, HUMAN FAECES, PROBIOTICS.

INTRODUCTION

Keeping in view the health benefits of probiotic bacteria, several scientists are actively engaged in inventing/ improving new strains of promoting gut bacteria, (Kechagia 2013; Ding, 2019).The microflora of gut get is involved in many biochemical processes and has various applications in human life. Previous studies have shown that maximum bacteria, isolated from the milk, were LAB (Jin2011; Dunlop and May 2015). It is a large group of bacteria used throughout the globe as probiotics. The group includes the microbes of common genera *Lactobacillus, Lactococcus, Aerococcus, Enterococcus, Pediococcus, Leuconostoc, Streptococcus, Sporo lactobacillus, Vagococcus* and *Carnobacterium*. Hence, LAB strains are bestowed with boons for infants and also for the adults (Bisht and Garg 2019; Cunningham 2021).

It has been observed that increased interest in probiotics in public, government organizations like Ministry of Food, Agriculture and Health, WHO and FAO and the industries dealing with medicines and healthy food, have led to greater focus on research on gut microbiome and LAB. Probiotics helps in enhancing the health by helping in digestion of food and maintains pH of the digestive system, production of useful products that helps in eliminating the bad microbes(Amaraand Shibl 2015).It is well known that 70% of the immunity is controlled by gut microbiome.It is also known that the efficiency and safeguard of various pathologies, gastrointestinal disorders, several allergies causing diseases, acute diarrhoea along with necrotizing enterocolitis help paediatric healthcare professionals with the help of probiotics strains(Martinelli et al. 2020).

The developments that are going with respect to science of microbiome are enabling the innovative research in the field of prebiotics and probiotics. The applications of probiotics and prebiotics have the capability to enhance the understanding as well as healthcare applications. Mixture of different LABs living in host gut provide huge health benefits (Swanson 2020).The probiotics are not only restricted up to the gut associated diseases alone, but also help in management of various acute and chronic disorders. This is because of the fact, that says probiotics are capable of modifying the microbial ecosystem of the intestine, increase the resistance functionality of the gut, provide anti-microbial substances, provide adherence to the mucosa and epithelium, enhance immunity response whether it is innate or acquired, (Nazi2018; Michaelet al. 2020; Cunningham 2021).

It was reviewed the powerful preventive and therapeutic function of probiotics for high serum cholesterol, allergic and HIV diseases, cancer and providing possible action mechanisms, (Nazi2018).Gut microbiome is responsible for

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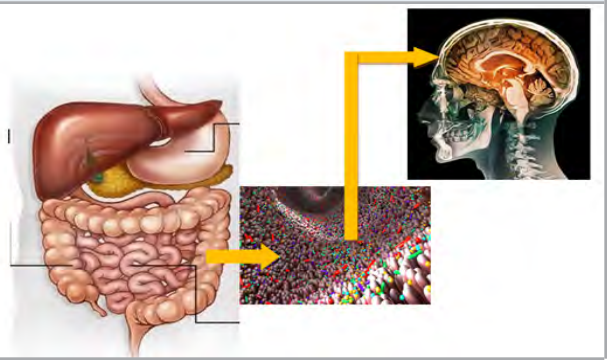
the digestion of food material and the fibres are digested to produce short chain fatty acids like butyric acid that cause happiness and can help in the treatment of depression too (Duchmann1999;Gregoire 2020; Michaelet al. 2020).In an investigation, it was confirmed that a great diversity of microbial populations is present within the saliva and human gut. Since, these microbes are of human origin, they may exhibit maximum functionality in the drugs and food which are there for human consumption. The living microbial organisms obtained from cultured milk and fermented foods are used for making foods for infants, also called as health friendly bacteria, which shows several health beneficial characteristics like for intolerance due to lactose, bowel diseases prevention, immune network system improvement, balance among the intestinal microbes, showing anti-hypertensive and anti-hypercholesterolemic, postmenopausal disorder alleviation, helps reducing traveller's diarrhoea etc (Balakrishnan2016;Hao et al.2018; Bazireh2020).

The work from our laboratories has demonstrated the human colostrum's is rich in LABs and their populations are greatly influenced with the diet of pregnant mothers (Arya 2020).At present, the most importantly used food agents are probiotics and prebiotics, that in combination act as symbiotic organisms. The dairy food products are regarded as the healthy product. Probiotic foods beneficially affect the host improving the life and induction of living microbial dietary agents in gastrointestinal flora, that regulate and stimulate the growth and development and the activation of catabolic mechanism of one or several health promoting bacteria in intestinal tract(Nagpal and Kumar 2012; Kailyn2020).Recent evidences suggest that gut microbiome contains more than 3000 different microbes, however, only few have been cultured till date.These microbes give the signal to the brain which controls all live activities of the body. Vedic teachings that Jaisa Anna Vaisa Man seems to be based on scientific principles because the gut microflora is controlled with the type and quality of the food and the thought process activates in the brain is under the control of gut microbiome which is depicted in Fig. 1 (Kelly, 2015; Evelyn 2019; Maria 2020).

Trillions of microbes have evolved with and continue to live on human beings. With the rapid advances in tools and technology in recent years, new knowledge and insight in cross-talk between the microbes and their hosts have gained. The role of microbiota and mechanisms involved in the progress and development of major human diseases, that include obesity, hypertension, cardiovascular disease, diabetes, cancer, Inflammatory Bowel Disease (IBD), gout, depression have been reviewed in previous studies (Ding 2019; Maria 2020).

The human microbiome comprises bacteria, archaea, viruses and eukaryotes which reside within and outside our body. These organisms impact human physiology, both in health and in disease, contributing to the enhancement or impairment of metabolic and immune functions. Microorganismsare known to colonise various sites on and in the body (Ogunrinola and John 2020).Gut microbes affect the physiology of their hosts. Studying their diversity and functions is thus one of utmost importance as it will open new avenues towards the discovery of new biomolecules and the treatment of diseases. Gut microbiome research is currently boosted by the unification of metagenomics, which has dominated the field in the last two decades, and cultivation, which is experiencing a renaissance (Thomas2021). Sehgal and Andreasson (2020) have found that there is bidirectional gut microbiota–brain communication in mood disorders. Effects of probiotics on brain connectivity and mental health outcomes and pregnancy related stress on gut microbiota in the newborn child has also been studied and positive relationship has been indicated (Sehgal and Andreasson 2020; Thomas 2021).

Figure 1: Transfer of signals from Gut Microbiome to Brain



The samples of Human Faeces (HF) were collected from 15different individual volunteers aseptically and were brought to the laboratory immediately in the School of Biological Engineering and Life Sciences, Department of Biotechnology, Shobhit Institute of Engineering and Technology, Meerut (UP) India. The individuals were told about the entire project and their consent was obtained. For the isolation of LAB, approximate 1 g of faeces samplewas quickly dissolved in 99mL of sterile distilled water from which dilution series (up to 10-8) was made. 0.1 mL suspension from dilutions10^{-6},$10^{-7}$and 10^{-8}were inoculated aseptically onto MRS agar plates by spread plate method. The plates were incubated at 37±1°C for 48 to 72h under anaerobic conditions using anaerobic gas jar. The plates were observed for bacterial colonies after incubation period, their morphology, color and texture were noted down carefully and colony forming unit per mL were counted using following formula.

$$\text{CFU/ml} = \frac{\text{Number of colonies x dilution Factor}}{\text{Volume Plated}}$$

For gram staining, smear was prepared on plain glass slide using a drop of saline and small amount of fresh bacterial culture from the plate. The smear was heat fixed gently and carefully, stained as per standard Gram's stain method and was observed under oil emulsion lens 10 x 100x of compound light microscope (Carl Zeiss Microscopy GmbH). As LAB are Gram positive in nature, all the isolates which showed purple (positive) color were

further processed for physiological and biochemical tests (Arya 2020).For catalase test, the 3% hydrogen peroxide solution was mixed gently over the surface of clean glass slide containing test microbial culture and was observed for formation of bubbles. Positive reaction was indicated by the presence of bubbles (Kumar and Kumar 2015, Asto et al. 2019). As LAB are catalase negative, all isolates that showed negative results were further tested for oxidase test.

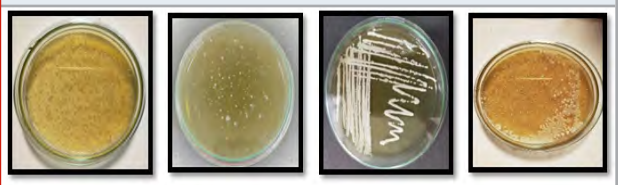
For oxidase test, Cytochrome C oxidase test was performed using a filter paper soaked with the freshly prepared solution of tetramethyl-p-phenyl diamine dihydrochloride over which fresh culture of test bacteria was gently rubbed using sterile nichrome wire loop. Change in color within 30 second showed positive reaction. (Kumari 2008). As LAB are oxidase negative. Sugar Fermentation tests were performed for each isolate to confirm their Genus and species as per the recommendations of Bergey's Manual of Systematic Bacteriology (9th edition).Disk containing 25 mg amount different sugars such as Maltose, Glucose, Lactose, Galactose, Mannitol, Xylose, Fructose was placed in 10mL of distilled water added and Durham tube was placed in inverted position. These were then autoclaved at 15Psi for 15 min andafter autoclaved 1 mL of inoculums of test isolate was added aseptically. These were incubated and observed for acid and gas production. These isolates were categorized on the basis of fermentation group (Koll 2010). The results are presented in (Table 2) and were interpreted as using Bergey's Manual of Systematic Bacteriology.

RESULTS AND DISCUSSION

All 15 HF samples examined in our study, showed positive growth of LAB on MRS agar plates. The characteristics of colonies formed on plates varied in the form, shape, size and colour (Table 1) and ranged between 3.5x 10^6 to4.5x 10^{10}..Sample no HF 7 showing the lowest number of colonies belonged to a person who was weaker and had suffered from gastric problem. HF10, HF15, HF11, HF5 and HF1 were collected from a healthy person taking good nutritious vegetable diet. In our preliminary studies, we have found that the diet seems to play significant role. Further experiments are being carried out in our laboratory with controlled diet.All the isolates tested in our study were found Gram's positive and negative for catalase and oxidase which suggested that the isolated colonies belonged to the lactic acid bacteria (LAB) group. Based on morphological characters, texture, color of the colony and physiological and biochemical tests including sugar fermentation coupled with gas production, we identified the isolated species as *Lactobacillus lactis, L. acidophilus, L. fermentumand Enterococcus faecium* (Fig.2; Table 2)as per the recommendations of Bergey's Manual of Systematic bacteriology.

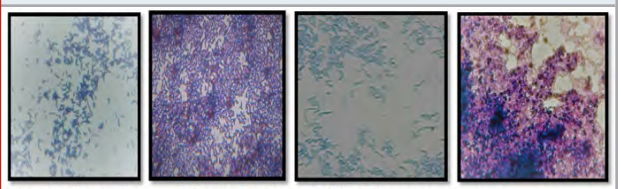
The microphotographs of the pure culture of identified species are shown in Fig 3.The cells of *L. lactis* were spherical to ovoid and found in pairs or short chains, these were Gram's positive and did not form spores.*L. acidophilus* showed rod shaped cells, Gram positive and fermented lactose. *L. fermentum fermented glucose, lactose, galactose, fructose* and sucrose (Table 2) and was Gram's positive rods while *Enterococcus faecium* fermented maltose, lactose, mannose, galactose, fructose and sucrose and showed Gram's positive reaction with rod shaped cells. It was found that *Lactobacillus acidophilus* was most common in all 15 samples and was detected in 80% of the samples followed by *L. lactis*, *Enterococcus faecium* and *L. fermentum* (Fig. 2, Table 2) on MRS agar medium under anaerobic/ partially aerobic conditions. Almedia (2021)have characterized functional and taxonomic groups of human gut microbiota and have presented a Unified Human Gastrointestinal Genome (UHGG) collection comprising 204,938 non-redundant genomes from 4,644 prokaryotes which encode >170 million proteins (Almedia2021).

Figure 2: Isolated LAB on MRS agar plates



L. lactis *L. fermentum* *L. acidophilus*

Figure 3: Microphotographs of isolated strains of LAB under 10 x 100X (original magnification)



Lactobacillus lactis *L. fermentum* *L. acidophilus* *Enterococcus faecium*

Roughly, 40 trillion microbes, consisting of more than 3000 species of several viruses, bacteria, archaea, fungi and other eukaryotes that live in and on our body and are extremely important for our health, however, few cause diseases and disorders (Wang 2018). Microbial biomass in/ on our body consists of 1 Kg (approximately) representing about 1000,000 genes while entire human genome consists of approximate 30,000 genes and the human microbial genome greatly influence the physiology and behaviour of human. Gut microbiome is the principal ecological niche and secretes various enzymes that are mainly responsible for the digestion of food and also accounts for 70% of the total immunity of the body (Stefan 2020). Lactic acid bacteria (LAB) constitute the major part of gut microbiome but these are difficult to culture because of the requirement of anaerobic / partially aerobic conditions for their culture. Most scientists have focussed on genomic characterization of gut microbiota and a large number of microbial species have been identified (Samuel 2019; Stefan 2020).

However, very few species have been cultured so far. The isolation, characterization and culture of 4 species of LAB from faeces as representative of gut microbiota of human in this paper suggest that gut microbes if cultured under anaerobic / partially aerobic conditions on MRS agar media,

can help in isolation of more and more species which can then be evaluated for their commercial exploitation. Diet influences the gut microbiome and the importance of plant-based diet in regulation of gut microbiome and its impact on human brain functions has been reviewed by (Medawar 2019; Angelis 2020). Gut bacteria can also help to manage depression (Pichler 2020; Chevalier 2020). Extraction of probiotic *Lactobacillus* and *Enterococcus* strains from faeces and human saliva was also reported in previos studies (Bazireh 2020).It is suggested that human gut microbiome need to be cultured so that their probiotic value may be evaluated and used for commercial purposes(Bazireh 2020).

Table 1. Morphological characteristics and quantitative number of isolated LAB.

Sample No.	Isolates No	Colour	Shape	Size	Margin	Opacity	Elevation	Texture	CFU/ mL
HF 1	SUB-101	Pale Yellow	Circular	Small	Entire	Opaque	Raised	Smooth	2.8×10^{10}
HF 2	SUB-102	Off white	Circular	Moderate	Entire	Transparent	Flat	Moist	3.5×10^{7}
HF 3	SUB-103	Creamy white	Circular	Large	Entire	Opaque	Convex	Dry	2.5×10^{7}
HF 4	SUB-104	Bright white	Circular	Small	Entire	Opaque	Flat	Powdery	3.9×10^{10}
HF 5	SUB-105	Pale yellow	Circular	Moderate	Entire	Transparent	Raised	Smooth	4.6×10^{9}
HF 6	SUB-106	Creamy white	Circular	Small	Entire	Transparent	Flat	Moist	4.1×10^{8}
HF 7	SUB-107	Pale yellow	Circular	Moderate	Entire	Opaque	Convex	Smooth	3.5×10^{6}
HF 8	SUB-108	Off white	Circular	Large	Entire	Opaque	Flat	Dry	2.6×10^{8}
HF 9	SUB-109	Pale yellow	Circular	Large	Entire	Transparent	Raised	Moist	2.9×10^{10}
HF10	SUB-110	Creamy white	Circular	Small	Entire	Opaque	Convex	Dry	4.5×10^{10}
HF11	SUB-111	Off white	Circular	Moderate	Entire	Transparent	Flat	Smooth	6.3×10^{9}
HF12	SUB-112	Creamy white	Circular	Small	Entire	Opaque	Convex	Dry	4.6×10^{8}
HF13	SUB-113	Pale yellow	Circular	Small	Entire	Opaque	Raised	Moist	4.5×10^{7}
HF14	SUB-114	Creamy white	Circular	Small	Entire	Opaque	Flat	Smooth	3.4×10^{7}
HF15	SUB-115	Pale yellow	Circular	Small	Entire	Transparent	Convex	Dry	4.8×10^{9}

Table 2. Characterization of different isolated strains based physiological and biochemical tests.

Species isolated	% Occurrence In 15 samples	Gram's staining	Catalase test	Oxidase test	Cell form	Fermented Sugars (+)	Unfermented Sugars (-)
Lactobacillus acidophilus	80.0%	+	-	-	Rod	Glu, Mal, lac, Gal	Man, Fru, Suc
L. lactis	66.67%	+	-	-	Coccobacillus	Glu, Mal, Lac, Man	Gal, Fru, Suc
L. fermentum	53.3%	+	-	-	Cocci	Glu, Mal, Fru, Suc	Lac, Man, Gal,
Enterococcus faecium	60.0%	+	-	-	Rod	Mal, Gal, Fru	Glu,Lac, Man, Suc

CONCLUSION

The findings of the present study suggest that the lactic acid bacteria can be isolated, characterized and cultured from human faeces as representative of gut microbiota on MRS agar plates under anaerobic/partially aerobic conditions. We have successfully isolated, characterized and cultured *Lactobacillus lactis*, *Lactobacillus acidophilus*, *Enterococcus faecium* and *Lactobacillus fermentum* from 15 human faeces samples.It may safely be concluded that probiotics play a vital role in the management of the health of human beings. Proper concentration and diversity of species of probiotics are necessary for the maintenance of the immunity of the ruminants.We have isolated, cultured and characterized LAB from human faeces where we found that immediate processing of the samples was extremely essential. Most of the studies in western countries are confined to genomics and proteomics of gut microbiome while the culture of microbes is essential for their further commercial utilization. With this view point, our studies are very important and we suggest that the microbiologists working on gut microbiome should make all prior preparations for inoculation before sampling and the time between the sampling and inoculation should be less than 10 minutes (personal experience of our entire group).

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Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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In vitro Antioxidant Activities of Lichen Species *Dirinaria applanata* and *Parmotrema andium* Collected from Similipal Biosphere Reserve, India

Bijayananda Sahoo, Satyabrata Dash, Sabyasachy Parida, Shubham Pradhan and Biswajit Rath*
Department of Biotechnology, Maharaja Sriram Chandra Bhanja Deo University, Baripada, Odisha, India

ABSTRACT

Lichens appear to be a promising source of antioxidants due to presence of numerous metabolites that can reduce free radicals. The Lichen species were obtained from Similipal biosphere Reserve (SBR). The antioxidant activity was carried out by DPPH, H_2O_2, FRAP scavenging assay and further TPC and TFC was also estimated. Among the lichens tested, *Dirinaria applanata* exhibited strong antioxidant activities than *Parmotrema andium*. The methanol and acetone extracts of *D. applanata* showed DPPH radical scavenging activities (IC_{50} value) as 471.16±0.85µg/ml and 519.79±1.29µg/ml whereas in *P. andium* IC_{50} value was 534.77±0.75µg/ml and 600.77±0.95µg/ml respectively. Similar result was also observed in H_2O_2 scavenging assay and FRAP. An interesting strong relationship between total phenolic and flavonoid contents and their antioxidant activities in both the Lichen species was marked as determined with respect to gallic acid and quercetin equivalents. The results indicates that the selected lichen species possess significant antioxidant activity which may be utilized as novel sources of natural antioxidant compounds.

KEY WORDS: ANTIOXIDANT, DPPH, FRAP, H_2O_2 , LICHEN.

INTRODUCTION

Lichen is a symbiotic organism that consists of a fungus (mycobiont) and a photosynthetic partner (photobiont) and are important constituents of many ecosystems (Obohv and Ademosun 2006; Bates et al. 2011; Hawksworth et al. 2020). Lichens are unanimously distributed in diverse climatic conditions spanning from the plains to the high mountains and from polar regions to the tropics and are susceptible to a variety of environmental stress (Felczykowska et al. 2017). As a result, they produce different secondary metabolites including some specific such as terpenes, depsides, depsidone, dibenzofuran, and xanthone which are unique to lichen species (Olivier-Jimenez et al. 2019; Stanojković 2019; Sabarwati et al. 2020). The secondary metabolites of lichen contain bioactive substances which have manifold biological activities such as antimicrobial, anticancer, anti-inflammatory and antioxidant properties (Maulidiyah et al. 2016; Mohammadi et al. 2020; Mohan et al. 2020; Nugraha et al. 2020; Studzińska-Sroka et al. 2021).

Antioxidants are compounds that can retain the quality of foods by delayed process of oxidation and protect from damage caused by free radical induced oxidative stress (Souri et al. 2008). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene toluene (BHT), tertbutylhydroquinone (TBHQ) and propyl gallate (PG) are widely accepted, but their use is being restricted nowadays due to their toxic and harmful side effects (Zhang et al. 2009; Maulidiyah et al. 2021). Thus, at present there is a growing interest towards natural antioxidants. So in the search for novel natural antioxidant sources, our target is aimed on lichens found in Similipal Biosphere Reserve (SBR) as it harbours a large variety of lichen species. Thus, the purpose of the present work is to assess the antioxidant activity of acetone and methanol extract of the lichens obtained from this region. Our investigation is the first report describing the evaluation of Similipal Biosphere Reserve lichen species as a source of natural antioxidant.

MATERIAL AND METHODS

For the collection of lichen samples, two lichen species, *Dirinaria applanata* and *Parmotrema andium* as shown in fig.1 were collected from various localities within Similipal Biosphere Reserve (SBR). Herbarium specimen of each species was maintained in research laboratory, Department of Biotechnology, Maharaja Sriram Chandra Bhanja Deo University, Baripada, Odisha for experimental purpose.

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The lichen species were identified following available monographs and standard methods (Awasthi 1988; Awasthi 1991; Orange et al. 2001). For the preparation of lichen extract, dried lichen samples were milled into fine powder using a sterile mortar and pestle. The powdered form of material was moved through the sieve (75 microns) and 10 g of dry fine powder of sample was mixed with 100 ml of solvent (acetone and methanol) on an orbital shaker and filtered using Whatman no. 1 filter paper. Then it was vaporised in a rotary evaporator at 45°C and preserved in deep freeze for subsequent use.

For the DPPH radical scavenging activity, the antiradical activity of two test lichen extracts was evaluated by DPPH (1,1-diphenyl-2-picryl-hydrazil) assay (Kosanic et al. 2011). One ml of DPPH solution (0.1mM) was added to 3 ml of different concentration (100-1000 µg/ml) of lichen extract and the mixture was kept for 30 min at room temperature and the absorbance was recorded at 517 nm using UV-Vis. Spectrophotometer (Systronics-119). Ascorbic acid was used as positive control. The hydrogen peroxide (H_2O_2) scavenging activity was determined by spectrophotometric analysis according to the method of Ruch et al. (1989). H_2O_2 solution was prepared in PO_4 buffer (1 M, pH 7.4) and 0.6 ml was added to the lichen extract of various concentrations (100-1000µg/ml). The optical density was recorded at 230 nm after 10 min. Ascorbic acid was used as standard against the test extract. The DPPH/ H_2O_2 radical scavenging activity was calculated by the following equation:

$$\% \text{ activity} = [(\text{control Abs} - \text{sample Abs}) / \text{control Abs}] \times 100$$

IC_{50} values were derived from the percentage of inhibition versus concentration plot and expressed in µg/ml.A lower IC_{50} meant better radical scavenging activity.

For the ferric reducing antioxidant power assay (FRAP), the ferric reducing antioxidant power of the tested lichen species was estimated according to the method of Oyaizu (1986). Various concentration of lichen extract (100, 250, 500, 750, 1000µg/ml) was added with 2.5 ml of PO_4 buffer (pH 6.6, 0.2M) and potassium ferricyanide (1%) and was kept at 50°C for 25 minutes. Then it was mixed with trichloro acetic acid (10%) and centrifuged at 3000g for 20 minutes. Finally, the obtained supernatant was thoroughly mixed with 2.5 ml distilled water alongwith 0.5 ml $FeCl_3$ (0.1%) and recorded at 700nm. butylated hydroxytoluene is used as positive control for the experiment. Total phenolic content (TPC) was estimated by Folin-Ciocalteu reagent following the method of Taga et al. (1984). The lichen extract (100µl) was mixed with 2ml of Sodium carbonate (2%). After 10 min, 500 µl of Folin's reagent was supplemented and then the reaction mixture was kept under dark for 20 minutes and the absorbance was measured at 650 nm. The result was expressed as µg GAE /g dry extract. Total flavonoid content (TFC) was determined according to the method of Zhishen et al. (1999). One ml of the lichen extract was mixed with 500 µl of Sodium nitrite and Aluminum chloride (10%c 300 µl). After 10 min, Sodium hydroxide (1M, 1ml) was mixed and the volume was made up to 5ml with distilled water. Then the mixture was incubated for 30 min and the absorbance was read at 510nm. The result was expressed as quercetin equivalent i.e µg QE /g dry extract.

Table 1. DPPH radical and H_2O_2 scavenging activity of methanol and acetone extract of *Dirinaria applanata* and *Parmotrema andium*

Lichen species	Solvent	DPPH radical scavenging activity IC_{50}(µg/ml)	H_2O_2 scavenging activity IC_{50} (µg/ml)
Dirinaria applanata	Methanol	471.16±0.85	554.57±0.90
	Acetone	519.79±1.29	707.74±0.79
Parmotrema andium	Methanol	534.77±0.75	592.25±0.93
	Acetone	600.77±0.95	755.34±0.86
	Gallic acid (standard)	326.61±0.94	341.52±0.87

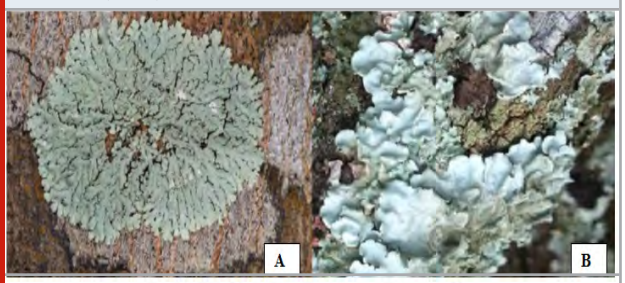
RESULTS AND DISCUSSION

DPPH and H_2O_2 scavenging assay: The scavenging of DPPH and H_2O_2 radicals by the lichen extracts was determined and shown in table1.The test lichen species extracts exhibited differential scavenging ability. However, methanol extract of *D. applanata* has strong DPPH and H_2O_2 scavenging activity with IC_{50} i.e., 471.16±0.85 µg/ml and 554.57±0.90 µg/ml respectively. The lowest IC_{50} of DPPH and $H_2O_2$was recorded in acetone extract of both the species. These results are in agreement with the literature where it was reported that many lichen extracts has their ability to scavenge free radicals (Behera et al. 2009; Manojlović et al. 2012; Hawrył et al. 2020).

Ferric reducing antioxidant power assay (FRAP): The experimental result on assay of Ferric reducing power of two lichen species was represented in fig 2. The results infer that the higher absorbance (700nm) of lichen extracts results in higher reducing power. Accordingly, more reducing power was recorded in methanolic extract of *Dirinaria applanata* (absorbance: 0.762±0.0008) as compared to *Parmotrema andium* (absorbance:0.673±0.0008). Comparing this result it was found that *Dirinaria applanata* showed more promising antioxidant activity than *Parmotrema andium*.

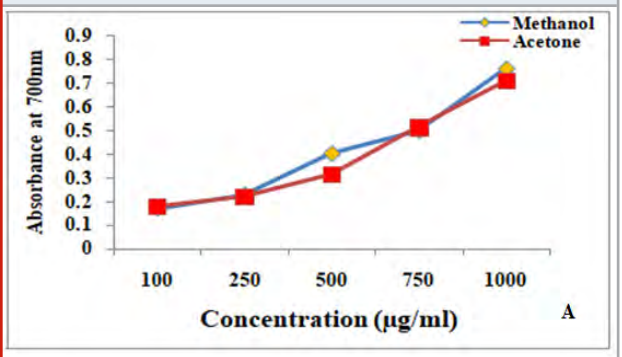
Likewise, Smitha et al. (2016) reported comparative reducing power activity with ethanol and methanol extracts of the *Ramalina pacifica* which was less when compared with *Rocella montagnei*. Similarly, the maximum reducing power was detected in methanol extract of *P. centrifuga* (Ranković and Kosanić, 2019; Aoussar et al. 2020; Hawrył et al. 2020).

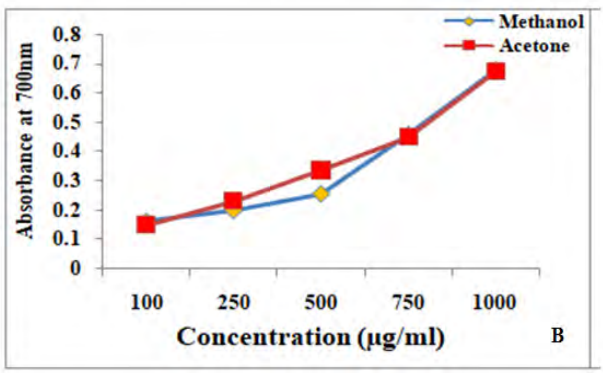
Figure 1: (A-B) Lichens collected from Similipal Biosphere Reserve (SBR)



A. *Dirinaria applanata* B. *Parmotrema andium*

Figure 2: Ferric reducing antioxidant power assay of A. *Dirinaria applanata* and B. *Parmotrema andium*





Total Phenolic and Flavonoid content (TPC and TFC): TP and TF content of methanol and acetone extracts in both the lichen species was determined in terms of gallic acid equivalent (µg GAE/ g of extract) and quercetin equivalent (µg QE/ g of extract) respectively and the findings was summarized in table 2. Maximum phenolic content was recorded in methanolic extract of *Dirinaria applanata* (68.96±0.60 µg/ml) while acetone extracts of *Parmotrema andium* showed the lowest phenolic content (48.56±0.97µg/ml) (Manojlović et al. 2021). Similarly high flavonoid content was also found in *Dirinaria applanata*, (38.22±0.89 µg/ml) as compared to *Parmotrema andium* (27.57±1.03 µg/ml). Both Phenolic and Flavonoid compounds have antioxidant characteristics due to their redox properties, which can assist in the absorption and neutralisation of free radicals, quenching of singlet and triplet oxygen and the decomposition of peroxides. Thus, total phenolic compounds in *Dirinaria applanata* were found to be more encouraging with higher level of TPC and TFC (Manojlović et al. 2021).

It was understood and established that the antioxidant activity might be cumulative effect of different natural components and not solely of its fractions. In our result, the antioxidant activity of the *Dirinaria applanata* and *Parmotrema andium* extract might be the resultant synergistic effect of various compounds present within the extract and more interestingly encouraging antioxidant potential of *Dirinaria applanata* is due to the associated action which contributes a higher antioxidant activity in its extracts (Sargsyan et al. 2021).

Table 2. TPC and TFC of *Dirinaria applanata* and *Parmotrema andium*

Lichen Species	Methanol	Acetone
TPC(µg/ml)		
Dirinaria applanata	68.96±0.60	57.24±0.96
Parmotrema andium	59.82±0.85	48.56±0.97
TFC(µg/ml)		
Dirinaria applanata	38.22±0.89	30.79±0.82
Parmotrema andium	32.54±0.53	27.57±1.03

CONCLUSION

The findings of the present study demonstrate that methanol extract of *Dirinaria applanata* has impressive antioxidant activities *in vitro*. In summary, the experimental results conclude that *Dirinaria applanata* and *Parmotrema andium* may act as an agent of potential natural sources of antioxidants. Results indicated that probably the higher amount of phenolic compounds in the lichen extracts are responsible for encouraging antioxidant properties that would be of interest in food and pharmaceutical industry. Hence further work needs to be carried out to isolate and purify the active components from these species to determine their antioxidant activity.

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Conflict of Interests: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Brucellosis: An Investigation of the Knowledge, Attitudes and Behaviors Among a Selected Population in Majmaah Saudi Arabia

Mohammed Alaidarous
Department of Medical Laboratory Sciences, Majmaah University, Majmaah, Saudi Arabia.

ABSTRACT

Brucellosis is a zoonotic bacterial disease carried by animals such as sheep, cows, and camels. It is transmitted to humans through the consumption of affected animals or their by-products. Dairy products form a major part of the Arabic diet and are very widely consumed in Saudi Arabia, where brucellosis is endemic. This study has analyzed the current knowledge, attitudes, and behaviors toward brucellosis of adults aged 18 years and older among Majmaah University students and staff. A cross-sectional study with 181 participants was conducted using an online survey during March–April 2021. The results showed that the majority of people knew about brucellosis and its transmission, but only a fraction of them took the required precautionary measures. This study highlights that knowledge alone is insufficient to promote healthy behaviors. It is thus important to raise awareness in a way that can convert knowledge into action.

KEY WORDS: ATTITUDES, BEHAVIORS, BRUCELLOSIS, KNOWLEDGE, UNPASTEURIZED DAIRY PRODUCTS.

INTRODUCTION

Brucellosis is an infectious disease that spreads to humans through the consumption of unpasteurized dairy products and contact with livestock. It is most common in Middle Eastern countries among farm families who routinely handle livestock and dairy products. The results of a cross-sectional survey of Israeli respondents that sought to understand the purchase and consumption patterns of dairy products indicated a high-risk association of brucellosis with the consumption of unregulated dairy products (Baron-Epel et al. 2018; Nejad et al. 2020).

A survey in Jordan by Musallam et al. (2015) analyzed farmers' hygiene practices and found that 60% of livestock keepers were not boiling dairy products before use, which is a critical risk indicator of brucellosis. The lack of awareness of dairy product hygiene affects not only farmers directly, but also the people who consume such products in the supply chain (Nejad et al. 2020). Peck et al. (2018) conducted interviews with 51 small-scale goat farmers in Thailand to examine their knowledge, attitude, and practices (KAP) associated with brucellosis and found a low perceived risk. The researchers' analysis indicated a critical gap in knowledge and attitudes toward safe farm practices. Education and awareness training for farmers was thus considered a priority to contain the brucellosis endemic in Thailand. Kiffner et al.'s (2019) KAP analysis of Tanzanian residents revealed a demographic correlation between zoonotic diseases such as brucellosis and pathogenic transmissions, signifying that a careless attitude, limited knowledge, and the consumption of raw milk and meat are risky practices. Children who are highly exposed to livestock, dogs, and zoonotic pathogens may transmit such pathogens to their families as well as the people in those specific regions (Cavalerie et al. 2021).

Evaluation of the weak KAPs of Tajikistan farmers found a risk of brucellosis in about 30% of households (Nejad et al. 2020). In a survey by Cloete et al. (2019), only 60% of a cattle-keeping community in South Africa had heard of brucellosis, leaving the whole community highly vulnerable to the disease. The significant degree of risk provides essential insights into the alarmingly low awareness of brucellosis in urban and semi-urban areas (Cavalerie et al. 2021). Al Jindan (2021) argued that the diverse ethnicity of the workforce is a critical reason for the prevalence of *Brucella* or brucellosis in the Kingdom of Saudi Arabia (KSA), suggesting that people from various backgrounds and countries are potential carriers of zoonotic diseases. Alkahtani et al. (2020) conducted an epidemiological study

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in a KSA hospital and found demographic correlations between brucellosis and infected people. The results of the study showed that, during summer, 40.5% of young males were infected with *Brucella* (Alkahtani et al. 2020). The critical lack of standard KAP in the Saudi region was considered the primary factor in this high prevalence as the dearth of community awareness, practice of drinking raw milk, and unhygienic manufacturing in rural communities has resulted in a high rate of brucellosis in KSA (Alkahtani et al. 2020; Cavalerie et al. 2021).

Qasim et al. (2020) performed a specific medical analysis to understand the causes, complications, and clinical features of brucellosis in the Riyadh region. Their results indicated that nearly 15% required hospitalization from the samples that consumed large amounts of unpasteurized camel's milk (Qasim et al. 2020). A significant number of KSA livestock keepers engage in direct contact with the animals in their care, which carries a critical risk of spreading brucellosis to their families, communities, and the wider population. Al Hashan et al. (2017) conducted a study to determine the magnitude of brucellosis infection and the associated KAPs among the children of Najran City in southern KSA. They found that the children habitually ingested raw milk and dairy products, had contact with animals, and were constantly exposured to endemic areas and thus recommended that the rural areas of southern KSA implement more strategic plans to curb brucellosis. Aloufi et al. (2016) offered an analysis of the disease's trend. They showed that KSA had the highest rate of human brucellosis during the late 1990s and that, even though case numbers had plunged, it is still counted as an endemic disease in the country. This indicates that inadequate adherence to protective KAPs is responsible for this major health problem. Hosting diverse travelers, importing livestock, "supporting birth in infected livestock," unhygienic meat packaging, and the ingestion of raw milk products are the reasons for the existing cases of brucellosis in KSA (Al Anazi et al. 2019; Edathodu et al. 2021).

It has been suggested that KSA needs stricter policies and better awareness of hygiene and farm livestock maintenance (Al Anazi et al. 2019). Bakheet and Alnakhli (2019) reported that the infection rate in KSA is 70 per 100,000 people, which is significantly higher than that in other developed countries. KSA's socioeconomic mobility continues to be a strong cause of the existing endemic (Bakheet and Alnakhli 2019). According to the Ministry of Health Saudi Arabia, treatment policies to cure Malta fever, which is caused by brucellosis, include a clinical examination, laboratory tests, and a six-week program of antibiotics. The prevention guidelines include cooking meat at 63°C –74°C, avoiding unpasteurized dairy products, taking precautions in the workplaces, promoting handwashing, and wearing gloves when in contact with farm animals (Edathodu et al. 2021).

Because brucellosis is endemic in KSA and there are no specific health policies for brucellosis, it is crucial that public health officials, veterinary authorities, and governmental agencies disseminate information to support the essential KAPs and thus reduce the number of cases. This will not only strengthen the medical policies of the country, but also ensure a healthy population (Edathodu et al. 2021). In the current study, I assessed the knowledge and attitudes of Majmaah University staff and students regarding brucellosis as well as the amount of unregulated (homemade) dairy products purchased and consumed. I aimed to determine whether exposure to past cases of brucellosis in the individuals' communities had affected their behaviors. The study results may contribute to the planning of effective public health interventions.

Table 1. Showing Participants' Characteristics.

Participants Characteristics	Mean ± SD*/ percentage (frequency)
Age (in years)	30.88 ± 12.64
Gender	
Female	50.3 (91)
Male	49.2 (89)
Did not report	0.6 (1)
Marital Status	
Not married	40.9 (74)
Married	59.1 (107)
Job Group	
Academic	26.0 (47)
Administrative	6.6 (12)
Student	67.4 (122)
Income	
<5,000–10,000 SAR	42.0 (76)
10,001–20,000 SAR	34.8 (63)
20,001–30,000 SAR	5.0 (9)
>30,000 SAR	13.8 (25)
Did not report	4.4 (8)
Town	
With cases of brucellosis	54.7 (99)
Without cases of brucellosis	40.3 (73)
Did not report	5.0 (9)
History of brucellosis infection in the family	
No	95.0 (172)
Yes	5.0 (9)
Education	
School level	9.9 (18)
Diploma	0.6 (1)
Bachelor	49.2 (89)
Master	22.1 (40)
PhD	18.2 (33)

*SD: Standard deviation

MATERIAL AND METHODS

The cross-sectional study included a random online survey, which was distributed to multiple departments at Majmaah University during March and April 2021. The study was

approved by the Majmaah University Ethical Committee (no. MUREC-Feb/COM-2021/22-1). The study population included adults (18 years and older) who were either staff or students at Majmaah University. The survey garnered 181 responses (59 staff, 122 students). All the statistical analyses were conducted using SPSS 26.0 (IBM) and Factor 10.10.03 (Universitat Rovira I Virgili, Tarragona, Spain). I employed descriptive statistics, bivariate correlation tests (Pearson's test for continuous variables and Spearman's test for categorical variables), the Mann–Whitney *U* test, and binary logistic regression for the analyses. The Mann–Whitney *U* test was used because the knowledge and attitude scores were not normally distributed, as determined by both the Kolmogorov–Smirnov test and the Shapiro–Wilk tests. Multivariate binary logistic regression was applied after the necessary data assumptions had been verified. For all four models, there were no cases of multicollinearity (tolerance >0.1, variance inflation factors <10.0, inter-independent variable correlation coefficients <0.7) (Baron-Epel et al. 2018).

The linearity of the relationship between the continuous independent variables and the logit transformation of the dependent variable was met for three of the models. In one model with "Eating cheeses in unsealed packaging from unregulated sources" as the dependent variable, no linear relationship existed between attitude (the independent variable) and the dependent variable. For this model, the attitude score was therefore dichotomized. Attitude scores of 0–3 was coded as 0, indicating no or less agreement with the item, and a score of 3.1–5.0 was coded as implying agreement with the item. Hosmer and Lemeshow statistics were used to determine the model fit. Nagelkerke's R^2 statistics indicated that the variance was accounted for by the model (Baron-Epel et al. 2018).

RESULTS AND DISCUSSION

Participants' characteristics: The average age of the participants was 30.88 ± 12.64 years (Table 1). The participation proportion was nearly identical for men and women. Most (59.1%) of the study participants were married, and two-third (67.4%) were students. More than 75% of the participants recorded a monthly income in the range of 5,000–20,000 SAR. While the majority (54.7%) of the respondents indicated that their city had cases of brucellosis infection, most (95.0%) reported not having had a brucellosis infection in the family (Table 1).

Table 2. Distribution of correct responses to knowledge items across responses.

No.	Knowledge items	Correct responses % (n)		
		Responses with or without reported cases of brucellosis		
		With	Without	Total
1	*Brucella* can pass from a sick livestock animal to its milk.[#]	82.8 (82)	90.4 (66)	86.0 (148)
2	*Brucella* can cause serious illness and death.[#]	81.8 (81)	79.5 (58)	80.8 (139)
3	In humans, medications can be used to treat brucellosis.[#]	93.8 (91)	97.2 (70)	95.3 (161)
4	*Brucella* can be killed in dairy products if they are boiled or pasteurized to at least 63°C.[#]	73.7 (73)	69.9 (51)	72.1 (124)
	Mean[#] (0 = no correct answers, 5 = all correct answers; range: 0–5)	3.33	3.36	2.89

[#]Not significant. Tests employed: chi-square for items of the knowledge tool; Mann–Whitney *U* test for the mean knowledge score.

Distribution of knowledge about brucellosis: All four items used to assess the respondents' knowledge of brucellosis were loaded onto a single component that explained 52.8% of the variance. The knowledge subscale had good reliability, as indicated by a McDonald's ordinal omega value of 0.72 and a value of 0.88 for the greatest lower bound to reliability. Table 2 shows the distribution of the correct responses to the four knowledge items for the responses with and without reported cases of brucellosis. The percentage of correct responses (95.3%–72.1%) was high for all four items concerning knowledge of brucellosis. There were no significant differences in the percentage of correct responses for any of the four items in the knowledge subsection for towns with or without cases of brucellosis (Table 2). Younger respondents were associated with greater knowledge (the sum of all the knowledge item scores; $r = –0.41$, $p < 0.001$). In the entire survey, a total of four questions were posed to assess the participants' knowledge of brucellosis. The mean knowledge level for all the participants was 2.89, indicating that the subjects were aware of brucellosis, especially those who were younger. In KSA, the younger population tends to consume unpasteurized dairy products from livestock animals, especially in rural areas. Majmaah city is considered as a rural area with many farms. A population is more likely to be at risk of *Brucella* infection where poor hygiene is practiced in farming areas (Alaidarous 2018; Alqahtani et al. 2021).

Table 3. Attitudes toward the factors that increase *Brucella* transmission.[#]

Items	Mean	SD	Percentage agreeing (scores 4–5) with item
Homemade cheese is tastier than cheese purchased in sealed, certified packaging.	3.23	1.41	40.7
The milk from well-known dairies is not as fresh as milk prepared at home.	3.27	1.44	45.6
Homemade cheeses are free of bacteria because I get them from individuals who keep things clean.	2.69	1.28	26.1
I trust the people I buy cheese from.	2.89	1.30	30.9
Brucella bacteria will be killed if I heat the milk to at least 63°C.	3.39	1.24	45.6
Whether I get sick is a matter of luck; it does not relate to what I eat or drink.	1.67	1.12	8.6
Mean	2.86	0.72	

[#]On a scale of 1–5 (1 = do not agree at all; 5 = agree very much).

Table 4. Behavior associated distribution among responses.

No.	Behavior Items	Correct responses % (n)		
		Responses with or without reported cases of brucellosis		
		With	Without	Total
1	Drinking milk	91.9 (91)	93.2 (68)	92.4 (159)
2	Eating cheese	92.9 (92)	97.3 (71)	94.8 (163)
3	Preparing cheese from unpasteurized milk at home	12.4 (12)	5.5 (4)	9.4 (16)
4	Preparing cheese from pasteurized milk at home	27.3 (27)	26.0 (19)	26.7 (46)
5	Buying milk from unregulated sources[#]	16.2 (16)	11.3 (8)	14.1 (24)
6	Buying cheese from unregulated sources	6.2 (6)	9.6 (7)	7.6 (13)
7	Eating cheeses with unsealed packaging from unregulated sources	9.1 (9)	6.9 (5)	8.2 (14)
8	Eating white cheeses from unregulated sources	8.2 (8)	5.5 (4)	7.0 (12)
9	Drinking unpasteurized milk	15.3 (15)	13.9 (10)	14.7 (25)
10	Asking whether the milk you drink is from a pasteurized source	25.3 (25)	25.0 (18)	25.1 (43)
11	Drinking milk directly from a livestock animal, such as a goat, sheep, camel, or cow	50.5 (50)	38.4 (28)	45.3 (78)
12	Owning or visiting a farm with livestock animals, such as goats, sheep, camels, or cows	46.5 (46)	39.7 (29)	43.6 (75)
13	Experienced slaughtering a livestock animal, such as a goat, sheep, camel, or cow	58.6 (58)	69.4 (50)	63.2 (108)

[#]Not significant. Test employed: chi-square for items of the behavior tool.

Attitudes toward factors enhancing the transmission of brucellosis: All six items used to assess attitudes toward the factors that enhance the transmission of brucellosis loaded onto a single component and explained 39.9% of the cumulative variance. The attitude subscale had satisfactory reliability, as indicated by a McDonald's ordinal omega value of 0.68 and a value of 0.79 for the greatest lower bound to reliability. A small portion (8.6%) of the participating university students and staff showed a deterministic attitude (last item of Table 3). A large percentage of the study participants preferred the cheese and milk products made at home. This was reflected in 40.7% reporting that homemade cheese is tastier and 45.6% answering that home-produced milk products are fresher than those of major dairies (Table 3). A large percentage (45.6%) agreed that heating milk to at least 63°C will

kill *Brucella* bacteria. The unmarried respondents were associated with a higher score in their attitude toward the factors that promote the increased transmission of brucellosis (sum of all the attitudes item scores/no. of items; $r = –0.20$, $p < 0.05$) (Nejad et al., 2020).

The majority of the participants preferred their own cheeses made at home to the pasteurized ones sold in sealed, certified packaging, but many also demonstrated an awareness of the need to heat milk to 63°C to kill pathogens. A handful maintained that sickness and health are matters of fate or luck and that no additional precautionary measures were needed to prevent illness. It was also observed that the unmarried subjects were more likely to pursue activities that promote the transmission of brucellosis. These results are consistent with reports showing that the populations in multiple regions in KSA consume unpasteurized dairy products, especially those from camels, as part of their culture or to demonstrate the absence of fear of possible consequences. This may relate to low knowledge or awareness regarding infection caused by consuming dairy products in these regions (Alaidarous 2018; Alqahtani et al. 2021).

Table 5. Association between knowledge, attitudes, and behaviors regarding the practice of buying and consuming dairy products.

Type of Behavior		Knowledge[a]		Attitude[a]	
		Mean	SD	Mean	SD
Buying milk from unregulated sources	Yes	3.5	0.66	3.19	0.75
	No	3.3	0.87	2.81	0.71
	p	0.33		0.04	
Buying white cheese from unregulated sources	Yes	2.78	1.05	3.04	0.71
	No	3.37	0.82	2.84	0.73
	p	0.02		0.26	
Eating cheeses that come in unsealed packaging from unregulated sources	Yes	3.13	0.80	3.24	0.56
	No	3.33	0.85	2.83	0.73
	p	0.32		0.02	
Eating white cheese bought from unregulated sources	Yes	2.86	1.10	3.15	0.59
	No	3.36	0.82	2.82	0.73
	p	0.07		0.06	

[a] Mann-Whitney U test.

Table 6. Multivariate binary regression models: Predictors of the purchase and consumption of milk and milk products from unregulated sources.

Buying milk from unregulated sources

		Odds ratio	p	Confidence interval
Age (in years)		0.94	0.36	0.81, 1.08
Gender[a]		0.27	0.03	0.08, 0.87
Marital status[b]		1.52	0.68	0.21, 10.84
Job group	Academic	1	-	-
Job group	Administration	0.91	0.94	0.08, 10.48
Job group	Student	0.70	0.71	0.11, 4.63
Income	<5,000–10,000 SAR	1	-	-
Income	10,001–20,000 SAR	0.72	0.66	0.17, 3.09
Income	20,001–30,000 SAR	5.05	0.19	0.45, 56.21
Income	>30,000 SAR	0.88	0.89	0.14, 5.73
Town[c]		1.93	0.30	0.56, 6.60
History of brucellosis infection in the family[d]		0.35	0.50	0.02, 7.18
Knowledge		1.75	0.19	0.76, 4.01
Attitude		2.84	0.01	1.27, 6.37

Hosmer and Lemeshow statistics: $p = 0.27$; Nagelkerke's $R^2 = 0.23$

Buying white cheese from unregulated sources

		Odds ratio	p	Confidence interval
Age (in years)		0.97	0.64	0.83, 1.12
Gender[a]		0.33	0.17	0.07, 1.58
Marital status[b]		0.21	0.15	0.02, 1.78
Job group	Academic	1	-	-
Job group	Administration	0.00	1.00	0.00, 0.00
Job group	Student	1.44	0.74	0.16, 12.96
Income	<5,000–10,000 SAR	1	-	-
Income	10,001–20,000 SAR	0.30	0.25	0.04, 2.33
Income	20,001–30,000 SAR	1.20	0.90	0.08, 17.91
Income	>30,000 SAR	0.88	0.92	0.08, 9.76
Town[c]		0.87	0.84	0.23, 3.36
History of brucellosis infection in the family[d]		1.01	0.99	0.06, 16.52
Knowledge		0.61	0.26	0.26, 1.44
Attitude		1.96	0.19	0.72, 5.32

Hosmer and Lemeshow statistics: $p = 0.79$; Nagelkerke's $R^2 = 0.22$

Eating white cheese bought from unregulated sources

		Odds ratio	p	Confidence interval
Age (in years)		0.97	0.74	0.79, 1.18
Gender[a]		0.15	0.05	0.03, 0.96
Marital status[b]		0.28	0.40	0.02, 5.35
Job group	Academic	1	-	-
Job group	Administration	0.00	1.00	0.00, 0.00
Job group	Student	1.03	0.98	0.07, 15.14
Income	<5,000–10,000 SAR	1	-	-
Income	10,001–20,000 SAR	0.11	0.10	0.01, 1.57
Income	20,001–30,000 SAR	1.22	0.90	0.05, 28.85
Income	>30,000 SAR	0.20	0.35	0.01, 5.67
Town[c]		3.61	0.15	0.63, 20.50
History of brucellosis infection in the family[d]		0.31	0.52	0.01, 10.97
Knowledge		0.87	0.79	0.32, 2.41
Attitude		4.11	0.03	1.12, 15.03

Hosmer and Lemeshow statistics: $p = 0.12$; Nagelkerke's $R^2 = 0.30$

Eating cheeses that come in unsealed packaging from unregulated sources

		Odds ratio	p	Confidence interval
Age (in years)		0.62	0.05	0.39, 1.00
Gender[a]		0.03	0.01	0.00, 0.45
Marital status[b]		0.04	0.11	0.00, 2.03
Job group	Academic	1	-	-
Job group	Administration	0.00	1.00	0.00, 0.00
Job group	Student	0.01	0.03	0.00, 0.65
Income	<5,000–10,000 SAR	1	-	-
Income	10,001–20,000 SAR	0.04	0.09	0.00, 1.69
Income	20,001–30,000 SAR	32.89	0.10	0.53, 2051.03
Income	>30,000 SAR	0.03	0.13	0.00, 2.94
Town[c]		2.55	0.31	0.42, 15.46
History of brucellosis infection in the family[d]		0.16	0.36	0.00, 8.15
Knowledge		1.17	0.79	0.39, 3.53
Attitude dichotomized[e]	No/less agreement	41.42	0.00	4.45, 385.61

Hosmer and Lemeshow statistics: $p = 0.14$; Nagelkerke's $R^2 = 0.52$

[a]Reference group: men
[b]Reference group: not married
[c]Reference group: city with no reported cases
[d]Reference group: no brucellosis infection in the family
[e]Reference group: no/less agreement with the items of the subscale showing attitude toward the factors enhancing the transmission of brucellosis
SAR: Saudi Arabian riyal

Behavior associated with buying and consuming milk products: All 13 items used to assess the purchase and consumption behaviors associated with milk products loaded onto two components and explained 46.7% of the variance. The behavior subscale had excellent reliability, as indicated by a McDonald's ordinal omega value of 0.98 and a value of 0.83 for the greatest lower bound to reliability. Most of the participants or their family members used milk (92.4%) and cheese (94.8%) (Table 4). Of those preparing cheese at home, a small proportion (9.4%) used unpasteurized milk while a relatively higher percentage (26.7%) used pasteurized milk. The purchase of milk (14.1%) and cheese (7.6%) from unregulated sources was low (Table 4). The consumption of cheese from an unsealed package (8.2%) or unregulated sources (7.0%) was also low, while the consumption of unpasteurized milk (14.7%) was substantial. When consuming milk products, more than a quarter of the respondents inquired whether they were made from pasteurized milk. The drinking of milk drawn directly from a livestock animal was very common among the study participants (45.3%), and they commonly owned or visited a farm with livestock animals (43.6%) (Nejad et al. 2020).

The majority (63.2%) of the participants or their family members were experts in slaughtering livestock animals. The analysis of the participants' behaviors associated with the purchase and consumption of milk and milk products revealed that the majority of people consumed milk and milk products. While many of the participants took precautionary measures to prevent the transmission of brucellosis, others did not inquire about the source of their milk or milk products (Nejad et al. 2020).

Moreover, they also did not know whether the milk and cheese they were consuming had been pasteurized. They also purchased milk items from unregulated sources. As many of the participants were students, married, and earning 5,000–10,000 SAR per month, they likely purchased such products to reduce costs. Those who purchased milk from unregulated sources and cheese in unsealed packaging also demonstrated an attitude that promotes the transmission of brucellosis. Studies have shown that the young population in KSA constitute the higher percentage with this attitude. Young people are more outgoing and like to experience new things. Awareness of infectious diseases and how to prevent them therefore needs to be promoted widely (Al Anazi et al. 2019; Nejad et al. 2020).

Association between knowledge, attitudes, and behaviors regarding the purchase and consumption of dairy products: A significantly higher score in attitude toward the factors that promote the transmission of brucellosis (U = 1367.5, p = 0.04) was evident among those who purchased milk from unregulated sources (Table 5) (Alqahtani et al. 2021).

A significantly higher score in attitude toward the factors that promote brucellosis transmission (U = 729, p = 0.02) was also observed in those (or their family members) who consumed cheeses presented in unsealed packaging from unregulated sources (Table 5). Although the participants displayed impressive behaviors, attitudes, and knowledge regarding brucellosis and its transmission, a gap remains in translating that knowledge into practice. Knowledge, if not implemented, cannot reduce instances of brucellosis in KSA. Because milk and milk products form a major part of the Saudi diet, it is important that people know they must purchase dairy and meat items only from well-known, certified sources that properly pasteurize their milk and cheeses (Alaidarous 2018; Alqahtani et al. 2021).

Multivariate analysis: Predictors of the purchase and consumption of milk and milk products from unregulated sources: Four distinct binary logistic regression models were run to identify the predictors of four aspects of the purchase and consumption of milk and milk products from unregulated sources. The model with nine predictors, namely, age, job, gender, knowledge, income, town (with or without cases of brucellosis), history of brucellosis infection in the family, and attitude toward the factors enhancing brucellosis transmission, was significant in comparison to the model with intercepts only for classifying those who reported purchasing milk from unregulated sources: $\chi 2$ (12, N = 153) = 21.4, $p < 0.05$. Male gender and a higher score in attitude toward the factors enhancing the transmission of brucellosis were associated with purchasing milk from unregulated sources (Table 6). This is consistent with previous studies showing that men in KSA have higher cases of *Brucella* infection than women (Alaidarous 2018; Edathodu et al. 2021).

Furthermore, the model with nine predictors (i.e., age, job, gender, knowledge, income, town [with or without cases of brucellosis], history of brucellosis infection in the family, and attitude toward the factors enhancing brucellosis transmission [dichotomized score]) was significant in comparison to the model with intercepts only for classifying those who recorded purchasing and consuming cheeses in unsealed packaging from unregulated sources: $\chi 2$ (12, N = 154) = 39.8, $p < 0.001$. Male gender, younger age, and stronger agreement in attitude toward the factors enhancing the transmission of brucellosis were associated with purchasing milk from unregulated sources (Table 6). The consumption of livestock animals and their products is a cultural practice in the region, with people both drinking cow, sheep, and camel milk and consuming their meat (Alaidarous 2018). However, it seems that the majority of people involved in the supply chain are unaware of this infection and how it can be prevented. The disease has become so prevalent that it is deemed endemic in KSA (Nejad et al. 2020). The primary cause of brucellosis in the country is the significant consumption of milk, yogurt, cheese, and meat products. Many people are unaware of the concept of pasteurization, in which food items are heated to a specific temperature to halts all pathogenic activity, thus making it safe to consume meat and dairy products (Alaidarous 2018; Nejad et al. 2020).

CONCLUSION

Although there has been a reduction in brucellosis cases in KSA, the findings of the present study support the need to encourage people in KSA to cook their meat products

thoroughly, so that the chances of infection can be reduced even further. Furthermore, in closing the gap between knowledge and practice, it is important to include all those in the supply chain who deal with dairy and poultry products at various stages so that not only consumers but also manufacturers can play a role in preventing the transmission of brucellosis.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Production of Total Reducing Sugars from *Bambusa balcooa* through Oxalic Acid Pretreatment

S. Sivamani,[1]* R. Kaveri[2] and S. Umaa Nandhini[2]

[1]Engineering Department, University of Technology and Applied Sciences, Salalah, Oman

[2]Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, India

ABSTRACT

This study aims to assess the applicability of organic acid pretreatment on culm of *Bambusa balcooa* for the production of maximum total reducing sugars. The experiments were performed by varying organic acids (Formic, acetic, pyruvic, maleic, malic, tartaric, adipic, citric and oxalic acids), concentration of oxalic acid (1-4% (w/v)), solvent to solid ratio (5-40 mL/g), pretreatment time (0-60 min) and temperature (75-135 ^{0}C) to maximize total reducing sugars (TRS) concentration using fractional factorial design based one-factor-at-a-time (OFAT) approach. Total reducing sugars concentration was estimated by using 3,5-dinitro salicylic acid (DNSA) method. Among various organic acids, oxalic acid exhibited higher reducing sugars concentration. The optimal values show that the maximum TRS of 48.33 g/L was achieved at oxalic acid concentration, solvent to solid ratio, time and temperature of 3% (w/v), 10 mL/g, 15 min and 121 ^{0}C for the pretreatment of cassava stem with oxalic acid. A low value of coefficient of variation (CV = 0.32%) showed that the optimal conditions were validated by experiments. Thus, *B. balcooa* could be used as a potential feedstock for the production of TRS by organic acid pretreatment.

KEY WORDS: BAMBOO, FRACTIONAL FACTORIAL DESIGN, OXALIC ACID, PRETREATMENT, TOTAL REDUCING SUGARS.

INTRODUCTION

Biofuels are major sustainable alternative to petroleum-based fuels due to current reliance on supplies from the organization of petroleum exporting countries, increased emissions of greenhouse gases in the atmosphere and depletion of oil reserves (Zahan and Kano 2018). Bioethanol produced from renewable resources emits fewer gases than fossil fuels and reduces the burden of carbon dioxide emissions to the atmosphere (Handler et al. 2016). When bioethanol is blended with petrol, the fuel mixture is oxygenated and burns more completely and reduces polluting emissions (Wu et al. 2020). Although bioethanol can be produced by chemical route, it is majorly produced through fermentation of sugar (Devi et al. 2021). The energy stored in the plants in the form of sugar is utilised for bioethanol production (Thatoi et al. 2016; Wu et al. 2020; Devi et al. 2021).

First generation biofuels are produced from starch and sugar. But it requires food crops such as sugarcane, corn, wheat, and sugar beet. Using food crops as raw material, first generation bioethanol threatens food supplies and biodiversity (Anushya et al. 2019; Sivamani et al. 2020). The alternative cheaper and polysaccharide-rich sources is required to explore as raw materials for bioethanol production to reduce the fuel-food conflicts, increase the available raw materials, and produce economically competitive with petroleum-based fuels. Thus, second generation biofuels can help solve the problems created by first generation biofuels (Vanitha et al. 2017). Second generation biofuels utilize lignocelluloses derived feedstocks that are abundant and less utilized renewable resources. These include the residues from agriculture and forestry (sugarcane bagasse, corn stover, straw, etc.) and energy crops (Chandrasekaran and Sivamani 2018; Sivamani et al. 2020). The plant residues consist of stems, leaves and husks of non-food crops. Several countries including South Africa are currently engaged in major research projects studying the utilization of lignocellulosic materials to produce bioethanol (Bensah et al. 2015; Sivamani et al. 2018). The lignocelluloses compound is rich in cellulose and hemicellulose, which are covered by lignin (Sivamani et al. 2021).

Lignocellulosic materials are hence recalcitrant to hydrolysis (saccharification) and require several steps before they are converted to bioethanol which makes the process somewhat complex (Chandrasekaran et al. 2017). During pretreatment, plant cell walls were pretreated to break the

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lignocellulosic matrix. Then, hemicellulose and cellulose present in lignocellulosic materials hydrolysed to their monomers, xylose and glucose predominantly. Finally, the monomeric sugar units were fermented using ethanologenic organisms to bioethanol (Sivamani et al. 2018; Sivamani et al. 2021).

Bamboo-based residues are one of the lignocellulosic materials that can be used as a feedstock for bioethanol production due to the relatively higher growth rate of the plants, their abundancy and availability in the tropics (Alzagameem et al. 2019). Bamboo is specifically utilised as a building material where the wood plays a major role (Shen et al. 2019). Bamboo plants are found notably in South Asia, Southeast Asia and East Asia for the economic and cultural significance used for building materials, as a food source and versatile raw material (Sathishkumar et al. 2020). Bambusa balcooa is an evergreen bamboo forming a dense clump of erect, woody stems. This species is one of the most important village bamboos used for construction.

The plant is widely cultivated on a small scale in Northeast India and Bangladesh and occasionally also outside this region (Banik 2015; Banik 2016; Sathishkumar et al. 2020). Tang et al. (2021) examined the potential *Bacillus velezensis* LC1 for degradation of polymers in bamboo to monomeric sugar units. The, they subjected the hydrolysate to ethanolic fermentation with *Saccharomyces cerevisiae* and *Escherichia coli* KO11. The degradation efficiencies were found as 59.90, 75.44 and 23.41% for cellulose, hemicellulose and lignin, respectively, and the ethanol yield was achieved at 10.44 g/L after 96 h. Yang et al. (2019) investigated the alkaline liquid hot water pretreatment of a bamboo species, *Neosinocalamus affinis*, by examining the effect of temperature and alkali dosage. Bioethanol yield of 4.8 g/L was achieved by separate hydrolysis and fermentation (SHF) at 0.5% (w/v) NaOH 1t 170 ^{0}C (Sathishkumar et al. 2020).

From the analysis of literature, the various pretreatment methods such as bacterial degradation, alkaline liquid hot water, modified alkaline hydrogen peroxide, autohydrolysis, alkaline extraction, steam explosion, green liquor (mixture of Na2S and Na_2CO_3), ultra-high-pressure explosion, hydrothermal, and chemical (acid or alkali) treatment (Li et al. 2015; Dai et al. 2020). Organic acid pretreatment was employed for cassava stem, corncob, wheat straw, Napier grass, water hyacinth and so on (Kootstra et al. 2009; Amnuaycheewa et al. 2017; Sivamani and Baskar 2018; Qiao et al. 2019; Tantayotai et al. 2019). But only limited literature is available on organic acid pretreatment of bamboo biomass (Li et al. 2014; Sindhu et al. 2014; Sathishkumar et al. 2020). Hence, in the current study, culm from B. balcooa was evaluated as a feedstock for organic acid pretreatment by varying different organic acids, organic acid concentration, solid to liquid ratio, pretreatment time and temperature.

MATERIAL AND METHODS

Culm from *Bambusa balcooa* was collected from Forest College and Research Institute, Mettupalayam (Longitude 11.19'N, Latitude of 77.56'E), Coimbatore district. Sulphuric acid, sodium hydroxide, acetic acid, trichloroacetic acid, oxalic acid, citric acid, tartaric acid, adipic acid, formic acid, malic acid, maleic acid, xylose, 3,5 dinitro salicylic acid, crystalline phenol, sodium sulphite, sodium hydroxide, potassium sodium tartrate, ethanol, toluene, glacial acetic acid, sodium chlorite , acetyl bromide, perchloric acid, nitric acids were procured from Finar chemicals Ltd. and HiMedia Laboratories Pvt. Ltd. *B. balcooa* was characterized for lignin, cellulose, hemicellulose and ash by using standard operating procedure reported elsewhere (Chandrasekaran et al. 2017). Different organic acids such as formic acid, acetic acid, malic acid, maleic acid, adipic acid, trichloro acetic acid, lactic acid, tartaric acid, oxalic acid, and citric acid were taken separately for the pretreatment.

30 mL of 1% (w/w) organic acid concentration was added to 3 g of culm of B. balcooa. The mixture was cooked in the domestic pressure cooker at 121 ^{0}C for 15 min. Then the samples were made up to 100 mL and total reducing sugars (TRS) content was estimated following dinitrosalicyclic acid (DNSA) method (Miller 1951). Due to higher productivity of sugars, oxalic acid was selected for further pretreatment. Oxalic acid solution was prepared in different concentrations (1, 2, 3 and 4% (w/v)). 30 mL of 1% (w/v) oxalic acid was added to 3 g of the sample. Pretreatment was carried out in the domestic pressure cooker at 121 ^{0}C for 15 min. These steps were repeated for 2, 3 and 4% oxalic acid solution (Pandian et al. 2016). Then, the samples were diluted to 100 mL and TRS content was estimated following dinitrosalicyclic acid (DNSA) method.

Solid to liquid ratio was varied by varying volume of solvent from 15, 30, 60, 90 and 120 mL for 3 g of the sample. 3 g of the culm sample was mixed with 15 mL of the 3% (w/v) oxalic acid solution was added. Pretreatment was carried out in the domestic pressure cooker at 121 ^{0}C for 15 min. These steps were repeated for 30, 60, 90 and 120 mL of 3% (w/v) oxalic acid solution. Then, the samples were diluted to 100 mL and TRS content was estimated following dinitrosalicyclic acid (DNSA) method. The pretreatment time was varied from 10, 20, 30 and 40 min for pretreatment of culm from *B. balcooa*. 30 mL of 3% (w/v) oxalic acid solution was prepared and added to 3 g of the culm sample. Pretreatment was carried out in the domestic pressure cooker at 121 ^{0}C for 15 min. These steps were repeated for 30, 45 and 60 min of pretreatment. Then, the samples were diluted to 100 mL and TRS content was estimated following dinitrosalicyclic acid (DNSA) method. The pretreatment temperature was varied from 75, 90, 105, 121 and 135 ^{0}C for pretreatment of culm from B. balcooa. 30 mL of 3% (w/v) oxalic acid solution was prepared and added to 3 g of the culm sample. Pretreatment was carried out in the domestic pressure cooker at 121 ^{0}C for 15 min.

These steps were repeated for 75, 90, 105 and 135 ^{0}C of pretreatment. Then, the samples were diluted to 100 mL and TRS content was estimated following dinitrosalicyclic acid (DNSA) method. The experiments were performed in triplicate under optimized conditions to validate the optimal conditions. Optimal experiment was performed by mixing 30 mL of 3% (w/v) oxalic acid solution and 3 g of

the sample. Pretreatment was carried out in the domestic pressure cooker at 121 ^{0}C for 15 min. Then, the samples were diluted to 100 mL and TRS content was estimated following dinitrosalicyclic acid (DNSA) method.

RESULTS AND DISCUSSION

Biochemical characterization of *B. balcooa*:

Table 1 shows the biochemical characterization of culm from *B. balcooa*. The results reveal that it contains 20% lignin, 48% cellulose 23% hemicellulose and 2.2% ash on a dry weight basis. The hemicellulose accounted to 23%, which is higher than hemicellulose obtained in Hongbin et al. (2014). The cellulose content (48%) is higher than Li et al. (2012) and Tippayawong et al. (2011) that has made *B. balcooa* suitable for ethanol production.

Organic acid pretreatment of *B. balcooa*: Formic acid, acetic acid, malic acid, maleic acid, adipic acid, trichloro acetic acid, lactic acid, tartaric acid, oxalic acid, and citric acid were used for the pretreatment of *B. balcooa*. Table 2 shows the pKa values of various oxalic acids used in this study. pKa represents dissociation constant of acid that describe the acidity of a particular molecule. It can be calculated from Henderson-Hasselbalch equation. The smaller the pKa value, strong acids have weak conjugate bases. Organic acids with single carboxylic group have one pKa value and acids with multiple carboxylic acid groups have multiple carboxylic values (Adcock 2001; Sathishkumar et al. 2020).

Table 1. Biochemical characterization of *B. balcooa*

Lignin	Hemicellulose	Cellulose	Ash	References
22.0	24.7	44.4	2.4	Vena et al. 2010
22.1	22.9	NA	NA	Hongbin et al.,2014
24.29	21.60	37.21	1.41	Li et al. 2012
27.1	26.5	40.7	1.2	Tippayawong et al. 2011
20	23	48	2.2	Present study

NA – Not available

Table 2. pKa values of different organic acids

Organic acid	pKa1	pKa2	pKa3
Formic acid	3.8	-	-
Acetic acid	4.75	-	-
Pyruvic acid	2.39	-	-
Oxalic acid	1.25	4.23	-
Maleic acid	2	6.25	-
Malic acid	3.4	5.11	-
Tartaric acid	2.89	4.4	-
Adipic acid	4.4	5.4	-
Citric acid	3.14	4.77	6.30

Figure 1: Effect of various organic acids on pretreatment of *B. balcooa*

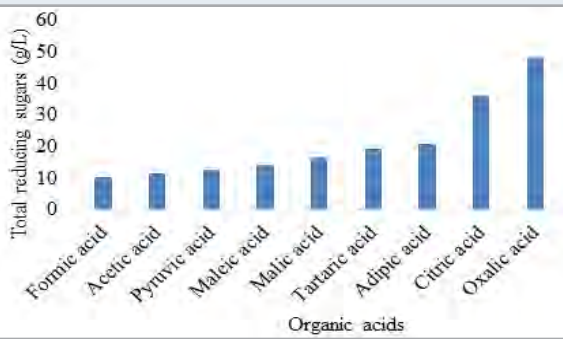


Figure 1 illustrates about the pretreatment of *B. balcooa* using various organic acids. Among nine different acids attempted, oxalic acid pretreated culm sample produced 48.3 g/L of reducing sugars. Hence, oxalic acid was selected for further experiments of pretreatment for maximum production of TRS. Li et al. (2014) reported that sulphuric acid, oxalic acid, and formic acid produced 56.46%, 56.68% and 61.64% of glucose, respectively, with sulphuric acid being generated higher amount of fermentation inhibitors than the samples pretreated with oxalic and formic acids (Sindhu et al. 2010; Sathishkumar et al. 2020).

Effect of concentration of oxalic acid on pretreatment of *B. balcooa*: Figure 2 exhibits the impact of concentration of oxalic acid on pretreatment of *B. balcooa*. When the concentration of oxalic acid solution increased from 1 to 3% (w/v), the concentration of TRS increased from 18.1 to 48.3 g/L. When the oxalic acid solution concentration exceeds 3% (w/v), the concentration of TRS remained constant. The TRS concentration does not exhibit significant variation with an increase in concentration of oxalic acid solution beyond 3% (w/v) (Jeong and Lee 2016; Sathishkumar et al. 2020).

Effect of solvent to solid ratio on pretreatment of *B. balcooa*: Figure 3 illustrates the influence of solvent to solid ratio on pretreatment of *B. balcooa*. When the solvent was not added to the feed mixture, the concentration of TRS was minimum at 10.3 g/L. When the solvent to solid ratio was increased to 10 mL/g, the TRS concentration was increased to 48.2 g/L. When the oxalic acid solution concentration

exceeds 10 mL/g, the concentration of TRS dropped as the volume of solvent increases or solid loading decreases. The TRS concentration exhibits declination with an increase in solvent to solid ratio beyond 10 mL/g (Song et al. 2020).

Figure 2: Effect of concentration of oxalic acid on pretreatment of *B. balcooa*

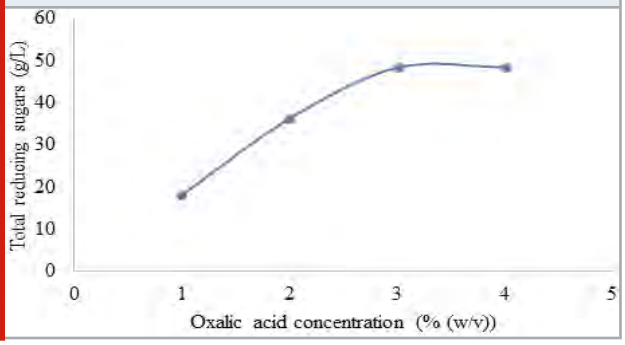
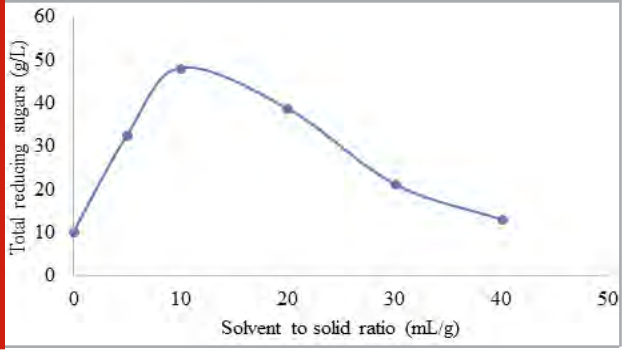


Figure 3: Effect of solvent to solid ratio on pretreatment of *B. balcooa*



Effect of time on pretreatment of *B. balcooa*: Figure 4 exhibits the impact of time on pretreatment of B. balcooa. As the pretreatment progresses, the concentration of TRS increases to 48.4 g/L. When the pretreatment time exceeds 15 min, the concentration of TRS dropped as the TRS further form furfural and other similar compounds. The TRS concentration demonstrates declination with an increase in pretreatment time beyond 15 min (Yuang et al. 2019; Sathishkumar et al. 2020).

Figure 4: Effect of time on pretreatment of *B. balcooa*

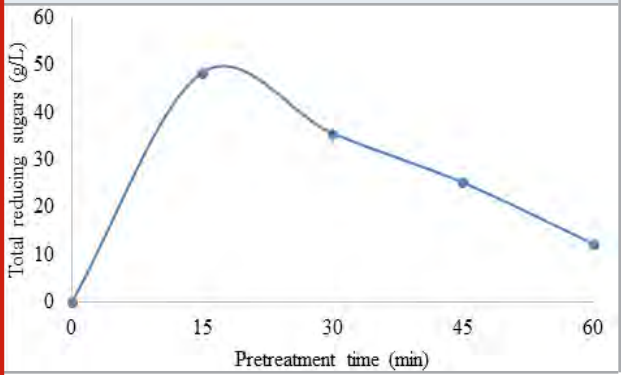


Figure 5: Effect of temperature on pretreatment of *B. balcooa*

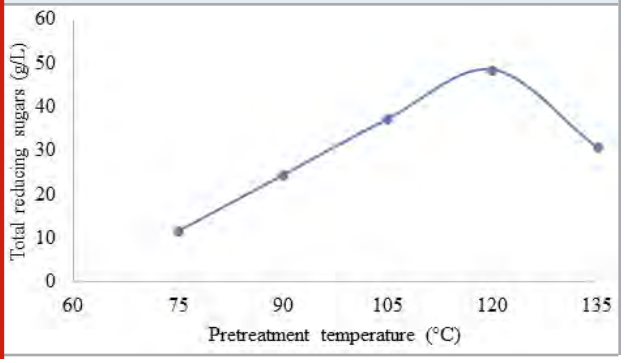


Table 3. Optimal conditions for the pretreatment of *B. balcooa* with oxalic acid

Run no.	Oxalic acid concentration (% (w/v))	Solvent to solid ratio (mL/g)	Time (min)	Temperature (^{0}C)	TRS concentration (g/L)	Mean	Standard deviation
1	3	10	15	121	48.3	48.33	0.15
2	3	10	15	121	48.5		
3	3	10	15	121	48.2		

Effect of temperature on pretreatment of *B. balcooa*: Figure 5 exhibits the influence of temperature on pretreatment of *B. balcooa*. When the pretreatment temperature increased from 75 to 121 ^{0}C, the concentration of TRS increased from 11.6 to 48.5 g/L. When the temperature exceeds 135 ^{0}C, the concentration of TRS decreases to 30.8 g/L. The TRS concentration exhibits declination with an increase in temperature beyond 121 ^{0}C (Huang et al. 2020).

Oxalic acid pretreatment of *B. balcooa* under optimized conditions: The optimal conditions were confirmed by performing experiments in triplicate under optimal conditions (Table 3). The mean±standard deviation between the TRS concentration obtained from the experiments was found to be 48.33±0.15 g/L. A low value of coefficient of variation (CV = 0.32%) showed that the optimal conditions were validated by experiments (Sathishkumar et al. 2020). Table 4 shows the various pretreatment methods employed for bamboo and compared the results obtained in the present study with the previous literature.

Table 4. Pretreatment methods employed for bamboo

Pretreatment methods	Process	Outcomes	Reference
Modified alkaline hydrogen peroxide	Simultaneous saccharification and fermentation	1 ton of ethanol produced per 5.6 ton of bamboo	Huang et al. (2020)
Autohydrolysis and alkaline extraction	Sequential two-stage pretreatment and fermentation	0.467 g ethanol per g hydrolysate	Yuan and Wen (2017)
Steam explosion followed by green liquor	Simultaneous saccharification and fermentation	20.3% ethanol yield	Gao et al. (2021)
Ultra-high-pressure explosion	Simultaneous saccharification and fermentation	Theoretical ethanol yield percentage of 89.7-95.1%	Jiang et al. (2016)
Oxalic acid pretreatment	-	48.33 g/L	Present study

CONCLUSION

The findings of the present study aimed to utilize *B. balcooa* as a potential feedstock to produce TRS by organic acid pretreatment. Oxalic acid produced maximum total reducing sugars among other carboxylic acids. The optimal values show that the maximum TRS of 48.33 g/L was achieved at oxalic acid concentration, solvent to solid ratio, time and temperature of 3% (w/v), 10 mL/g, 15 min and 121 ^{0}C for the pretreatment of cassava stem with oxalic acid. Thus, *B. balcooa* could be used as a potential feedstock for the production of TRS by organic acid pretreatment.

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Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

Author Contribution Information: S. Sivamani: Conceptualization, Methodology, Writing - review & editing; R. Kaveri: Methodology, Investigation, Writing - Original draft; S. Umaa Nandhini: Methodology, Investigation, Writing - Original draft;

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On the Efficacy of the Gene, Juxtaposed with Another Zinc Finger Protein 1 (JAZF1) in the Development of Type 2 Diabetes Mellitus among Indians

Yamini Goyal, Amit K. Verma and Kapil Dev*
Department of Biotechnology, Jamia Millia Islamia, New Delhi, India.

ABSTRACT

Type 2 diabetes mellitus (T2DM) is a chronic disorder characterized by pancreatic beta-cell dysfunction and insulin resistance. The present study was designed to understand the association of genetic variations in the JAZF1 gene with T2DM in the Indian population. The polymorphic study was conducted by PCR-RFLP methods. Further, the biochemical parameters were collected for statistical analysis on the semi-structured questionnaire, and correlation with the polymorphism was done by using SPSS software. The significant differences were observed between T2DM cases and controls in triglycerides, HbA1c, T-cholesterol, LDL-C, BMI, systolic and diastolic BP, PPG, FPG, while no significant differences were observed in HDL-C, WHR. Our results suggested that the JAZF1 rs864745 variant is significantly associated with T2D among the Indian population. The present study concludes that the association of genetic variations and biochemical factors play a vital role in T2DM risk and its prevalence.

KEY WORDS: HYPERGLYCAEMIA, INDIAN POPULATION, JAZF1, TYPE 2 DIABETES MELLITUS.

INTRODUCTION

During the last couple of decades, the prevalence of diabetes has increased drastically all over the world and now diabetes disease has become a worldwide public health problem. According to the International Diabetic Federation (IDF-2017), a total of 8.8% of the World's population was suffering from diabetes and this population of 425 million is estimated to further increase to 628.6 million people by (2045). Diabetes has established itself as one the fastest growing disease in humans and has become an epidemic with a 48% increase in the last 30 years. Its prevalence has continuously increased in the adults 20-79 years' age group from 151 million in (2000), to 285 million in (2009) to 382 million in 2013 and 424.9 million in (2017) (Zimmet 2017; Brussels and Belgium 2019).

In India, 72.9 million people are suffering from diabetes and by (2045) this patients count is expected to be 134.3 million. Diabetes accounts for high morbidity and mortality due to complications like renal failure, amputations, cardiovascular disease, and cerebrovascular events (Schulze et al. 2004; Park et al. 2020). Nearly half of those affected are undiagnosed. Furthermore, among all major ethnic groups, Asian Indians have one of the highest incidences of pre-diabetes and diabetes, and the transition from pre-diabetes to diabetes occurs more quickly in this community (Anjana et al. 2011). Long-term diabetes has major problems, some of which are fatal (Alam et al. 2021).

The successful discovery of common (SNPs) contributing to diabetes susceptibility has been made possible by technological advances in molecular biology. Genome-wide techniques, such as (GWAS), have found statistically significant links between certain genetic regions and T2DM risk (Basile et al. 2014). In humans, several JAZF1 single nucleotide polymorphisms (SNPs) have been linked to T2DM and IR-related disorders. The replacement of Asparagine for Alanine is caused by a well-known missense mutation of rs1635852 (C to T substitution). The rs1635852-T risk allele in JAZF1 was linked to T2DM in meta-analysis research involving approximately 1 million participants (Fogarty et al. 2013; Mahajan et al. 2014). Patients with the T risk allele had lower JAZF1 mRNA expression and higher protein complex binding (Fogarty et al. 2013). It's worth noting that T risk allele carriers had lower insulin secretion due to transcriptional suppression of PDX1, a key transcription regulator for beta-cell formation and regeneration (Zhu et al. 2017).

Another explanation is that rs1635852 mutations cause insulin exocytosis to be reduced by binding to miR-96 (Li et al. 2016). However, not only is there a scarcity of information about the rs864745 variant of JAZF1, but its relationship

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to T2DM in the Indian population is also unknown. The goal of this case-control study was to see if the JAZF1 variation polymorphism (rs864745) was associated with an increased risk of T2DM. Thus, the present study analyses the significance of the association of genetic of JAZF1 gene polymorphism in the risk of development T2DM as well as the correlation with the clinical biochemical parameters in the Indian population.

MATERIAL AND METHODS

The present study includes a total of 300 persons and was conducted at Medical Biotechnology Laboratory, Dept. of Biotechnology, Jamia Millia Islamia (JMI) (A Central University), New Delhi, amongst these 300 individuals, 200 individuals were newly diagnosed T2DM cases the and remaining 100 healthy controls. Patients with T2DM and healthy controls were chosen on basis of the inclusion and exclusion criteria. This study was performed only after the due approval of the institutional ethical committee, JMI, New Delhi. Patients included in the present study were examined and collection of samples was done only after informed consent from all study participants. Information of patients was taken in standardized questionnaires. Isolated DNA was amplified to determine JAZF1 rs864745 (A/G) genotype by a particular set of primers; forward primer: 5'- GAGCCATATAAGTGATGCTCAAA-3' and reverse primer: 5'- GGTTGTCAGGCTTTCCATGT-3' using thermal cycler. The amplified DNA product of 378 bp was viewed with an ultraviolet (UV) transilluminator. JAZF1 rs864745 (A/G) polymorphism was identified by the SSPI restriction enzyme identifying the sequence of DNA. DNA band showed 378 bp A allele- uncut, G allele- 338 + 40 bp. Frequencies of genotypes between patients (cases) and healthy individuals were assessed by Chi-square test and those values which were <0.05, were evaluated by Fisher-exact test (Gong et al. 2021).

The link between JAZF1 rs864745 (A/G) and T2DM risk was projected by calculating the odds ratios (OR) value with 95% confidence intervals (CI). P-value <0.05 considered significant (Morris 2018). The study has been conducted only after the due clearance and approval from the Ethics committee of Jamia Millia Islamia (vide Proposal No. 26/11/273/JMI/IEC/2019). As part of the mandatory standardized ethical norm, written informed consent was taken from the person before inclusion in the research work.

RESULTS AND DISCUSSION

Genotype and allele frequencies of JAZF1 gene polymorphism in patients and controls: Table 3.1 illustrates genotypes and alleles frequencies, odds ratios, 95% confidence intervals, and P values for the three 'JAZF1gene polymorphism among T2D patients and controls. JAZF1 (rs864745) showed a high percentage of Homozygous mutant GG 55 (27.5%) in patients as compared to controls GG 10 (10%A). A 2% difference was observed in the case of heterozygous AG in patients (28%) as compared to control (26%). A low percentage of homozygous AA (44.5) was observed in patients as compared to controls (64%). The odds ratio of JAZF1 genotype AG (heterozygous) and GG (Mutant Homozygous) with AA (wild homozygous), 1.54 (0.88-2.72), and 3.95 (1.87-6.70) were observed, respectively. We observed a significant difference in the frequency of risk allele 'G' among patients and controls (p<0.0001).

Table 3.1. Genotypic and allelic frequencies of JAZF1 gene polymorphism among T2D cases and controls.

Gene/SNP ID	Genotype/ Allele	Cases (n=200)	Control (n=100)	Odd Ratio (95% CI)	P-value
JAZF1 (rs864745)	AA	89 (44.5%)	64 (64%)	Ref	Ref
	AG	56 (28%)	26 (26%)	1.54 (0.88 - 2.72)	0.12
	GG	55 (27.5%)	10 (10%)	3.95 (1.87 – 6.70)	<0.001*
	P-value < 0.001*				
	A (Normal allele)	234 (58.5%)	154 (77%)	2.37 (1.61 - 3.48)	<0.0001*
	G (Risk allele)	166 (41.5%)	46 (23%)		

Note: * = P-value < 0.05 considered significant.

The frequencies, odds ratios, and P-values of the JAZF1 (rs864745) genotypes among T2D patients and control subjects under dominant and recessive models: Table 3.2 shows the frequencies, OR and, p-values of dominant and recessive models of JAZF1 (rs864745) among T2D patients and controls. There is a significant difference was observed between the two groups under the dominant and recessive models (p-value < 0.001).

Comparative analysis of the Biochemical parameters in T2D cases and controls: Diabetes is a multifactorial disorder and along with genotype, different factors come into play to develop this condition. Tables 3 illustrates comparative analysis of biochemical factors of T2D patients and controls in Delhi population. A significant association was observed in all levels among different JAZF1 genotype except HDL-C and WHR.

Table 3.2. Frequencies, OR, and p-values of dominant and recessive models of JAZF1 (rs864745) among T2D patients and controls

Model	Genotype/ Allele	Cases (n=200)	Control (n=100)	Odd Ratio (95% CI)	P-value
Recessive	GG	55	10	3.41 (1.65 – 7.03)	<0.0001*
	AG + AA	145	90		
Dominant	AG + GG	111	36	2.2 (1.35 – 3.63)	<0.001*
	AA	89	64		

Note: * = P-value < 0.05 considered significant.

HbA1c - haemoglobin A1c test, LDL - Low density lipoprotein, HDL – High-density lipoprotein, BMI - Body mass index, BP - Blood pressure, PPG - Postprandial plasma glucose, FPG - Fasting plasma glucose, WHR – Waist to Hip Ratio.

GWAS has been identified in more than 150 different loci associated with type 2 diabetes (Suzuki et al. 2019). Environmental factors are associated with T2DM onset which includes inactive/sedentary lifestyle, obesity, and stress (Adeghate et al. 2006). We analyzed various demographic and clinical parameters and significant differences were observed between T2DM patients and healthy controls in triglycerides, HbA1c, T-cholesterol, LDL-C, BMI, systolic and diastolic BP, PPG, FPG, while no significant differences were observed in parameters such as HDL-C, WHR, among T2DM patients and controls (Alam et al. 2021).

Table 3.3. Comparative analysis of clinicopathological parameters among T2D patients and controls.

Factors	T2DM patients	Controls	P-values
Number	200	100	--
Age (in years)	42.4 ± 9.3	40.7 ± 8.2	
Triglycerides (mg/dl)	347.7 ± 98.5	139.7 ± 6.1	<0.001*
HbA1c	7.9 ± 0.9	5.5 ± 0.7	<0.001*
T-Cholesterol (mg/dl)	242.6 ± 14.8	151.3 ± 19.1	<0.001*
LDL-C (mg/dl)	189.4 ± 28.7	104.3 ± 20.1	<0.001*
HDL-C (mg/dl)	47.1 ± 10.9	45.9 ± 9.1	0.180
BMI (kg/m2)	29.9 ± 4.9	25.1 ± 1.9	<0.001*
Systolic BP (mmHg)	144.9 ± 16.8	105.7 ± 9.9	<0.001*
Diastolic BP (mmHg)	101.9 ± 16.1	76.1 ± 11.2	<0.001*
PPG	229.8 ± 36.2	135.8 ± 4.9	<0.001*
FPG (mg/dl)	160.4 ± 25.7	89.8 ± 6.9	<0.001*
WHR	1.0 ± 0.2	1.0 ± 0.1	1.0

Note- Data presented as Mean ± SD; P-value (*) <0.05 considered significant.

According to the CARRS (Centre for Cardio-metabolic Risk Reduction in South Asia) Study, the total prevalence of diabetes in three major cities in South Asia was 22.8 percent (21.5-24.1 percent), 25.2 percent (23.6-26.8 percent), and 16.3 percent (15.2-17.3 percent) (Deepa et al. 2015). Diabetes prevalence varies significantly depending on where you live (less in rural areas) and your socioeconomic status (less in low socio-economic stratum). Diabetes prevalence ranged from 3% in rural Jharkhand, east India, to 13.7 percent in metropolitan Tamil Nadu, south India, according to the ICMR-INDIAB research (Anjana et al. 2011). Men (3.33 per 1000 per year) have been reported to have a faster rate of increase in diabetes prevalence than women (0.88 per 1000 per year) (Mishra and Khurana, 2011).

The human JAZF1 gene has five exons and is found on chromosome 7p15.2. JAZF1 is a 243-amino-acid protein with a predicted molecular mass of 27 kDa. JAZF1 is made up of three zinc-finger domains with a repeating Cx(4)C2H and a ligand-binding domain (residues 341-583) (Koontz et al. 2001; Nakajima et al. 2004). Endometrial stromal tumors are linked to chromosomal abnormalities involving this gene (Koontz et al. 2001). Different protein isoforms are encoded by alternatively spliced variants, which have been

described. Not all varieties, however, have been adequately characterized (Alam et al. 2021).

The human JAZF1 protein shares 90% homology with that of chimps, monkeys, mice, and pigs, implying that JAZF1 may have a comparable biological function in diverse species (Yang et al. 2015). In the present study, we examined the association of gene polymorphism in the JAZF1 gene to the risk of T2DM in the Indian population. JAZF1 rs864745 (A/G) variant association with T2DM has been reported by various studies among numerous populations (Koontz et al. 2001; Nakajima et al. 2004). Besides lifestyle and environmental risk factors, type 2 diabetes mellitus also has an established genetic predisposition (Zhang et al. 2019; Alam et al. 2021).

Our results suggested that the JAZF1 rs864745 variant is significantly associated with T2D among the Indian population. It was observed that GG genotype frequency was significantly higher in T2DM cases as compared to healthy controls. Allelic frequency of G allele was higher in T2DM cases in comparison to healthy controls. We found significant relations of JAZF1 polymorphism and T2DM risk under dominant and recessive models. Similar results were observed in studies conducted among Chinese and Iranian populations (Han et al. 2010; Soltanian et al. 2020; Alam et al. 2021). The link between the rs864745 variation and T2DM, as well as the mechanism behind it, has been explored. The probability of developing T2DM was 2.32 times higher among Uyghurs with the rs864745-C risk allele (Song et al. 2015). Subjects with the rs864745-T risk allele, on the other hand, were found to have a lower risk of GDM (Stuebe et al. 2014). Based on mechanism, rs864745-T polymorphisms in the JAZF1 gene are linked to lower JAZF1 mRNA expression and insulin secretion (Grarup et al. 2008; Zano et al. 2020).

Increased fasting plasma insulin concentration is connected to rs864745-T polymorphisms, according to an autosomal genomic scan (Grarup et al. 2008). Several published research, on the other hand, have discovered a link between the rs864745 gene variant and T2DM-related illnesses. To begin, the rs864745-T variant of the JAZF1 gene is substantially linked to arteriolosclerosis in neuropathologic studies (Chou et al. 2013). The Saudi population with the JAZF1 rs864745-G risk allele had lower BMI and waist circumference (Alharbi et al. 2015). The G-risk allele has also been linked to T2DM and lowered eGFR, which is consistent with lower JAZF1 gene expression in the peripheral blood of DN patients (Chen et al. 2013; Peng et al. 2017). Several studies have already been published that show a link between the JAZF1 (rs864745) mutation and T2DM, particularly in industrialized nations, but additional research is needed to use this gene as a biomarker (Zano et al. 2020). Environmental, metabolic, and genetic factors all play a role in the development of T2DM, according to previous research (Geng and Huang 2020).

CONCLUSION

The findings of the present study concluded that JAZF1 gene polymorphism was found to be associated with the risk of T2DM in the Indian population. Our study concludes association of genetic and biochemical factors plays a significant role in potential risk associated with the prevalence of T2DM and the JAZF1 gene may increase the severity of T2DM specifically in the Indian Population.

Conflict of Interests: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

Author Contributions: Conceptualization was carried out by KD; Methodlogy, YG Formal analysis, YG Data Curation, YG Wrote original draft, YG, AKV; Review and editing, KD, YG, AKV; Supervision, KD.

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Feeding Behavior of Blackbuck, Chinkara and Spotted Deer in Captivity at Lal Sohanra National Park Bahawalpur, Pakistan

Saddam Hussain,[1] Irfan Ashraf,[1] Rabia Mehboob,[1] Sehrish Rana Rajpoot,[2] Muhammad Wasim Tasleem*[3] and Esha Gulfreen[4]

[1]Department of Forestry, Range and Wildlife Management, University College of Agriculture and Environmental Sciences, Islamia University of Bahawalpur, Bahawalpur, Pakistan

[2]University College of Conventional Medicine, Islamia University Bahawalpur, Bahawalpur, Pakistan

[3]Department of Zoology, Islamia University Bahawalpur, Bahawalpur, Pakistan

[4]Department of Zoology, University of Agriculture Faisalabad, Faisalabad, Pakistan

ABSTRACT

Lal Suhanra National Park is one of the important protected areas of Pakistan. From many years endangered species of deer are being raised in captivity at Lal Suhanra National Park. In this study, we have observed the blackbuck, chinkara and spotted deer which are highly endangered. The the findings of study showed that blackbuck, chinkara and spotted deer eat daily any of the seasonal grasses like e.g., Maize (Zea mays), Jantar (Sesbania bisbinosa), Berseem (*Trifolium alexandrinum*) Bajra (*Pennsietum glaucum*) 4 to 6 kg, 3-5 kg and 4-5 kg respectively. Softened parts of the plants were also being eaten, which included Jandi (*Prosopis spicigera*), Kikar (*Acacia* sp.), Sheesham (*Dalbergia sissoo*), Lamb (*Aristida depressa*), Gorkha (*Lasiurus hirsutus*), Khawai (*Cymbopogon jawarancusa*), Murat (*Panicum antidotale*), Dhaman (*Cenchrus pennisetiformis*), Lana (*Haloxylon recurvum*), Ber (*Zyziphus* sp.), Katran (*Cymbopogon martinii*), Khiri (*Euphorbia prostrata*), Khip (*Leptadenia pyrotechnica*), Chag (*Crotalaria burhia*), Dele (*Capparis decidua*), Phel (*Neslia* sp.), Ghandeel (*Eleusine flagillifera*), Ak (*Callotropis* sp.) and Jal (*Salvadora oleoides*). While the all showed the similar amount of parched channa consumption. Softened plant parts of various species were also being given in their feeding. It is concluded that these species can live on the variety of fodders where the deer eat little amount of grass in a single day for survival as compared to other fodder types.

KEY WORDS: BLACKBUCK, CHINKARA, FEEDING BEHAVIOR, LAL SOHANRA NATIONAL PARK, SPOTTED DEER.

INTRODUCTION

Pakistan is a country which includes a wide range of environmental conditions starting from Karakorum, Himalayan, Hindu Kush, Indus plains, coastal halts and desert variations to the second highest peak in the world (K2) in the North to Southern sea levels. Pakistan has a rich variety of its fauna and flora, as well as other wildlife habitats and related landscapes. Continuing to be a special and enticing biodiversity artistic endeavor, in Pakistan the species come primarily from Ethiopia since its transitional zone is flanked by two of the six most important zoogeographic zones, the Palearctic and also the Oriental. The National Parks management, through the protection of ecosystems and threatened species, has an important role in biodiversity conservation. It also serves as a fauna and flora reservoir that can restore lands where numerous species have vanished (Chishty et al. 2021).

On 26 October 1972, Lal Suhanra National Park (LSNP) was declared a National Park by government following a 1971 Wildlife Enquiry Committee recommendation. It originally occupied a land of 313.549 km2 from which the desert occupied 209,319 km2, the forest plantation covered 84.880 km^2 and the forestry reservoir was 19.339 km^2 and was expanded to 226.80 km2. It is known for its diversity of animals that obviously includes Black buck, Antelope

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Nilgai and Rhino. LSNP is Pakistan 's primary biosphere reserve (Salahud et al. 2021).

The UNESCO-recognized "Human and Biosphere" (MAB) plan for sustainable development includes biosphere reserves. These are suitable for researching and demonstrating groundbreaking sustainability methodologies between local and international norms. Such biosphere reserves are capable of performing three related purposes-conservation, growth and research (UNESCO 2011). The large lake in the center of the park is a wonderful wintering ground, ideal for bird-watching (Maan and Chaudhary 2001; Roy and Mistry 2021).

Black buck (*Antilope cervicapra*) belongs to the Antelope genus that has been observed in Cholistan. It was recognized as among the most graceful animals, as a native Cholistan species. Female Blackbucks need large grasses as well as small bushes to supply fawns (Roy and Mistry 2021). In the first week, the fawns lie in the grass and bush and the nursing mothers go to the nurseries within a few hours. Fawns are very weak and can be quick to predated within the first few weeks. For the proper growth of young Blackbucks, therefore, it is important to also have undisturbed open spaces with the small covers and grass covers. At the age of around two weeks, the fawns join mother as well as other groups (Khan and Khan 2018; Rai 2021).

The Chinkara (*Gazella gazella*) is another important specie of deer family, inhabiting the LSNP. The failure to maintain some reticulo-rumen cellulolytic bacteria that promote digestion of fibrous feed is due to the preferences of foods with low fiber leaves and high nutrient requirements. Found in Cholistan, Chinkara consumes fruits of plants, leaves and flowers. It only grazes in the night and can cover several kilometers before it comes back to the desert early in the morning. In winter, it always prefers desert grasses, but feeds on the green *Calligonum polygonoides* as well as *Acacia jacquemontii* leaves. Food also comes from crops grown such as Brassica sp., Sorghum sp. As well as Sorghum sp (Salahud et al. 2021).

The Spotted Deer (*Axis axis*), often referred to as Chital, is a universal representative of deer family which is present in India and Pakistan region. It is found throughout the whole area except for in the farthest north. Spotted deer is considered as grazers and also browsers. In the morning and at night, they sleep, rest mostly in heat of midday. They eat almost every kind of plants, but their favorite diet is grass. A curious behavior is that this animal also consumes antlers for its rich nutrients. These are very afraid and anxious creatures who keep an eye on chasing predators at all times. Between summers and winters, they are bread twice a year, most usually in summer (Roy and Mistry 2021). The aim of present study feeding behavior of blackbuck, chinkara and spotted deer in captivity at Lal Sohanra national park Bahawalpur, Pakistan.

MATERIAL AND METHODS

The present study was conducted in Lal Suhanrai National Park (29024'N-72001'E), which is located in the east of Bahawalpur, approximately 36 kms away in the province of Punjab (Khan et al. 2018). 2 distinct sites (S 1 and S 2) have been selected for the current research. The study was conducted during the months of January to June, 2020. The blackbuck, chinkara and spotted deer in captivity were observed for their feeding behavior. The weight of fodder was measured by weighing machine and the data on types of fodders were collected on daily basis. The use of different fodders were also weighed and noted. The data of six month was collected and their averages were measured using Software / excel for different types of fodders and relative use. The different vegetation types which were the flora of this area were also recorded, when these plants were offered to the different species of deer.

RESULTS AND DISCUSSION

Lal Suhanra National Park is an important protected area present in Bahawalpur district of Punjab, Pakistan. This Park contains important mammal species which is considered endangered in other parts of the world. Blackbucks (*Antilope cervicapra*), Chinkara (*Gazella gazelle*) and Spotted deer (*Axis axis*) are some of the important mammal species of LSNP. Study indicated that Blackbucks, Chinkara and spotted deer consume fodder of seasonally grasses (Maize; *Zea mays, Jantar; Sesbania bisbinosa, Burseem; Trifolium alexandrinum*, and *Bajra; Pennsietum glaucum*) of 4-6 kg, 3-5 kg, 4-5, kg each individual in a day respectively. Also, it has been observed that parched channa was also being consumed with total amount of 250 to 500 g. Grasses that grow after the rain in enclosures were also used for feed. Most of the area of enclosure has become barren due to fast grazing. Soft bark trees, branches and fallen leaves were also used, along with softened parts of the plants including Jandi (Botanical name: *Prosopis spicigera*), Kikar (*Acacia* sp.), Sheesham (*Dalbergia sissoo*), Lamb (Aristida depressa), Gorkha (*Lasiurus hirsutus*), Khawai (*Cymbopogon jawarancusa*), Murat (*Panicum antidotale*), Dhaman (*Cenchrus pennisetiformis*), Lana (*Haloxylon recurvum*), Ber (*Zyziphus* sp.), Katran (*Cymbopogon martinii*), Khiri (*Euphorbia prostrata*), Khip (*Leptadenia pyrotechnica*), Chag (*Crotalaria burhia*), Dele (*Capparis decidua*), Phel (*Neslia* sp.), Ghandeel (*Eleusine flagillif era*), Ak (*Callotropis* sp.) and Jal (*Salvadora oleoides*).

A previous study has also present on deer raising in Cholistan desert by Khan and Khan (2016) and they suggested that mainly semi-arid as well as arid Pakistan is marked by low soil humidity as well as floral cover. A few other areas, such as Cholistan, in which economic choices are extremely limited, are extremely arid. No other activity in this part of the word is profitable in the present geographical place, except the growing of those animals which in arid conditions can thrive better. Together with other livestock, such as sheep, goats and camels, Cholistan 's climate allows deer to be raised. Promoting this exercise in the area can prove to be a perfect moonlighting business in several ways (Bhaskar et al. 2021).

In addition to providing people with an excellent source of income, it meets the increasing demand for meat. Deer meat is very common with many people, particularly those

of the upper economy with high purchasing power. So, deer farming will serve as a boost to Cholistan's economic circumstances. Deer farms along with goat, sheep as well as cattle farms can originally be established as limited enterprises. Owners can start up as a small business while still keeping a full-time job elsewhere. Deer herds require much less care and space than conventional live herds. Deer farming is on the other hand in a fragile condition everywhere practiced. Cholistan is some kind of region in which geographical circumstances allow this sector to develop as well as manage much better (Arandhara et al. 2021). Their study examines various aspects with possible futures of deer farming in the Cholistan desert, based on data gathered during field visits to Cholistan and various secondary sources (Arandhara et al. 2021).

It also provides a short spacious time view with deer farming around the world utilizing theoretical approach. It shows that certain deer species, including Chinkara, Chital and Blackbuck, can survive well in Cholistan, which can be comparatively cheaper than those of other animals (De et al. 2021). Feeding behavior of Blackbucks, Chinkara and Spotted deer is quite same. Although their amount given at a time may vary. So, from study indicated that Blackbucks consume Fodder of 4 to 6 kilograms per Blackbuck in a day. Also, it has been observed that parched channa was also being consumed with total amount of 250 to 500 g. but in case of Chinkara, they consume Fodder of total amount 3 to 5 kg and parched channa 250 to 350 g per Chinkara in a day. And in case of Spotted deer, they were consuming the same amount of Fodder (3 to 5 kg) and parched channa (250 to 350 kg) as it was given to the Chinkara because they inhabit the same enclosure (De et al. 2021).

Table 1. Food supplements and their quantity of Blackbuck, chinkara and spotted deer

Sr. No.	Common name	Botanical name	Black buck	Chinkara	Spotted deer
1	Maize	*Zea mays*	4-6 kg	3-5 kg	4-5 kg
2	Jantar	*Sesbania bisbinosa*			
3	Burseem	*Trifolium alexandrinum*			
4	Bajra	*Pennsietum Glaucum*			
5	Parched channa	*Cicer arietinum*	250-500 gm	250-350 gm	250-350 gm

Table 2. Common and Botanical names of the Vegetation

Sr. No.	Common name	Botanical name
1	Jandi	*Prosopis spicigera*
2	Kikar	*Acacia* sp
3	Sheesham	*Dalbergia sissoo*
4	Lamb	*Aristida depressa*
5	Gorkha	*Lasiurus hirsutus*
6	Khawai	*Cymbopogon jawarancusa*
7	Murat	*Panicum antidotale*
8	Dhaman	*Cenchrus pennisetiformis*
9	Lana	*Haloxylon recurvum*
10	Ber	*Zyziphus* sp.
11	Katran	*Cymbopogon martini*
12	Khiri	*Euphorbia prostrata*
13	Khip	*Leptadenia pyrotechnica*
14	Chag	*Crotalaria burhia*
15	Dele	*Capparis decidua*
16	Phel	*Neslia* sp
17	Ghandeel	*Eleusine flagillif era*
18	Ak	*Callotropis* sp.
19	Jal	*Salvadora oleoides*

The food habits of Blackbucks have indicated that on average a Blackbuck consumed around 7 lb (3-2 kg) per animal of fodder and maize per day. By taking into account the growth and development of plant and animals, a 1 acre (4,047 sq m) of desert will sustain 16.7 livestock and a 1 acre (36.6), for one day. The planned 1,280 acres enclosure includes 63% desert and 37% woodland, which are expected to be around 88 animals each year using those statistics (Bist et al. 2021). This is obviously an easy solution, but there was no time to get plant growth and die-off estimates. In addition, a large plant was palatable, but it's not very preferred. In Bharatpur, Rajasthan (India) and Kanha Park, Blackbuck have rarely been found to be navigated, because there was a lot of grass. Of course, they prefered certain grass not inevitably consumed in relation to the amount available: for example, Haloxylon recurvum, an abundant species, ate grass especially when woody plants were not readily accessible. Continuous heavy pasture and navigation will decrease annual plant growth and many of the most favored plant species will eventually vanish, leaving less food sources with likely lower nutritional content as has been reasoned by De et al. (2021).The carrying capacity of an enclosure may therefore be reduced. 'Imported' protein and other nutrients are greater in the fodder and maize than natural plants available (Sharma 2021).

CONCLUSION

The findings of the present study has concluded that these species can live on the variety of fodders. And all the deer eat little amount of grass in a single day for survival. These species had a very expensive meat value so we can rare them on little expenses.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly

available due to privacy, but are available from the corresponding author on reasonable request.

Conflict of Interest: There is no conflict of interest.

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Restorative Effects of Capsaicin Encapsulated Chitosan Nanoparticles on Chemically-Induced Hormone Receptor-Positive Mammary Carcinoma in *Sprague-Dawley* Rats

Dhamodharan Kalaiyarasi, Vengaimaran Manobharathi and Sankaran Mirunalini*
Department of Biochemistry and Biotechnology, Annamalai University, Chidambaram, Tamil Nadu, India

ABSTRACT

The clinical use of capsaicin has been confined due to its low-grade solubility and frail bioavailability. So, we examined the impact of capsaicin encapsulated chitosan nanoparticles (CAP@CS-NP) on chemically induced hormone receptor-positive mammary carcinoma. The mammary tumor was induced by a single dose of 7,12-Dimethylbenz(a)anthracene (DMBA) 25mg/kg b.wt. injected subcutaneously near the mammary gland. After 7 weeks, CAP@CS-NP 4mg/kg b.wt. was administered orally to tumor-bearing rats. Furthermore, sex hormones levels, ER and PR expression, mast cell population, and molecular docking studies were carried out. Tumor-bearing rats flashed significantly hoisted levels of sex hormones, mast cell population, ER, and PR expression. Administration of CAP@CS-NP 4mg/kg b.wt. restored the levels of sex hormones, mast cell population, ER, and PR expression to near-normal levels. Additionally, molecular docking revealed good binding affinity and best glide scores. These findings suggest that nano encapsulation of CAP@CS-NP 4mg/kg b.wt. administration can regulate hormonal expression to treat hormone receptor-positive mammary carcinoma..

KEY WORDS: BREAST CANCER, CAPSAICIN, DMBA, ESTROGEN RECEPTOR, PROGESTERONE RECEPTOR.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women across the globe, a pioneering cause of cancer-related fatality. According to GLOBOCAN 2020, female breast cancer comprises 11.7% of all cancer cases and 6.9% of all cancer mortality worldwide (Sung et al. 2020). Approximately two-thirds of all diagnosed breast cancers are categorized as hormone-dependent (Subramani et al. 2017). Female ovaries generate and release two groups of sex hormones, estrogen and progesterone, and their nuclear receptors are dubbed as estrogen receptors (ER) and progesterone receptors (PR). It offers a pivotal role in forming and growing both normal and cancerous mammary epithelium (Subramani et al. 2017). Long-term and high-level exposure of these hormones has been strongly associated to an elevated peril of breast cancer. Early menarche, late menopause, late pregnancy and nulliparity are all facets that increase hormone secretion uncontrollably. Polycyclic Aromatic Hydrocarbons (PAHs) are recognized as endocrine disruptors, which intervene with the homeostasis of organisms through mimicking endogenous hormones (Zhang et al. 2016; Kerdelhue et al. 2016; Sung et al. 2020).

Synthetic PAH, DMBA is a popularly researched model chemical carcinogen for the induction of mammary tumor in rodents. Mammary tumors thus induced are hormone-dependent adenocarcinomas emerge from terminal end buds on inadequately differentiated mammary glands (Russo and Russo 1978). A recent study by Alvarado et al. evaluated the immune expression of the prognostic factors ER and PR in DMBA-induced rat mammary tumors to know the model that best suits women's breast cancer (Alvarado et al. 2017). Current breast cancer treatments are chemotherapy, radiotherapy, hormone therapy and surgery (Sung et al. 2020).

Among these, hormonal therapy is crucial therapy to treat hormone receptor-positive breast cancer (Araki and Miyoshi 2018). Tamoxifen, anastrozole, exemestane, fulvestrant, goserelin, letrozole, leuprorelin, megestrol and toremifene are commonly used hormone therapy drugs. However, none has been proven optimal due to resistance and adverse side effects. This situation warrants the need for new anti-cancer

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drugs with potent and lower side effects for the treatment of mammary cancer (O'Reilly et al. 2020). Natural spices are a promising source for curing a variety of chronic ailments with their antioxidant, anti-inflammatory, antimicrobial and anti-cancer powers. It may also be used to relieve the adverse effects of cancer medications, such as fatigue, nausea, vomiting, indigestion and metallic taste (Ijpma et al. 2015; Zheng et al. 2016). Chili pepper (*Capsicum annum*) is a consistently consumed spice around the globe (Idrees et al. 2020). Capsaicin (CAP), the strong pungent component of chili peppers, exerts great anti-cancer effect in a vast number of malignancies (Clark and Lee 2016; Kalaiyarasi and Mirunalini 2021).

However, a major vital hindrance in the clinical practice of CAP is poor bioavailability due to its weak aqueous solubility that leads to constricted therapeutic potential and unsuccessful outcomes. The nanotechnology-based cancer therapy furnishes a promising solution to enhance the aqueous solubility and bioavailability of hydrophobic anti-cancer agents (Guo et al. 2011; Kalaiyarasi et al. 2021). Nanoparticle (NP) drug delivery systems, especially drug encapsulation with biodegradable polymeric NPs, have recently attained lots of attention due to their high cellular uptake, superior permeability and retention effect, and reduced cancer cell drug resistance (Mi et al. 2012; Tomasina et al. 2013). Chitosan (CS) is a natural-based polymer obtained from the exoskeleton of shrimps and other sea crustaceans. It is one of the rarest positively charged natural biopolymers in the world (Sogias et al. 2008; Taherian et al. 2021).

Due to its excellent physiochemical properties (non-toxic, bioadhesive, biocompatible and biodegradable) make CS a strong candidate for novel drug delivery systems, biosensors, edible films and nanofibers (Sogias et al. 2008; Taherian et al. 2021). In the current study, we aimed to investigate the anti-estrogen, and anti-progesterone effect of CAP encapsulated chitosan nanoparticles (CAP@CS-NP) on chemically induced hormone receptor-positive mammary carcinoma in female *Sprague-Dawley* rats.

MATERIAL AND METHODS

Capsaicin, 7,12-dimethylbenz(a)anthracene (DMBA), chitosan, sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich Co.Ltd. The primary antibodies for ER and PR were procured from Santa Cruz Biotech, USA. All other chemicals used were of analytical grade purchased from local commercial sources. CAP@CS-NP was synthesized by a novel method of ionic gelation with TPP solution (Gelling agent) and characterized by UV–visible spectroscopy, SEM analysis, FT-IR analysis and in vitro drug release (Arulmozhi et al. 2013). For *in vivo* experiment, 8 to10 weeks old female *Sprague-Dawley* rats (weight 130–150g) were purchased from Biogen Laboratory Animal Facility, Bangalore, India. Rats were maintained under controlled conditions of temperature 24 ± 2°C, humidity 50 ± 10% and photoperiod of 12 h (dark/light cycle), and bare access to a standard pellet diet and water provided in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, Chidambaram, Tamil Nadu, India. This study was approved by the Institutional Animal Ethics Committee (IAEC), regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India (Reg No. 160/1999/CPCSEA and Proposal No. 1203). Total numbers of 36 rats were randomly divided into 6 groups, each containing 6 rats (n = 6).

Group I rats served as control (normal untreated rat). Group II, III, IV & V rats received a single subcutaneous injection of DMBA 25mg/kg b.wt. (near the mammary gland) at the first week of the experiment. After 7 weeks, the tumor-bearing groups III, IV & V rats were treated with CAP 8mg/kg b.wt., CAP@CS-NP 4mg/kg b.wt. and CS-NP 5mg/kg b.wt. for 21 days (thrice per week). Group VI rats received bare Free CAP@NP for 21 days (thrice per week). The doses were fixed based on previous research studies (Jung et al. 2006; Koleva et al. 2013; Anandakumar et al. 2015). The experiment was terminated at the end of the 14th week, all the rats were sacrificed. Levels of Estradiol and Progesterone in plasma were measured by using Enzyme-linked immunosorbent assay (ELISA) kits (LifeSpan BioSciences Inc, USA) as per the manufacturer's instructions.

For immunohistochemical analysis, five micron-sized mammary tissue sections were embedded on poly-L-lysine coated glass slides. First, tissue slides were deparaffinized using xylene (5 min) and rehydrated in graded alcohol (10 min), washed in double-distilled water (5 min). Then the sections were incubated with 1% H_2O_2 in double distilled water at 22 oC (15 min) to quench the endogenous peroxidase activity, rinsed with Tris–HCl containing 150 mM NaCl and 1X TBS buffer at 22 °C (1 hr). After washing with 1X TBS buffer, the sections were incubated with primary antibodies ER and PR overnight at 4 oC. Followed by incubation, the respective secondary antibodies IgG-HRP conjugates for 1 hr at 4 °C. After that, slides were washed with 1X PBS then reactivity was developed with 0.03% of diaminobenzene and H_2O_2. Finally, the slides were visualized under a microscope (40×). For histopathological analysis, mammary tissues were sliced, immersed in 10% neutral buffered formalin for fixation, dehydrated with graded ethanol solutions, and then embedded in paraffin. Paraffin-embedded mammary tissue sections (3–5 µm) were cut using a microtome and stained with Toluidine blue. Then, slides were observed under a light microscope (40×).

Additionally, molecular docking study was carried out using Schrödinger software (Maestro V9.5). The structure of CAP ligand molecule was retrieved from the PubChem databases (https://pubchem.ncbi.nlm.nih.gov/), and crystal structure of ER targets (PDB ID: 1X7R, 4PPS, 4ZN7, 5KCU) and PR targets (PDB ID: 1SQN, 1SR7, 3D9O, 4OAR) were retrieved from the protein databank (PDB) (http://www.rcsb.org/pdb). Ligprep and Maestro-Glide were used to prepare ligand and receptor grid for docking algorithm. Subsequently, a Glide extra precision (XP) visualizer is used to explore the interaction between the CAP ligand and active targets (ER and PR), as well as the binding distance of various amino acid residues. The data were expressed

as mean ± standard deviation (SD). Statistical analysis was carried out using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA). The comparisons between groups were done using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A value of $P< 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Today's pharmaceutical research requires the discovery of secure and potent inhibitors with lower side effects, which are pivotal for cancer therapeutics. Over the past few years, we have been engaged in the creation of new nano formulated anti-cancer drugs against oral cancer and breast cancer (Arulmozhi et al. 2013; Isabella and Mirunalini 2017). In this direction, we have demonstrated the potency of CAP encapsulated chitosan nanoparticles against hormone receptor-positive (hormone-dependent) breast cancer. Hormones are signaling molecules secreted by glands in multicellular organisms and transmitted to distant organs through the circulatory system to bridle physiology and behavior (Bohra and Bhateja 2015). There are two types of hormones strongly related to hormone-dependent breast cancer (Trabert et al. 2020).

The first is estrogen, a crucial female sex hormone secreted in the ovary, placenta and adrenal cortex, promotes the growth and development of female genital organs and female secondary sex characteristics and triggers endometrial development. It is also considered one of the important etiological factors of mammary tumors. The three major endogenous estrogens with estrogenic hormonal activity are estrone (E1), estradiol (E2) and estriol (E3). Out of them, estradiol is the strongest biologically active hormone in mammary tissue and a major growth regulator for the mammary cancer subset (Russo and Russo 2006). The second hormone is progesterone, secreted by the corpus luteum in the ovary and is committed in both the menstrual cycle and early stages of pregnancy (Trabert et al. 2020). Higher circulating progesterone levels were closely linked to an escalating peril of breast cancer (Khan 2020).

Chemical carcinogen-induced cancer models in experimental rodents are a valuable resource. DMBA is a top-notch toxic which chemically promotes mammary cancer in the rat model. It renders ductal epithelial cells hyperplasia and atypical hyperplasticity and carcinogenesis of the terminal ducts. The tumor engendered by this chemical model resembles a human hormone-dependent breast tumor in terms of histology and hormone response profiles (Abba et al. 2016). In the current study, we found that DMBA-induced rats exhibit significantly elevated estradiol and progesterone levels in plasma (Group II) compared with the control rats (Group I). However, CAP 8mg/kg b.wt. (Group III) and CAP@CS-NP 4mg/kg b.wt. (Group IV) administration to tumor-bearing rats significantly altered these hormones levels to near normalcy. No changes were noted in CS-NP 5mg/kg b.wt. (Group V) treated rats when compared with DMBA induced rats (Group II). Although, no significant differences were detected in Free CAP@NP (Group VI) alone treated rats when compared to control rats (Group I) (Khan 2020).

Table 1. Effect of CAP and CAP@CS-NP on sex hormones in plasma of control and experimental rats

Groups	Estradiol (pg/mL)	Progesterone (ng/mL)
Control (I)	35.03±1.18	21.60±1.39
DMBA (II)	52.28±2.78###	32.18±2.26###
DMBA+ CAP (III)	44.29±2.18***	27.84±1.99**
DMBA+ CAP@CS-NP (IV)	38.33±1.72***	23.73±1.52***
DMBA+ CS-NP (V)	50.31±2.78	31.15±2.26
Free CAP@NP (VI)	36.28±1.33	22.91±1.41

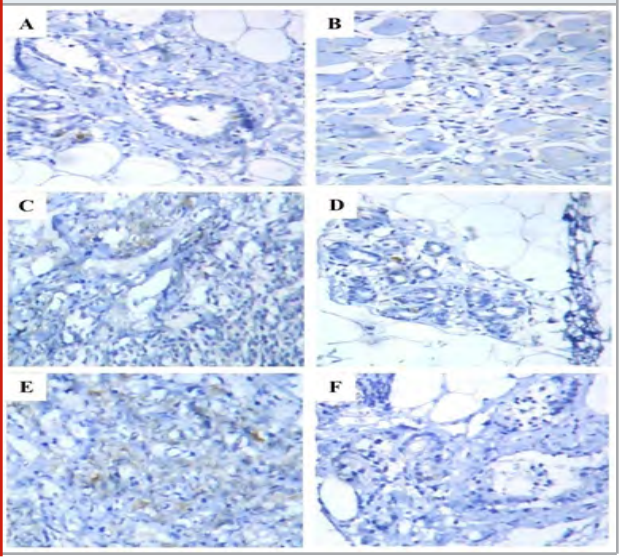
Values are expressed as mean ± SD for six rats in each group. Significant levels are ###$P < 0.001$ when compared with control group and **$P < 0.01$, ***$P < 0.001$ when compared with DMBA group.

CAP@CS-NP 4mg/kg b.wt. was found to be more efficient than CAP 8mg/kg b.wt. in reducing sex hormones levels (Table 1). Our findings agree with the previous report of (El-Aziz 2005) which proved that melatonin treated rats showed near-normal levels of endocrine hormones when compared with DMBA rats (El-Aziz 2005). Hormone receptor, a receptor molecule that sticks to a pertinent hormone and is mainly expressed in the female reproductive organs of humans. ER and PR are the most routinely studied markers in mammary carcinoma (Althuis et al. 2004). IHC examination of DMBA-induced tumor-bearing rats (Group II) (B) were displayed increased expression of ER and PR when compared to control rats (Group I) (A), confirming the origins of hormone positive-receptor mammary carcinoma. Contrastingly, administration of CAP 8mg/kg b.wt. (Group III) (C) and CAP@CS-NP 4mg/kg b.wt. (Group IV) (D) dramatically reduced the expression of ER and PR when compared with DMBA induced rats (Group II) (B). No alterations in the ER and PR expression of CS-NP 5mg/kg b.wt. (Group V) (E) treated rats when compared with DMBA induced rats (Group II) (B) (Khan 2020).

Even though no modifications were noticed in Free CAP@NP (Group VI) (F) alone treated rats when compared to

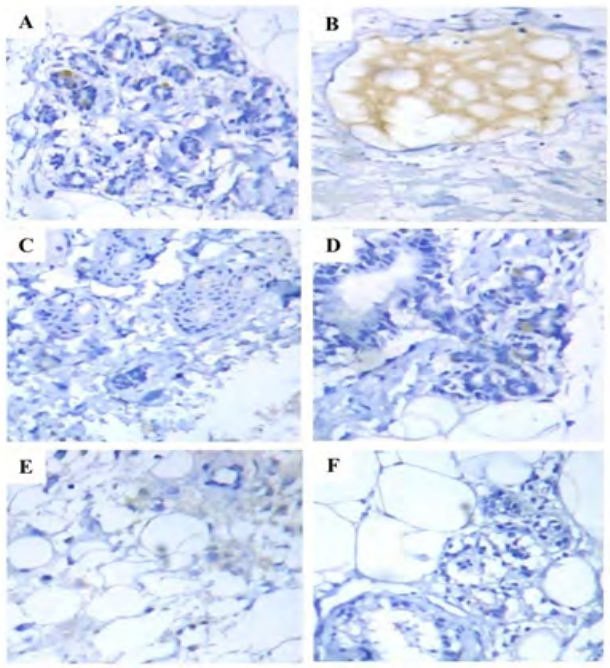
control rats (Group I) (A). Notably, CAP@CS-NP 4mg/kg b.wt. was shown to be more proficient than CAP 8mg/kg b.wt. in inhibiting abnormal hormone expression (Figure 1 (A–F) and Figure 2 (A–F)). Moreover, our result was also collaborating with previous studies of Isabella et al., who discovered that oral supplementation of DIM@CS-NP in tumor-bearing rats reduced the expression of ER and PR status (Isabella et al. 2018). Mast cells (MCs) represent a controversial constituent of the stromal compartment of breast cancer and a potent proangiogenic factor that promotes tumor development. The populations of MCs were favorably correlated with ER and PR expression (Glajcar et al. 2017; Aponte-Lopez et al. 2018; Khan 2020).

Figure 1: Immunohistochemical analysis of ER expression in the mammary tissues of control and experimental rats. Immunohistochemical on mammary tissues of control (A) and Free CAP@NP (F) alone treated rats showed normal mammary tissue staining; Mammary tissues of DMBA induced (B) and CS-NP 5mg/kg b.wt. (E) treated rats showed increased expression of ER; Mammary tissues of CAP 8mg/kg b.wt. (C) and CAP@CS-NP 4mg/kg b.wt. (D) treated rats showed diminished expression of ER as compared to DMBA induced rats (B).



Our histopathological report clearly shows the excessive mast cell population in the mammary tissues of DMBA induced rats (Group II) (B) when compared with the control rats (Group I) (A). Conversely, CAP 8mg/kg b.wt. (Group III) (C) and CAP@CS-NP 4mg/kg b.wt. (Group IV) (D) administration to tumor-bearing rats greatly diminished the levels of a mast cell population when compared with DMBA induced rats (Group II) (B). No conversions were specified in CS-NP 5mg/kg b.wt. (Group V) (E) treated rats when compared with DMBA induced rats (Group II) (B). However, no differences were spotted in Free CAP@NP (Group VI) (F) alone treated rats when compared to control

Figure 2: Immunohistochemical analysis of PR expression in the mammary tissues of control and experimental rats. Immunohistochemical on mammary tissues of control (A) and Free CAP@NP (F) alone treated rats showed normal mammary tissue staining; Mammary tissues of DMBA induced (B) and CS-NP 5mg/kg b.wt. (E) treated rats showed increased expression of PR; Mammary tissues of CAP 8mg/kg b.wt. (C) and CAP@CS-NP 4mg/kg b.wt. (D) treated rats showed diminished expression of PR as compared to DMBA induced rats (B).



rats (Group I) (A). Noteworthy, CAP@CS-NP 4mg/kg b.wt. was more suitable than CAP 8mg/kg b.wt. in alleviating the mast cell population (Figure 3 (A–F)), which is in agreement with earlier research of pathological markers in mammary tissues (Arivazhagan et al. 2014; Khan 2020).

Figure 4 and 5 shows the 2D Structure binding interactions of CAP ligand with four distinct ER and four distinct PR breast cancer targets. The binding modes of the docked CAP compound with ER and PR targets get an excellent Glide score (-9.01, -8.92, -10.21, -8.17, -8.93, -9.24, -8.77, -7.79 kcal/mol), binding energy, lipophilic evidence and a number of hydrogen bond, according to the data (Table 2). Likewise, CAP ligand hydrogen bond, amine, and functional groups have interacted with PHE 404, GLU 353, LEU 346, GLY 521, LEU 718, ARG 766, ASN 719, GLU A: 725, and ASN A: 719 amino acid residues in ER and PR targets. As a result, these interaction modes seem to be the most crucial notion in drug discovery at the preclinical stage. our findings concur with the previous report of Acharya et al. who reported that furanocoumarin phytochemicals have the best docking confirmation with ER, PR, EGFR, and mTOR (Acharya et al. 2019; Khan 2020; Maruthanila et al. 2019; Elancheran et al. 2019).

Figure 3: Histopathological analysis of mast cell population in the mammary tissues of control and experimental rats. Histopathological on mammary tissues of control (A) and Free CAP@NP (F) alone treated rats showed normal mammary tissue staining; Mammary tissues of DMBA induced (B) and CS-NP 5mg/kg b.wt. (E) treated rats showed increased levels of mast cell population; Mammary tissues of CAP 8mg/kg b.wt. (C) and CAP@CS-NP 4mg/kg b.wt. (D) treated rats showed decreased levels of the mast cell population as compared to DMBA induced rats (B).

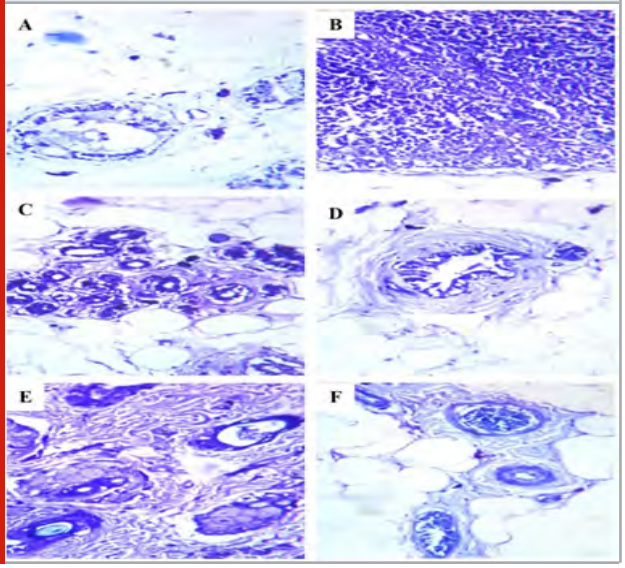


Figure 4: 2D Structure binding interactions of CAP ligand with ER targets.

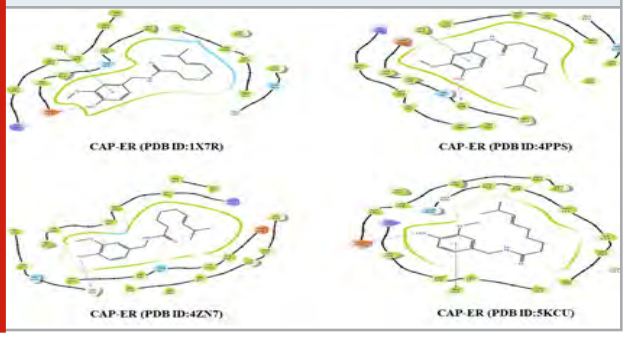


Figure 5: 2D Structure binding interactions of CAP ligand with PR targets.

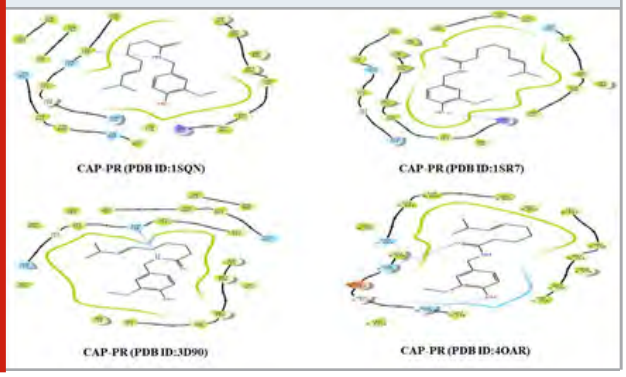


Table 2. Molecular docking result of CAP ligand with ER and PR targets

S.No	Ligand	Receptor	PDB ID	GScore	Lipophilic Evdw	Phob En	Hbond	Electro	Site map	Low MW	Rot Penal
1.	CAP	ER	1X7R	-9.01	-6.21	-1.91	-0.48	-0.29	-0.4	-0.48	0.76
2.	CAP	ER	4PPS	-8.92	-6.12	-1.63	-0.96	-0.1	-0.4	-0.48	0.76
3.	CAP	ER	4ZN7	-10.21	-6.41	-2.69	-1.01	-0.14	-0.24	-0.48	0.76
4.	CAP	ER	5KCU	-8.17	-5.25	-2.11	-0.57	-0.2	-0.32	-0.48	0.76
5.	CAP	PR	1SQN	-8.93	-6.28	-1.73	-0.78	-0.13	-0.29	-0.48	0.76
6.	CAP	PR	1SR7	-9.24	-6.55	-1.99	-0.48	-0.25	-0.25	-0.48	0.76
7.	CAP	PR	3D90	-8.77	-6.15	-1.14	-1.18	-0.23	-0.35	-0.48	0.76
8.	CAP	PR	4OAR	-7.79	-4.82	-0.6	-1.74	-0.69	-0.22	-0.48	0.76

CONCLUSION

The findings of the present study shed light on the therapeutic prospect of nano-formulated drugs in the treatment of breast cancer. In cases of breast carcinomas, the role of sex hormones is well recognized. The antiestrogen and antiprogesterone activity of CAP@CS-NP was proven by the reduction of ER and PR expression. In addition, an alteration in the mast cell population implies anti-inflammatory activity. As a consequence, the current study clearly establishes the efficacy of CAP@CS-NP against DMBA induced hormone receptor-positive mammary carcinoma in rats. Also, the molecular docking study bolsters this research.

Ethical Clearance: This study was approved by the Institutional Animal Ethics Committee (IAEC), regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India (Reg No. 160/1999/CPCSEA and Proposal No. 1203)

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

Conflict of Interest Statement: Authors declare no conflict of interests to disclose.

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Carvacrol Prevents Bisphenol A-Induced Behavioral Changes And Oxidative Stress in Zebrafish Through Modulating Brain Antioxidant Defense Mechanism

Bichandarkoil Jayaram Pratima, Ravichandran Ragunath and Namasivayam Nalini*
Department of Biochemistry and Biotechnology, Faculty of Science,
Annamalai University, Chidambaram, Tamil Nadu, India

ABSTRACT

It has been discovered that bisphenol A (BPA), an established anthropogenic xenoestrogen, is a causal factor in developing cancer, cognitive impairment, neurotoxicity, oxidative stress, and other harmful effects in humans and other species. Although there is some research into the mechanisms of BPA-induced toxicity, it is unclear whether there is a chance of amelioration through natural intervention. Zebrafish (*Danio rerio*) were used in this study after waterborne exposure to BPA, to assess whether carvacrol co-supplementation could reduce the destructive potential of the compound. All the chemicals and reagents utilized in the current investigations were purchased from Sigma-Aldrich, Ottochem, India. 5-7month-old zebrafish were acquired from a local fish store in Kolathur, Chennai and kept in a 50-L tank at a constant temperature of 25±2ºC. There were no animal ethical issues involved to carry out this research. Laboratory studies were conducted to determine whether the antioxidant nature of carvacrol might protect the zebrafish brain from BPA-induced altered behavioural responses and oxidative stress. The current data demonstrates that carvacrol is effective in alleviating the altered behavioural response caused by BPA. Biochemical investigations in the zebrafish brain suggest that carvacrol may have therapeutic potential in treating oxidative stress induced by BPA. In addition, the zebrafish brain is protected by carvacrol against BPA-induced toxicity. These preliminary data suggest that carvacrol may be a helpful intervention in treating BPA-induced toxicity in zebrafish by inhibiting the reactive oxygen species production. Novel therapeutic approaches for treating BPA-induced predisposition to severe illnesses could emerge from future research on signaling cascades.

KEY WORDS: BISPHENOL A, CARVACROL, OXIDATIVE STRESS, TOXICITY, ZEBRAFISH.

INTRODUCTION

The demand for consumer goods has increased the usage of synthetic polymers in the current manufacturing of high-quality plastic and micro-plastic materials. Their uncontrolled release into the environment poses hazard to human health in the form of the appearance of significant health problems in the future due to the release of these substances into the atmosphere. Because of its widespread use since its inception in the (1950s), bisphenol A (BPA), a synthetic compound with polymeric nature, an analogue of bisphenol (BP), is used in the production of plastics. BPA is the most frequently encountered chemical in the synthesis of epoxy and polycarbonate resins (Ansari et al. 2009; Abdalla et al. 2013). The discharge of sewage effluents and waste seepage have been identified as probable sources of BPA in surface water and groundwater. As an additional point of reference, BPA contamination has been evident in the dust and human urine, providing further evidence of its omnipotence in the environment. The estrogenic properties of bisphenol A (BPA) make it a potent anthropogenic xenoestrogen in the endocrine-disrupting chemicals (EDC) class. These estrogenic characteristics can make it a potential endocrine disruptor (Bencan et al. 2009; Ahn et al. 2015). Bisphenol A (BPA) is lipophilic, allowing it to pass through the placenta and the blood-brain barrier and even into the breast milk (Calafat et al. 2005; Canesi et al. 2015; Ben-Jonathan et al. 2016; Barboza et al. 2020).

According to the opposing viewpoint, many studies have discovered that BPA exposure is associated with various adverse effects such as depression, cognition failure, cancer, inflammation, reproductive problems, and increased stress due to oxidation. A further concern is that increased oxidative stress has contributed to various health problems such as cardiovascular disease, ageing, cancer, and inflammation. BPA's repercussions and oxidative stress have been widely researched in both *in vitro* and *in vivo* models

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focusing on organs such as the liver, colon, pancreas, and testes (Ahn et al. 2015; Barboza et al. 2020). Nevertheless, the pathological expression of this stress in the brain has remained a mystery until now (Bindhumol et al. 2003). BPA's increased production of reactive oxygen species (ROS) has been indicated in several earlier studies as a possible contributor to increased oxidative stress (Crain et al. 2007; Egan et al. 2009; Dong et al. 2014; Joseph et al. 2015; Eid et al. 2015; Cassar et al. 2020). As a result, further research into the potential involvement of increased BPA exposure in developing the brain stress pattern upon oxidation is required (Corrales et al. 2015; Costa et al. 2016; Das et al. 2020).

The development of significant health issues resulting from BPA discharge in water bodies near human habitation regions poses a considerable health risk to the general public and should be considered. Zebrafish (*Danio rerio*) is currently considered an excellent animal model for various preclinical studies because it exhibits a clear behavioural pattern and the response to different chemical interventions and stress conditions, including those induced by therapy (Ishisaka et al. 2011; Kajta et al. 2013; Chin-Chan et al. 2015; El-Horany et al. 2016). Natural intervention as a prophylactic/therapeutic strategy may be a feasible alternative to alleviate BPA-induced toxicity. Carvacrol (CVC) is a monoterpenoid phenol available in the essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), pepperwort (*Lepidium flavum*) and many other plants.Several studies have demonstrated that carvacrol (CVC) has anti-inflammatory, antibacterial, antioxidant, and anticancer effects (Das et al. 2020).

As a phyto-additive in dietary supplements, carvacrol has shown to have considerable antioxidant activity and has been used successfully in animal studies to boost the antioxidant status of animals (Inadera et al. 2015; Gassman et al. 2017; Gao et al. 2018). It is most commonly used in conjugation with thymol. Many preclinical models of cancer have demonstrated that CVC has anticancer properties that are mediated by proapoptotic pathways (Kabuto et al. 2003; Heo et al. 2004; Flint et al. 2012). Research on carvacrol preventive efficacy on BPA-induced behavioural pattern changes and oxidation changes is unexplored. Therefore, in the current experiment, zebrafish was used as an *in vivo* model to determine the harmful effects of BPA on the brain antioxidant defense system and the alleviatory effect of carvacrol on BPA-induced changes in the brain of zebrafish (Das et al. 2020).

METHODOLOGY

All the chemicals and reagents utilized in the current investigations were purchased from Sigma-Aldrich, Ottochem, India. 5-7month-old zebrafish were acquired from a local fish store in Kolathur, Chennai and kept in a 50-L tank at a constant temperature of 25±2ºC. The 12–12 h light and dark cycle were maintained in the laboratory for zebrafish maintenance. There were no animal ethical issues involved to carry out this research. For toxicity testing and dose standardization the LC_{50} of BPA was analyzed, the BPA solution was prepared by dissolving it in 100% EtOH and vigorously mixing the solution. In the end, EtOH concentration was made 0.003 percent (v/v) in all experimental groups for acute toxicity testing and BPA dose-response study. The dose-response analysis revealed that BPA caused 100 percent mortality at a concentration of 38.04 M, while the fatal concentration for BPA was estimated to be 28.28 M. The findings of this test revealed that behavioural paradigm shift occurred at a dose of 20.52 M after 96 hours, indicating that the drug was harmful. Thus, in this study, BPA dosage of 20.52 M was potent to explore the changes in zebrafish brain and nervous system.

The increasing load of BPA in the environment prompted us to investigate the effects of waterborne exposure to BPA at a substantially higher concentration than the environmentally relevant dose. Carvacrol standardization was done to determine the toxicity of carvacrol and to evaluate the LC_{50} and also to assess the preventive dose of carvacrol for its protective effects on BPA-induced toxicity. Following a dose-dependent investigation, the carvacrol LC_{50} was found to be 55.83 µM. The behavioural study revealed a quick swing in its pattern at a dose of 12.82 µM. Because of this, carvacrol concentration of 2.96 µM was utilized in the current investigation to encase its protective effects in zebrafish. Zebrafish were grouped according to five different experimental strategies: naive, control, BPA, carvacrol, and BPA + carvacrol. Each group consisted of ten mature zebrafish, which were housed in a 15-litre experimental aquarium. According to the experimental paradigm, zebrafish from the relevant experimental groups were exposed to BPA and carvacrol for a total of 21 days (Fig.1).

In the behavioural evaluation by light and dark test it is suggested that the zebrafish have a strong preference for darker environments which is evident through the light/dark preference test (LDT). LDT was performed in the current investigation once the required experimental setup had been completed for 21 days. The adult zebrafish were moved separately and individually to the dark chamber of the apparatus after one minute of adaption in the light zone with the separating door screened between each zone. This was captured on a 5-minute video recorder after the separating gate was removed and the behavioural shifts were observed (Magno et al. 2015, Sera et al. 1999). The exploratory behaviour of zebrafish was done through the Novel Tank Test (NTT). NTT is the most frequently recommended method for analyzing zebrafish exploratory behaviour. It was discovered that zebrafish had a strong preference for spending most of their time in the bottom of the novel tank. The exploratory behaviour of zebrafish was assessed in this study (Bencan et al. 2009; Egan et al. 2009).

As soon as the studies were completed and the zebrafish behaviour had been assessed, they were sacrificed, and their brains were separated and stored at 4ºC for biochemical investigation. The zebrafish brains were used for the biochemical analysis, which was performed three-fold for each experiment (Mohanty et al. 2016). The brain samples were carefully homogenized using a glass homogenizer at 4ºC with an ice-cold RIPA buffer, then incubated at 4ºC for 25 min, and then centrifugated for 20 minutes 12,000

RPM. It was necessary to collect the supernatant, divide it and store it at -20ºC until used. Protein carbonylation determines how many carbonyls remain after oxidation (Mohanty et al. 2016). The supernatant was separated from 10% homogenate and centrifuged at 12,000 rpm for 20 min. The supernatant was then treated with 0.5 mL 10 mM DNPH (2,4-dinitrophenylhydrazine) in 2 M HCl for 1 h at room temperature, followed by 15 minutes of vortexing every 15 minutes. To obtain the final product, 0.5 mL of 20% trichloroacetic acid was combined and centrifuged at 11,000 g for 10 minutes at 4ºC. The pellet was washed three times with 1 mL of ethanol–ethyl acetate (1:1) to remove unreactive reagents before drying. A spectrophotometer measured the concentration of carbonyls in the pellet protein at 366 nm. The samples were incubated with 2 M HCl to obtain a blank test. Carbonyl content was determined using the aliphatic hydrazone molar extinction coefficient, and results were expressed as nMole/mg carbonyl.

Lipid peroxidation test.Thymidine TBARS formation is considered a distinguishing characteristic of the peroxidation of lipids (Mohanty et al. 2017). For the most part, 100 ml of the supernatant from the brain was combined with 3.8 ml of the anti-TBARS and incubated at 95ºC for 60 minutes, after which it was centrifuged at 10,000 g for 10 minutes to remove any remaining reagent. At this point, a pink chromogen formed was evaluated at 532 nm in an ultraviolet spectrophotometer. The results were expressed in terms of moles of TBARS generated per milligram of protein.

The activity of the catalase enzyme was determined using the procedure previously described. Catalase degrades H_2O_2, and the amount of H_2O_2 degraded was measured in 15-second intervals for up to 2 minutes with a spectrophotometer at a wavelength of 240nm. nanokatal/mg protein was used to express the catalase activity, where one nano katal (nkatal) is equal to one mole of H_2O_2 consumed per second in the reaction mixture, and one milligram of protein (mg protein) was used to express the catalase activity measured in milligrams. Tissue glutathione (GSH) can be used to detect low levels of cytosolic oxidative stress in the tissue. The GSH level in zebrafish brain tissue homogenate was determined in this study using the procedure that has previously been described. Phosphoric acid solution was mixed with approximately 200 mL of brain supernatant, and the solution was centrifuged at 4000 g for 15 minutes at 4ºC. A 30-minute incubation at room temperature with 5, 5-dithiobis-2-nitrobenzoic acid resulted in the generation of supernatant, which was then used to measure GSH. Once this was done, a spectrophotometric measurement at 412 nm was performed, and the amount of GSH present was expressed as micromoles per gram of tissue.

The glutathione reductase assay was carried out according to the previously published technique for GR activity assessment in zebrafish brain (Sarkar et al. 2014). To determine the degree of change in GSSG to GSH, a spectrophotometric measurement at 340 nm was taken, and the degree of change in GSH was calculated. When measuring glutathione reductase activity, the molar extinction coefficient of NADPH is utilized. This value is given as nmoles NADPH oxidized/min/mg protein. Using a previously developed methodology GST activity was determined (Pabst et al. 1974). To determine the amount of this enzyme (GST) present in brain tissue, it was previously necessary to observe the reaction between glutathione GSH and GST. The substrate CDNB (1-chloro-2,4-dinitrobenzene) was measured at an absorbance of 340 nm. The GSH-CDNB conjugate was used to determine the molar extinction coefficient, which was used to measure GST activity. The resultant value was reported as nanomoles of CDNB conjugate formed per minute per milligram of protein in the sample (nmole CDNB conjugate).

Our methods for calculating total SOD activity have been somewhat adjusted from the method established by (Beauchamp C 1971). The reaction mixture contained 2.9 mL 50 mM Na-phosphate buffer, 2 mM riboflavin, 10 mM EDTA, 75 mM Nitro Blue tetrazolium, 13 mM methionine, and 100 mL brain tissue aliquot which were added to a 100 mL flask. Further incubation was done at 30°C for 10 minutes to study its absorbance at 560 nm. In this work, one unit of SOD enzyme activity was defined as the amount of sample protein required to block the NBT by 50%. For statistical analysis the mean and standard mean of the mean was used to represent all of the data. Comparing the outcomes of the different groups was done using one-way analysis of variance followed by DMRT test for comparisons between the naive and control carvacrol groups and between the BPA and the BPA + carvacrol groups. In all groups, *p*-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Carvacrol co-supplementation improved the behaviour of BPA-induced groups, which was associated with a reduction in BPA exposure: The behaviour was significantly altered in zebrafish after its exposure to BPA in the aquatic condition, which was evident by the increase in time spent in the lighted environment than naive and control fish (Fig. 2a & 2b). Furthermore, latency to enter in the black zone in LDT compared to naive and control groups was very much evident after BPA exposure (Fig. 2c). Carvacrol significantly reduced the behaviour alterations in the BPA + carvacrol group compared to the BPA group of zebrafish.

The antioxidant carvacrol helps zebrafish recover their bottom-dwelling and explorative behaviours after being supplemented with BPA: Transition to the top-zone and the time spent in the entire zone increased in the groups exposed to BPA compared to groups 1 and 2 (Fig. 3a & b). Also, when compared to groups 1 and 2, BPA-exposed group had significantly lower latency to top zone entry (Fig. 3c). The addition of carvacrol to the BPA-exposed group reduced the time spent in the top zone, the number of transitions to the entire area, and the latency to enter the top site. The findings of the current study suggest that carvacrol may protect against BPA-induced behavioural changes.

Carvacrol co-supplementation has been shown to improve the symptoms of BPA-induced oxidative stress:

Compared to other groups, BPA exposure for 21 days increased LPX and protein carbonylation levels significantly (Fig. 4a & b). Catalase activity in zebrafish brains were reduced considerably after BPA exposure (Fig. 4c). The primary results of the current work reveal that the increased ROS production in the zebrafish brain resulted in the protein and lipid component breakdown compared to the control and naive groups. When used as a preventive supplement, carvacrol reduced the levels of protein carbonylation, lipid peroxidation and CAT activity in zebrafish brains exposed to BPA.

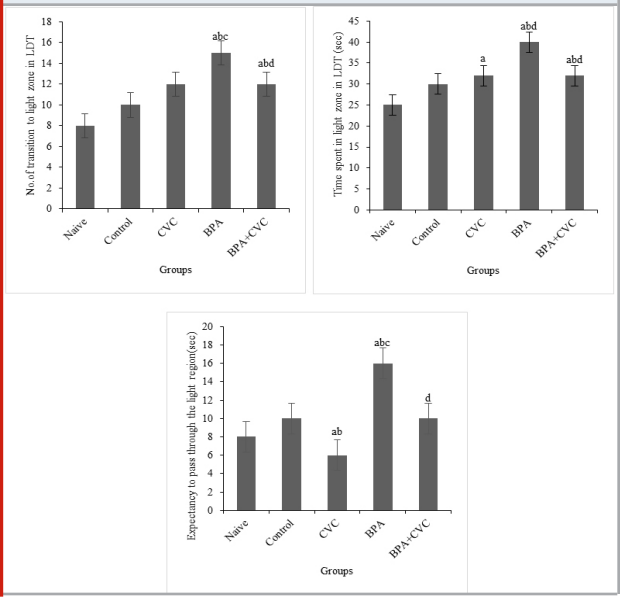
Carvacrol co-supplementation can reverse BPA-induced changes in glutathione production: The levels of BPA considerably reduced glutathione reductase activities (GR) and superoxide dismutase (SOD) activities in the brain of zebrafish (Fig. 5a, 5b, 5c and 5d) compared to naive and control zebrafish groups. According to the current study, BPA causes oxidative stress in zebrafish brains, which results in changes in antioxidant levels compared with naive and control groups. Previous studies have supported the protective role of flavonoids in restored neuronal redox equilibrium against oxidative stress. A standard concentration of carvacrol for waterborne complementation has also been proposed to deduce the function of carvacrol as a plausible mechanism of action against BPA-induced toxicity. Our research has shown that BPA has a significant influence on the antioxidant state of the zebrafish brains. As a result, carvacrol is shown to drastically reduce the ROS in the zebrafish brain by increasing antioxidants and the free radical scavenging enzymes in the cellular environment as a preventive supplement against oxidative stress induced by BPA.

Figure 1: Experimental design. The schmetic reprensentation showing time of BPA exposure, carvacrol co-supplementation neurobehaviroal assement.

BPA in the environment can be a major danger to human health in developing serious health concerns. BPA is largely an anthropogenic toxin. Therefore, the current study seeks to determine the toxicity of BPA and protective effect of carvacrol on toxicity of BPA. To investigate the impact of increased BPA load on zebrafish brains, the concentration of BPA selected for this study was 20.52 M, significantly higher than the environmental relevance level in aquatic bodies. Also, the preventive efficacy of carvacrol at the concentration of 2.96 µM was studied against BPA-induced toxicity. According to the current findings, carvacrol has both ameliorative and protective effects on BPA-induced oxidative stress-mediated behavioural change and toxicity (Kelly et al. 1998; Kawato et al. 2004; Kang et al. 2006; Kang et al. 2007; Lorber et al. 2015; Kuo et al. 2017; Cassar et al. 2020). Overall, we discovered that carvacrol lowers BPA-induced oxidative stress and recovers zebrafish scototaxis and bottom-dwelling behaviour.

NTT demonstrated that co-supplementing carvacrol following waterborne BPA exposure significantly changed the bottom-dwelling habit of zebrafish when compared with the other groups. Compared to other groups, carvacrol administered group dramatically put back the altered behavioural changes generated by BPA administration, as demonstrated by a considerable decrease in the frequency of movement to the brightly lit zone and the amount of time spent in the light zone in LDT (Nishikawa et al. 2010; Nagel et al. 2013; Negri-Cesi et al. 2015; Murata et al. 2018). By modifying the bottom-dwelling behaviour in zebrafish and also from the preliminary data it is evident that BPA has neurotoxic potential in zebrafish (Rochester et al. 2013; Rennekamp et al. 2015). We looked at the levels of various oxidants and antioxidant enzymes in the zebrafish brain to confirm that the BPA-induced altered behavioural response is due to increased oxidative stress and see if carvacrol could protect against BPA-induced oxidative stress (Cassar et al. 2020).

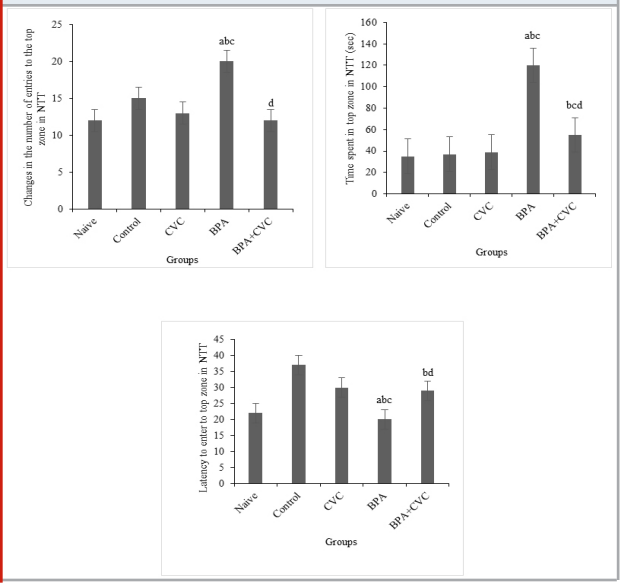
Figure 2: The light/dark test (LDT). [a] Graphs depicting the number of transitions to light zone, [b] amount of time spent, [c] and expectancy to pass through the light region after BPA exposure and carvacrol supplementation. The mean and SEM are used to express the values. a, b, c, d represents $p < 0.05$ when compared to the naïve, control, carvacrol and BPA group respectively.



When supplemented with carvacrol, it lowered ROS and lipid peroxidation in the zebrafish brain (Sangai et al. 2014). According to the findings and further investigation we found that this compound reverses the reductions in antioxidant levels and the free radical scavenging enzyme system caused by BPA, hence lowering oxidative stress in the zebrafish brain (Winston et al. 1991; Wong et al. 2017; Xu et al. 2019). This study suggests that persistent waterborne exposure to

BPA increased free radical production in zebrafish brains while simultaneously decreasing glutathione reductase function. The zebrafish brain had low glutathione (GSH). To keep the cell environment healthy, a lively balance between glutathione synthesis and oxidation is required. Thus, glutathione reductase is vital in regulating oxidative stress. According to our findings, carvacrol can reduce the effects of BPA on glutathione reductase activity in the zebrafish brain, which has implications for the regulation of GSH levels in the zebrafish brain and other tissues (redox balance) (Xu et al. 2019; Cassar et al. 2020).

Figure 3: Novel Tank Test (NTT). [a] Graphs depicting changes in number of entries to the top zone, [b]time spent in the top zone of the tank, and [c] Expectancy to enter the top zone of the tank exposure to BPA and carvacrol. The values are given as mean ± SEM. a, b, c, d represents *p*< 0.05 when compared to the naïve, control, carvacrol and BPA group respectively.



The antioxidant capacity of carvacrol is thought to be enhanced by its ability to regulate the level of cytosolic GSH, which is responsible for its protective effects. Following prior publications, our results suggest that carvacrol greatly reduces the increase in LPX in the zebrafish brain, potentially through an increase in GSH levels and superoxide dismutase activity (Eid et al. 2015). As a result, the simultaneous activation of GPX and CAT activity is essential for protection against oxidative stress, which is consistent with the involvement of SOD in superoxide radical detoxification. Under the initial assertion, our results demonstrate that carvacrol greatly alleviated the BPA-induced downregulation of CAT activity in the zebrafish brain (Zimmers et al. 2014; Yamazaki et al. 2015). Based on our findings, carvacrol appears to be a suitable supplement for zebrafish suffering from BPA-induced oxidative stress, resulting in altered behavioural responses. Additional research on behaviour was carried out to better understand the potential consequences of BPA exposure and establish preventive efficacy of carvacrol (Zhou et al. 2017; Barboza et al. 2020).

Figure 4: Assay for the parameters of oxidative stress. [a] Graphs depicting changes TBARS levels, [b] changes in the amount of protein carbonyl, and [c] changes in the catalase activity in the zebrafish brain following BPA exposure and carvacrol supplementation. The values are given as mean ± SEM. a, b, c, d represents *p*< 0.05 when compared to the naïve, control, carvacrol and BPA group respectively.

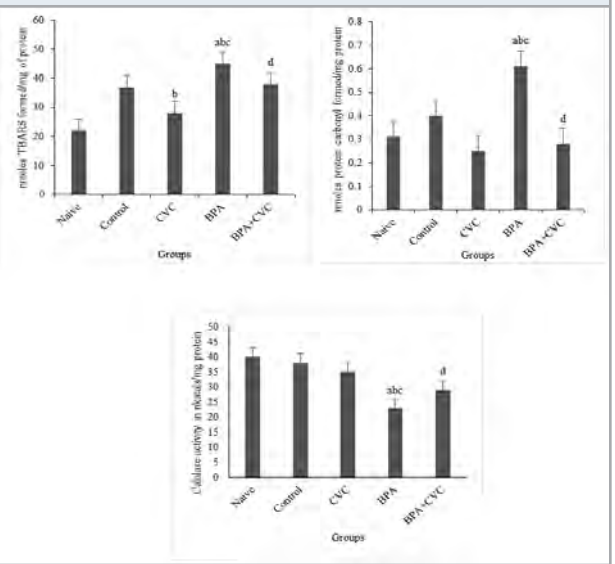
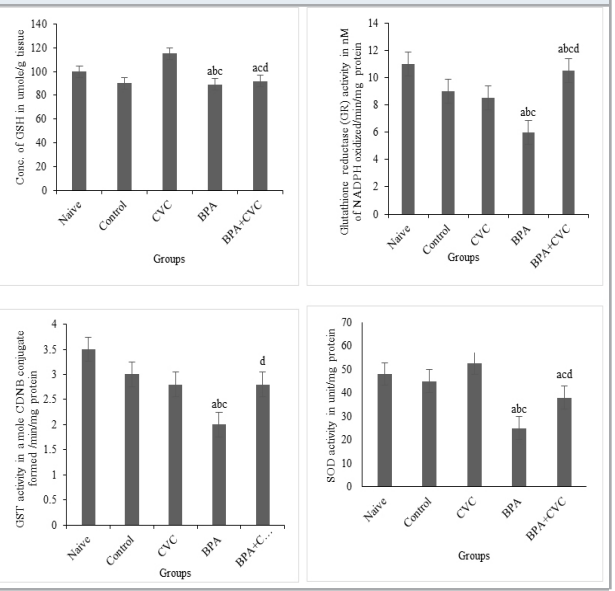


Figure 5: Enzymatic assay for free radical scavenging. [a] Graphs depicting changes in GSH concentration, [b] Glutathione reductase activity, [c] GST activity, and [d] superoxide dismutase activity in zebrafish brain after chronic BPA exposure and carvacrol supplementation. The values are given as mean ± SEM. a, b, c, d represents *p*< 0.05 when compared to the naïve, control, carvacrol and BPA group respectively.



Our findings indicate that carvacrol co-supplementation greatly mitigated the behavioural pattern changes due to BPA exposure. To put it succinctly, human populations of developing and undeveloped countries have become indiscriminate users of plasticizers (microplastics, including BPA) in recent years. As shown in the current study, carvacrol has a better and protective effect on the behavioral changes, toxicity, and oxidative stress caused by BPA. On the whole, we found that carvacrol reduces oxidative stress due to BPA, restores zebrafish behaviour changes. The NTT revealed a significant improvement in the bottom-life habit of zebrafish when compared to naive and control groups (Serra et al. 1999; Rahal et al. 2014). The altered behaviour in LDT caused by BPA was significantly reversed by the carvacrol co-supplementation, which is evident by the sudden downfall in the of the number of light zone transitions and time spent in light zone following administration of BPA. These findings strongly support the hypothesis that toxic potential of BPA in zebrafish by altering behaviour can be alleviated by using carvacrol (Barboza et al. 2020).

CONCLUSION

The findings of the present study suggest that the toxicity in brain caused by BPA is protected by carvacrol in zebrafish. According to the current research, increased oxidative stress caused altered behavioural responses in zebrafish. Carvacrol has been shown to effectively scavenge ROS and hydroxy radicals after prolonged aquatic exposure to BPA, it is also effective when used therapeutically. Carvacrol increases GSH levels and antioxidant enzymes in the cell, which may help protect against BPA-induced brain damage. Based on the findings of this study, carvacrol may be used to treat BPA-induced behavioural changes and oxidative stress. Novel therapeutic approaches for treating BPA-induced predisposition to severe illnesses could emerge from future research on signaling cascades.

Ethical Statement: This work was done in the year 2019 and at that time as per the guidelines of CPCSEA for experimentation, there was no ethical issue involved. Also, this is a PhD research work carried out by the research scholar who registered for PhD in the year 2018. Only in the year, 2021 ethical has been added for fish experimentation. Also, with regard to this, the institutional animal ethics committee has issued a circular on ethical clearance for a fish experiment only from Feb 2022. Since the work was done before the CPSCEA decision on fish ethics. There are no ethical issues involved to carry out the research.

Conflict of interests: Authors declare no conflicts of interests to disclose.

Data Availability Statement: The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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Dr. Ruchi Shivle	MSSN/BBRC	Department of Zoology Devi Ahilya Vishvidyalaya, Indore India
Dr. Kirti Dubey	MSSN/BBRC	Department of Fisheries Holkar Science College Indore MP India
Dr. AT Kalse	FSSN / BBRC	Department of Life Sciences North Eastern Maharashtra University, Jalgaon (MS)
Dr. F Kabinwar	FSSN / BBRC	Professor of Oncology, University of California, Los Angeles, USA
Dr Neelu Qayyumi PhD	MSSN/BBRC	Professor and Head Life Sciences Mittal College Opposite Bhopal Memorial Hospital Bhopal India Hospital Research Centre (BMHRC), Navi Bagh, Karond, Bhopal, 462008 India
Dr. Arjun Deb	FSSN / BBRC	Professor of Zoology Lumding College Lumding Assam, India
Dr. Z.Pir	FSSN / BBRC	Department of Biosciences University of Kashmir, Srinagar India
Dr. Razia Sultan	FSSN / BBRC	Department of Zoology Devi Ahilya Vish Vidyalaya , Indore India
Dr. Thingujam I. Devi	FSSN / BBRC	Institute of Bioresources and Sustainable Development, Imphal, Manipur
Dr. I Onyesom	FSSN / BBRC	Professor of Microbiology Abraska, Delta State Nigeria
Dr. K. Sudhakar	FSSN / BBRC	Assistant Professor Energy Centre, Maulana Azad National Institute of Technology Bhopal, India.
Dr. Ravi Jain	FSSN / BBRC	Associate Professor of Physics Samrat Ashok Technical Institute Vidisha India
Dr Shiv Kumar Jayant	MSSN/ BBRC	Department of Biochemistry All India Institute of Medical Sciences Bhopal India
Prof. C Rama Mohan	MSSN/BBRC	Narayana Engineering College, Jawaharlal Technological University Anantpur AP India
Dr. Sushil Kumar Upadhyay D. Phil	FSSN/BBRC	Assistant Professor, Department of Biotechnology Maharishi Markandeshwar (Deemed to be University) Mullana Ambala 133207 (Haryana) India

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Date…….….....….....…….

Department……..... Institution:.…….....City:.....Country…….....……

Email:…….....

Author 2 Name…….....Orcid Id.....…..…….....Signature……..…….

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