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Continued From Back Cover

Bioscience Biotechnology Research Communications
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BBRC
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Research Communications

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Bioecological Assessment of Arable Soil Pollution: A Case Study of Belgorod Region

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ABSTRACT

The Belgorod Region is one of the agricultural production leaders in the European part of Russia. The area is located in the forest-steppe and steppe zones, which differ in a significant level of arable soils pollution due to the long-lasting and intense agriculture. The work was aimed at a private (score by Cu, Cd, Pb, Zn and Cs) and integrated assessment of arable soil in view of 21 administrative units and individual agricultural commodity producers, identification of areas with high risk of contamination of agricultural products, which would allow us to recommend an adapted set of cultivated crops in crop rotations. A list of six agricultural crops was identified as a priority for agro-environmental monitoring of crop production quality. The elements of priority soil-ecological monitoring have been identified for steppe arable soils (Cu, Cd, Cs, Pb, Zn). The features of heavy metals translocation from soil to plants in specific soil and climatic conditions made it possible to recommend agronomic and technological methods aimed at obtaining environmentally friendly crop products.

KEY WORDS: HEAVY METALS, TRACE ELEMENTS, AGRICULTURAL SOIL, SOIL-PLANT SYSTEM, TRANSLOCATION

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INTRODUCTION

In order to search for agricultural solutions should remain technology-neutral it is necessary multiple paths to improving the production, food security and environmental performance of agriculture (Foley et al., 2011). A prolonged use of fertilizers has resulted in a change in the mineral composition of soils and plants by increasing the proportion of heavy metals (Protasova and Kopayeva, 1985). A number of studies have focused on the heavy metal content of arable soil (Fan et al., 2013; Briki et al., 2015; Wang et al., 2015; Baran et al., 2018; Volungevičius, et al., 2019). However, it should be borne in mind that even some of heavy metals are essential for humans and for them to apply term "trace elements" equal to "micronutrients" (Duffus, 2002).

The Russian Federation plans to lay down 80.5 mln. hectares with cultivated plants and get a grain yield of 110 mln. t. in 2019. Central Federal District (CFD) is an administrative unit that includes 18 regions of Russia with a total area of 650205 km^2 (3.8% of the country). This territory contains a significant part of the country's agricultural zone. The Belgorod Region (about 2 mln. ha agricultural land) is a part of CFD and it occupies 7% of the area under cultivation but provides 21% of agricultural production (2nd place on this indicator in CFD). There are 14 pig-growing companies, 9 poultry companies and 128 cattle breeding farms in the region.A tense environmental situation in the Belgorod Region is due to a range of problems related with the use of natural resources. A basic list of environmental resource issues includes the following: excess air emissions (Poletaev and Kornilov, 2017; Lisetskii and Borovlev, 2019), river degradation (Solov'eva et al., 2015; Grigoreva and Buryak, 2016; Marinina, 2018), reduction of forest areas (Chendev et al., 2016; Ukrainskij et al., 2016, 2017), dehumification of soil's plough layer (Lisetskii, 2012), soil erosion (Shtompel' et al., 1998), changes in soil biogeochemistry due to biological removal (Zelenskaya et al., 2018), deterioration of soil structure and erosion resistance (Lisetskii, 2008), increase in land areas disturbed by open pit mining (Lisetskii, 2018) and disproportion in land ratio (Lisetskii, 1998; Marinina, 2017; Martsinevskaya et al., 2018).

In addition, the Belgorod Region was exposed to radioactive contamination because of the accident at Chernobyl Nuclear Power Plant (1986). The eastern areas were most affected, where about 140 thous. ha of arable land was contaminated with Cs^{137} in the range from 1-5 Ku km^{-2}. Particularly significant changes have occurred with the fertility of arable soils and the development of erosion (60% of the territory). On wild land, the arrival of organic matter was 12 t ha^{-1}, and in agrocenoses (cereal crops), it decreased to 4 t ha^{-1} (Lisetskii, 1992). The monitoring soil organic matter (SOM) of soil organic matter in the arable layer under increasing anthropogenic stresses shows that this process is cyclical with SOM being decreased in general (Lisetskii, 2007). The soil cover on the slopes, which was relatively homogeneous before ploughing, has become to be deeply contrasting. Slope agricultural landscapes are distinguished by the fact that the masses of chemical elements, which are carried away from autonomous landscapes, are included in biogeochemical processes in subordinate ecogeosystems (Dobrovolskiy, 2003; Kalinitchenko, 2016).

Soil, the living terrestrial skin of the Earth, plays a central role in supporting life (Tecon and Or, 2017). The physico-chemical properties of various types of soils largely determine the composition and quantity of microorganisms, which forms a certain biological balance which can change under the influence of anthropogenic effects (Polyanskaya et al., 2016; Borisov and Shishlina, 2017; Lisetskii and Vladimirov, 2019). Composition and activity of microorganisms have a positive effect on the bioavailability of the metal polluted soil, in particular Cd and Ni (Ahmed et al., 2017).

MATERIALS AND METHODS

Study area: The automorphic soils of the Belgorod Region are characterized in a number of integrating monographs (Solovichenko, 2005; Solovichenko et al., 2007). Chernozem is the most common soil in the Belgorod Region. They predominate on the plateau and cover significant areas of riverine and ravine slopes. The total area of Chernozem is 2090.8 thous. ha or 77.1% of the entire territory of the region (27100 km^2), of which 327.6 thous. ha is located on the slopes. Among Chernozems subtypes typical (979.1 thous. ha) and leached (631 thous. ha) ones prevail, the third place in terms of area is occupied by ordinary ones (318.9 thous. ha). In addition, 97.6 thous. ha are occupied by specific residual-carbonate Chernozems on tight carbonate rocks (chalk, marl) and 64.2 thous. ha – by podzolized Chernozems. Grey forest soils (14.6%) are the second type by distribution.

The soil cover of the Belgorod Region steppe zone which, according to our measurements, occupies 12% of the region's territory is represented by ordinary Chernozems (208.2 thous. ha), ordinary carbonate Chernozems (74.5 thous. ha) and ordinary alkaline Chernozems (39.2 thous. ha) which in total occupy 11.8% of the total area of the region. These soil subtypes have been ploughed by 90% (Solovichenko, 2005). Ordinary back soils become dominant soils in the south-eastern part of the Belgorod Region (Rovensky, Veidelevsky districts and southern parts of Valuysky, Krasnogvardeisky and Alekseevsky districts).

In terms of nutrient-supplying capacity ordinary Chernozems do not significantly differ from typical Chernozems but the mobility of these elements is not high enough. In addition, ordinary Chernozems have insufficient phosphorus supply. It has been previously shown (Kiriluk, 2006) that Chernozems tend to differ by the average trace elements content at the subtype level: for example, in comparison with ordinary Chernozems, typical Chernozems are more enriched in Zn (81 vs 34 mg kg^{-1}) but somewhat depleted Cu (34 vs 37 mg kg^{-1}).

Data used: The field studies were carried out within the steppe part of the Belgorod Region in order to determine the regional standard for the content of the group of heavy metals including Pb, Cu, Zn, Cd, and Cs^{137}. An insight into the distribution of trace elements (micronutrients) and heavy metals over the arable land is provided by the results of five-year rounds of the agrochemical survey by Belgorodsky Agrochemical Service Centre (Lukin, 2004). The data on the content of gross forms of heavy metals were determined in the monitoring system of arable soils using samples taken from the wells. A concentrated HNO (1:1) extract with added HO was used by the laboratory. This is due to the fact that when determining the total content of heavy metals in the soil they use 1M HNO, 1M HCl and other extracts, but the obtained data make it impossible to determine the degree of territory contamination with heavy metals due to the lack of MAC. The total cadmium (extragent 5 MHNO3) was determined by atomic emission spectrometry. The total content of crop production element was determined using the methods generally accepted by the agrochemical service (Methodological guidelines ..., 1992; Sychev et al., 2006). Cs^{137} measurements were performed in soils and plants by gamma spectrometry. The energy resolution of the gamma-ray spectrometer with an energy of 662 keV is not less than 10%; the lower detection limit was at least 2 Bq kg^{-1}.

Methods: In ecogeochemistry any assessments of environmental contamination hazard in terms of landscape components are carried out using three main standards of comparison: hygiene standards (maximum allowable concentration – MAC), tentatively permissible concentrations (TPC) as well as background geochemical levels and chemical Clarks (Kasimov and Vlasov, 2015). Each of these standards has its own advantages and disadvantages. A cumulative pollution index (Z_c) used to characterize the effect of a group of elements is equal to the sum of concentration coefficients of selected chemical elements (K_{MAC}), and is calculated as per the formula

$$Z_c = K_{MAC1} + ... + K_{MACn}, \quad (1)$$

Where n – the number of chemical elements.

RESULTS AND DISCUSSION

The role of micronutrients and their redistribution in the landscape

The "elements of life", i.e. those which are required for the normal life of plants and animals, includes Cu, Zn and Cd. Although their content in plants and soil generally does not exceed thousandths of a percent, in the process of feeding these trace elements are already very important as micronutrients, because they complement the action of the main components of fertilizers: N, P, K. Several functions are performed in plants by Cu and Zn: they are associated with proteins and organelles, fixed in large molecules, and Cd is involved in accumulation and transfer processes (Kiriluk, 2006). It should also be noted that the relative content of Pb tightly bound in soils is 80-90%, and more than half of the total mass of Zn in the soil is part of complexes with organic matter and is sorbed by Fe hydroxide films (Dobrovolskiy, 2003).

Assessment of environmental risk of pollution arable soil

Under the conditions of active manifestation of soil erosion by water and with a high proportion of eroded soils in the southeast of the Belgorod Region (Rovensky district – 63.9%, Veydelevsky district – 57.0%) which exceeds the 53.6% (Solovichenko, 2005) average erosion in the Belgorod Region, one should take into account the migration mobility in solid runoff fine silt and clay. It has been established (Tanasienko et al., 2011) that the particle size distribution of solid runoff products is heavier than that of the original soil: heaving is due to decreased content of coarse silt and fine silt (63-2 μm) fractions therein and increased amount of clay fraction (2 μm). As the data Table 1 has shown, for humus-accumulative horizon the geochemical accumulation coefficient (Kac), which was determined by the ratio of the concentrations of elements in the clay to the soil, had the highest values for Zn and Pb.

Based on the values of the geochemical accumulation coefficient, any soils with moderate erosion degree can become the most active solid runoff-related suppliers of such elements as Zn, Pb, Cr and Sr to accumulation zones (gully bottoms, floodplains, etc.). Such land in the steppe zone is often used for vegetable crops.The rationale for in-soil MAC is highly dependent on the specific soil and environmental situation. In different soils, the behavior of incoming heavy metals is largely determined by the genetic properties of soils (soil solution reaction, redox potential, humus composition and amount, buffering, etc.), modern dynamics of soil processes and chemical properties of pollutant metals (Obukhov et al., 1980). Therefore, in different countries, the MAC levels in soils (mg kg^{-1}) differ but they are Zn – 300; Pb – 100; Cu –

Table 1. Background content of heavy metals (mg kg^{-1}) and enrichment factor (EF) values in the soils of the steppe zone of the Belgorod region (Veydelevsky district. Chernozem ordinary loamy, virgin)

Elements	Horizon A, 0-36 cm		EF	Horizon AB, 36-48 cm		EF
	soil	< 0.001 mm		soil	< 0.001 mm	
Pb	17.69	38.06	2.2	11.81	46.96	4.0
Cu	50.18	71.65	1.4	49.04	62.06	1.3
Zn	51.29	199.77	3.9	52.49	343.14	6.5
Co	11.94	13.25	1.1	30.97	52.09	1.7
Ni	35.07	85.39	2.4	44.51	129.84	2.9
Cr	81.39	130.75	1.6	95.74	215.99	2.3
Sr	48.30	369.20	7.6	71.76	703.84	9.8
V	81.32	167.22	2.1	90.45	230.29	2.5
As	4.92	11.17	2.3	5.77	14.97	2.6

Note: EF is enrichment factor.

100; Cd – 3-5 on the average (Kiriluk, 2006). These limits are mainly higher than those standards, which were previously adopted in the USSR, and now used in Russia.

We have adopted the following MAC levels in soils (mg kg^{-1}): Zn – 70; Pb – 32; Cu – 50; Cd – 2. The cesium MAC level for soils cesium (Cs) has not been officially established, but regional studies (Lukin, 2004) indicate that cesium concentration of up to 1.5 Ku km^{-2} is allowed to be present in the soil and it is considered harmless. With Cs Clarke in soils 5.0 mg kg^{-1} its normal content is considered to be 1-14 (average 5) mg kg^{-1} (Kiriluk, 2006). Over the period that has passed since the accident at Chernobyl NPP about one third of artificial radionuclides have already decayed, (the half-life of Cs^{137} is 28.5 years). In the Belgorod Region the level of soil pollution in Cs^{137} is characterized as low (Geographical atlas...,

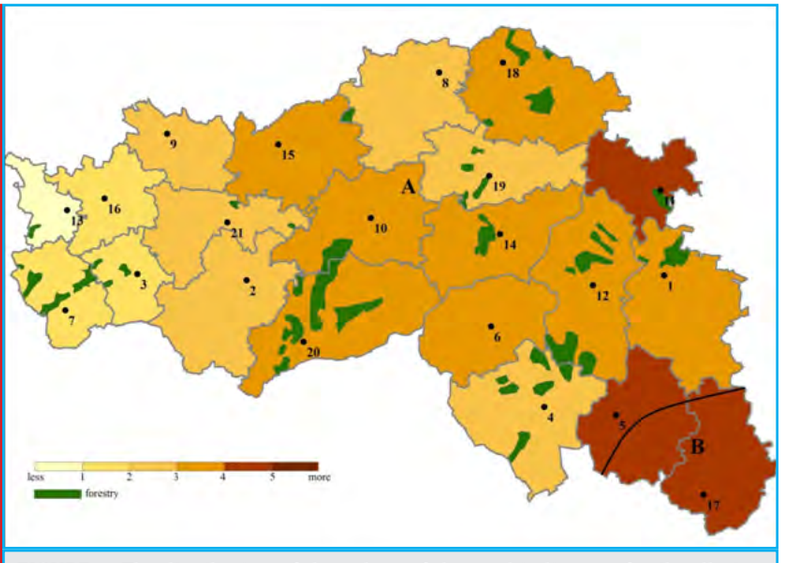

FIGURE 1. The distribution of the values of the total indicator of soil pollution (Zc) on the territory of the Belgorod region in the contours of agricultural enterprises: A – forest-steppe zone (88 % of the territory of the Belgorod region); B – steppe zone (12 % of the territory of the Belgorod region).
1 – Alekseevsky; 2 – Belgorodsky; 3 – Borisovsky; 4 – Valuysky; 5 – Veidelevsky; 6 – Volokonovsky; 7 – Graivoronsky; 8 – Gubkinsky; 9 – Ivnyansky; 10 – Korochansky; 11 – Krasnensky; 12 – Krasnogvardeisky; 13 – Krasnojaruzhsky; 14 – Novooskolsky; 15 – Prokhorovsky; 16 – Rakityansky; 17 – Rovensky; 18 – Starooskolsky; 19 – Chernyansky; 20 – Shebekinsky; 21 – Yakovlevsky

2018), and therefore normal technologies can be used in crop production.

We have conducted a soil pollution assessment in the Belgorod Region (Fig. 1) for the first time, and it showed that the north-western forest-steppe areas of the region (Krasnoyruzhsky, Grayvoronsky and Borisovsky) are least contaminated with heavy metals. In-oil concentrations of Cs and heavy metals, which exceed the MAC level, can be observed in the Rovensky (by Cu), Veidelevsky (by Zn) and Krasnensky regions.

The weighted average content of four heavy metals and cesium in the arable land of the Belgorod Region is shown in the Table 2. This is more generalized data, which was obtained using GIS-technologies in the context of municipal districts, which can contribute to the development of managerial decisions when introducing agroecological measures.

The steppe areas of the Belgorod Region (Rovensky and Veidelevsky) are characterized by excess average regional concentrations of all five elements (Table 2) under evaluation, and the total soil pollution index (Z_c) is consequently at 1.8 and 1.5 times higher than in the region on the average. The Krasnensky and Alekseevsky municipal districts are characterized by noticeably increased average regional concentrations of cesium – 2.3 and 2.2 times, respectively. The value of the total index of arable soils contamination (Z_c) exceeds the regional average level (2.7) for only 11 municipal districts (out of 21), and an excess of $Z_c > 3.5$ is observed in four districts only (Rovensky, Veidelevsky, Krasnensky and Alekseevsky). All these districts are located in the southeast of the Belgorod Region, i.e. in the steppe zone.

Soil contamination with heavy metals and assessment of their toxicity for cultivated crops

The phytotoxicity of the metals examined by us can be represented as a ranked series of toxicity (Kloke, 1980): Pb>Cu>Cd>Zn. The fact that there are significant ter-

Table 2. The weighted average content of heavy metals and cesium in the arable land of the Belgorod region

No	Municipal districts	Arable land, km^2	Zn	Pb	Cu	Cd	Cs	Zc
			mg kg^{-1}				Bq km^{-2}*	
1	Alekseevsky	1765	59.3	18.5	15.3	0.9	83.0	3.9
2	Belgorodsky	1628	57.3	14.1	13.8	0.8	21.2	2.2
3	Borisovsky	650	35.7	13.0	11.3	0.2	16.8	1.4
4	Valuysky	1709	48.7	14.5	13.7	0.6	43.5	2.1
5	Veidelevsky	1353	74.2	22.4	27.6	1.1	57.1	4.1
6	Volokonovsky	1288	52.9	17.2	55.5	0.6	42.7	3.1
7	Graivoronsky	854	35.0	12.6	10.8	0.2	17.1	1.5
8	Gubkinsky	1527	50.0	12.9	12.6	0.7	18.3	2.2
9	Ivnyansky	871	48.9	14.1	11.6	0.7	17.5	2.1
10	Korochansky	1455	63.6	15.6	15.4	0.9	44.7	3.1
11	Krasnensky	867	68.4	19.4	15.7	1.3	85.8	4.3
12	Krasnogvardeisky	1763	70.8	15.8	14.5	0.8	45.8	3.4
13	Krasnojaruzhsky	479	19.8	8.9	6.9	0.1	7.7	0.9
14	Novooskolsky	1401	54.6	16.9	15.8	0.8	38.2	3.3
15	Prokhorovsky	1380	56.2	15.4	34.4	0.9	31.4	3.2
16	Rakityansky	901	35.1	11.2	11.6	0.1	16.9	1.6
17	Rovensky	1369	67.7	22.3	65.7	1.1	57.3	4.8
18	Starooskolsky	1693	63.5	14.8	64.2	0.7	32.1	3.5
19	Chernyansky	1192	53.7	15.1	14.7	0.9	37.1	2.4
20	Shebekinsky	1866	61.3	16.4	15.0	0.9	46.2	3.4
21	Yakovlevsky	1089	60.1	14.8	13.9	0.8	17.6	2.3
	By region	27100	56.7	16.1	14.1	0.7	37.9	2.7
MAC**		–	70	32	50	2	–	–

Note: * 1 Ku km^{-2} = 37 Bq km^{-2}. ** MAC is maximum allowable concentration.

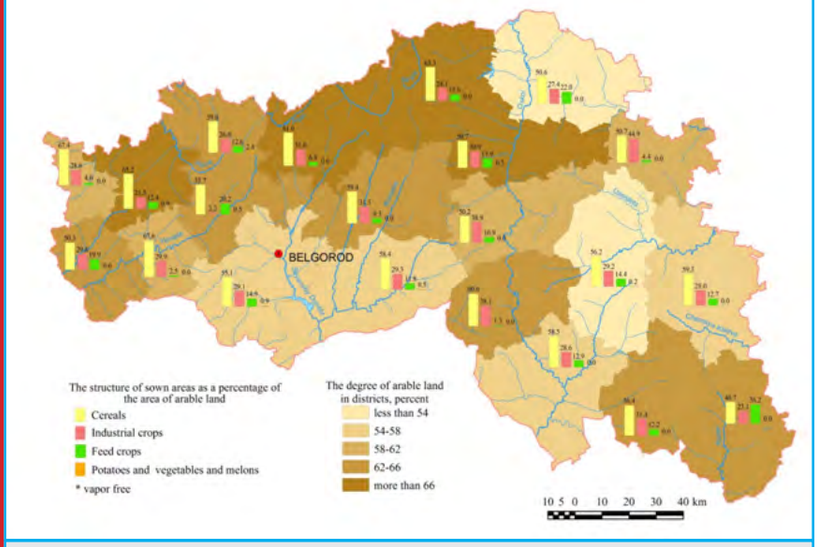

FIGURE 2. Plowing of land in the Belgorod region

ritorial differences observed in the Belgorod region for the formation of dangerous zones for agriculture and livestock breeding (on pastures) in terms of heavy metals concentration in soils makes it necessary to have cultivated areas agroecologically differentiated subject to a set of rotation crops, and in the future, possibly also with regard to their varietal characteristics. For example, it is shown that differences in cadmium uptake in various maize hybrids can be up to 13–18 times (Chernikov et al., 2000).

In the Belgorod region the sown areas are structurally dominated by (in thousand ha): spring grain crops (3552.2), fodder crops (3251.0), winter crops (2601.3), industrial crops (1944.5), legumes (302.4) (Fig. 2). Among industrial crops, large areas are occupied by sugar beet, which is inferior to other crops in resistance to the accumulation of heavy metals.

When considering the soil-plant block in agroecosystems it is important to note that the features of soil-to-plants translocation of heavy metals depend on the genetic properties of the soil, the behaviour of heavy metals and the biological characteristics of cultivated plants. Plants have certain mechanisms that prevent the accumulation of heavy metals in the reproductive organs and assimilate storage organs. The highest content of heavy metals is generally found in the roots, followed by stems and leaves, and finally by the following: seeds, tubers. As experiments with wheat (Il'in, 2007) from heavily contaminated soil have showed, only 0.5% of the heavy metal reserve was supplied to the whole phytomass at the end of the vegetation period. Thanks to the protective (barrier) capabilities of plants the following occurred: 90% of the metals turned to be trapped in the roots and about 10% of them were present in stems and leaves, and only 0.1% penetrated into the grain, which does not exceed the established limits (Il'in, 2007). Due to the translocation of trace elements from the soil, their content in grain crops can be as follows: Pb – 34-69%; Cd – 45-83%.

The content of heavy metals in root crops is comparable to their content in leaves and stems (Lukin, 2004). When growing food crops on soils with a high content of heavy metals, you should avoid provide them with plants the leaves (lettuce, spinach, onions, sorrel, etc.), stems and roots of which are used for food (Lukin, 2004). The data Table 2 show that in the steppe regions of the Belgorod Region (Rovensky, Veidelevsky) there is a ranked series of those heavy metals and Cs whose concentrations in steppe soils are higher than in the region on the average has the form: Cu, Cd, Cs, Pb, Zn. To reduce the risk of soil contamination by these heavy metals, you should apply agronomic and technological protective arrangements – the selection of less sensitive (tolerant) agricultural crops, the use of different plant parts with due account for their different ability to accumulate metals, etc. The plants can be arranged in descending order subject to their resistance to the toxic effects produced by heavy metals: herbs – cereals – industrial crops – potatoes – sugar beets (Chernikov et al., 2000).

Special environmental requirements are also needed for grass-arable rotations the share of which has significantly increased in the Belgorod Region with the implementation of the arable land biologization program. You may not use contaminated soils for feed growing

because livestock are fed with the parts of plants being in the phase when they accumulate very many metals (Chernikov et al., 2000).

CONCLUSION

The Belgorod Region is one of the leading Russian regions in the production of crop and livestock products. The specific soil and climatic conditions in the steppe part of the region (12% of the territory) have led to increased concentrations of a number of heavy metals (Cu, Cd, Pb, Zn) and Cs in arable soils. A large-scale regional program for arable land biologization has resulted in increased share of grass-arable rotations and tilled land the products of which are used in animal husbandry.Given the current structure of cultivated areas in the Belgorod Region, the following list of agricultural crops should be considered as a priority for agro-environmental monitoring of the quality of the main products: winter wheat, barley, sunflower, sugar beets, corn, and peas.With due regard for the multicriteria approach to the determination of the phytotoxicity of heavy metals and radionuclides (Kiriluk, 2006) for hazardous territorial areas in crop production, it is necessary to take into account different sensitivity of cultivated plants to metals as well as the influence of soil properties subject to the practice in the application of fertilizers and ameliorants.

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Feed utilization and growth of tilapia, *Oreochromis niloticus* fingerlings fed with three composed feeds formulated with locally available raw materials

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ABSTRACT

This research was designed to enhance availability of quality feeds for fish farmers to improve growth performance of Nile tilapia *Oreochromis niloticus* fingerlings. Three different isonitrogenous (38% crude protein) composed feeds (SG1, SG2 and G) were formulated with the local available low-cost raw materials of Côte d'Ivoire three agro-ecological fish farming areas. Eight weeks feeding trial was conducted with *Oreochromis niloticus* fingerlings initial mean weight 9.07±1.65 g with the three feeds formulated. Fish were randomly stocked at 6 fish/m2 in three earthen ponds with triplicate on a semi-intensive fish farm and fed at 10-5% of body weight three times per day with feeds produced. At the end of trial, daily weight gain values recorded varied between 0.56±0.06 and 0.91±0.09 g/day and feed conversion ratio values ranged between 1.38±0.14 and 2.06±0.23. The highest growth and feed efficiency performances were observed from the fish fed feeds G and SG2. Highest significantly values of fish carcass crude protein (16.66 – 17.10 %) and gross energy (8.23-8.26%) were recorded from fish fed feeds G and SG2 too. Carcass lipid content of the three fish groups fed ranged between 10.51±0.81 (SG1) and 11.58±0.93% (G) and did not differ significantly. Growth and survival results promote the formulation and the use of local quality feeds to improve the breeding of the tilapia *O. niloticus* in the semi-intensive fish farms.

KEY WORDS: AGRO-ECOLOGICAL AREAS, FEEDS, FEED INGREDIENTS, FINGERLING, GROWTH PERFORMANCE, *OREOCHROMIS NILOTICUS*

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INTRODUCTION

Cichlid fish species such as *Oreochromis niloticus* has many attributes that make him ideal for aquaculture (Elsayed, 2006). So, it's one of the first fish species aquacultured and is still the most farmed freshwater fish in Africa. Today, pond culture of *Oreochromis niloticus* is the most widespread type of aquaculture in this continent (Dereje et al., 2015). In Côte d'Ivoire, the semi-intensive pond system is the main type of commercial fish production of this fish and majority of fish farmers in semi-intensive system are farmers (FAO, 2008; Yao et al., 2017a,b). So, inaccessibility of high cost (1.38 – 2.16 USD/kg) imported commercial adapted fish feeds for *O. niloticus* fingerlings feeding affect growth and production of fish farming (Koumi et al., 2015; Koumi et al., 2016; Yao et al., 2017b).

In fact, nutritional quality of feed sellers' commercial feeds, national industrial commercial feeds, fish farmer's feeds and all agro-industrial byproduct used for *O. niloticus* feeding vary greatly and not always met the requirement of all stage of *O. niloticus* growth (Koumi et al., 2015).Moreover, the good growth management and feeding practices of *O. niloticus* fingerling enhance fish growth and is very important in this fish production. It is reported that *O. niloticus* fingerlings feeds must contain 35-40% protein, 4-10% lipid, around 25% carbohydrates, maximum of 8% of fiber with the feed protein energy ratio value ranged between 18-20 mg/kJ for attain indicated growth and good yield on farms (Guillaume et al., 1999; Gabriel et al., 2007; Lazard, 2007; Médale and Kaushik, 2009; Abdel-Tawwab et al., 2010). The high protein requirement (35-40 %) for *O. niloticus* fingerlings feeds has an economic impact on the production of juvenile tilapia, because of expensive cost of most of protein sources ingredients used in aquaculture feeds. Currently, one of the challenges of fish farming is to formulate competitive fish feeds with local available low cost raw materials in order to improve quality feeds use and reduce fish production costs. So, the use of local agro-industrial by-products as components of fish feeds have become increasingly common (Goddard et al., 2008; Agbo et al., 2011; Workagegn et al., 2014; Abarike et al., 2016).

In addition, Côte d'Ivoire, due to its strong agro-economic character and high industrial level has a large variety of products, sub products, agro-industrial by-products and food wastes that could be used in fish feeds manufacturing. Their adequate use in requirement feeds production could be the solution of the high price of first stages fish feeds and difficulty to supply high cost commercial feeds of *Oreochromis niloticus* to the majority of fish farmers.

The purpose of this study is to propose to the majority of fish farmers low-cost compound feeds formulated with local available raw materials of their area which cover requirement needs of *O. niloticus* fingerlings growth.

MATERIALS AND METHODS

Description of the study area: Three Côte d'Ivoire agro-ecological areas were the main basis of choice of fish feeds raw materials and comparison of feeds formulated and their influence on the feed's efficiency and fish growth in this study. Those are Guinean, Sudano-Guinean 1 and Sudano-Guinean 2 areas, which are the main zones of high concentration of fish farmers in Côte d'Ivoire (Yao et al., 2017a). Feeding trial was conducted on a large (two dams, fifty ponds) private fish farm located near of Azaguié town in Department of Agboville and Agneby-Tiassa region located to 40 km from Abidjan, the economic capital of Côte d'Ivoire.

Feed ingredients: Availability, price per kilogram and nutritional composition of the different raw materials usable in fish feeds were evaluated in the preliminary survey in the Guinean, Sudano-Guinean 1 and Sudano-Guinean 2 areas. Based on these parameters, the six different local feeds ingredients were selected for the three feeds formulation and production. So, imported fish meal (55% protein content) and cotton seed oil cake were both used in Sudano-Guinean and Guinean areas, cottonseed oil cake and soybean meal were used in the three selected agro-ecological areas (Guinean, Sudano-Guinean 1 and Sudano-Guinean 2 areas), when local fish meal (42% protein content) was used only in Sudano-Guinean 2 (SG2) area and wheat bran was used in only in the Guinean (G) area too.

Formulated feeds: Three isonitrogenous 38% protein content fish feeds were formulated with the selected raw materials by area used linear programming method based on the *Oreochromis niloticus* fingerlings feeds requirement. The centesimal compositions of the three formulated feeds are shown in Table 1. After formulation, the different levels of selected fish ingredients were weighted, then mixed and the determination of proximate

Table 1. Centesimal compositions of the three feeds formulated with the local available raw materials in Guinean, Sudano-Guinean 1 and Sudano-Guinean 2 areas

Ingredients (%)	Formulated Feeds		
	SG1	SG2	G
Imported fish meal (55 % protein content)	30	-	30
Local fish meal (42% protein content)	-	45	-
Cotton seed oil cake	20	20	15
White rice bran	20	-	15
Wheat bran	-	-	10
Soybean meal	30	35	30

FIGURE 1. a) 38% protein content *Oreochromis niloticus* fingerlings feed presented in fine flour form, b) *Oreochromis niloticus* fingerlings before the feeding trial, c) Earthen ponds on an Azaguié fish farm, d) *Oreochromis niloticus* fingerlings sample, e) Soxhlet extraction with hexane as solvant

compositions of the different mixes was done following the standard methods given by Association of Official Analytical Chemists (AOAC 1995; 2003). All feeds formulated were presented in flour form and stored in labeled bags in a cool dry place and used throughout the experimental period.

Fish and feeding trial: The feeding trial was performed with *Oreochromis niloticus* fingerlings initial mean weight 9.07±1.65 g males purchased from tilapia fingerlings fish farmer distributor of Azaguié town. The ponds used were completely drained and exposed to sunlight for dry during one week, then there were refilled with fresh water provided by the dams before fish stocking. *O. niloticus* fingerlings were randomly stocked of 6 fish per square meter in nine similar dimensions (328.67±29.67 m^2) earthen ponds. After the one-week acclimation period of fish, feeding trial was conducted from July to September with the semi-intensive recommended management practices. Figure 1 presents some photographies of the feeding trial. *O. niloticus* fingerlings were fed three times daily 09:00, 13:00 and 17:00 GMT at 10-5 % body weight during two months. Temperature, pH, dissolved oxygen, TDS (total dissolved solids), ORP (Potential redox) and conductivity were monitored every week throughout the feeding period using multiparameter HANNA. Every 2 weeks, a sample of 30 % of fish by ponds were randomly collected, individually weighed and measured, and then monthly total fish biomass of each pond was determined to adjust accordingly the feeding ratio. At the end of feeding trial, all the fish by pond were removed for individual weight and length measured. The total quantity of fish feed used by pond was recorded.

Determination of growth and feed efficiency parameters

Growth and feed utilization parameters were calculated using the following formulas:

Weight gain: WG (g)

$$WG = Final\ mean\ weight\ of\ fish - Initial\ mean\ weight\ of\ fish$$

Mean daily weight gain: MWG (g/day)

$$MWG = \frac{Weight\ gain}{Duration\ of\ feeding\ trial\ in\ days}$$

Biomass gain: BG (kg)

$$BG = Final\ fish\ biomass - Initial\ fish\ biomass$$

Specific growth rate: SGR (%/day)

$$SGR = \frac{Ln\ final\ mean\ weight - Ln\ initial\ mean\ weight}{duration\ of\ feeding\ trial\ in\ days} \times 100$$

Survival rate: SR (%)

$$SR = \frac{Initial\ number\ of\ fish\ stocked - Number\ of\ fish\ died}{Initial\ number\ of\ fish\ stocked} \times 100$$

Feed Conversion Ratio: FCR

$$FCR = \frac{Total\ weight\ of\ feed\ consumed\ by\ fish}{Wet\ fish\ biomass\ gain}$$

Protein Efficiency Ratio: PER

$$PER = \frac{Wet\ fish\ biomass\ gain}{Total\ dietary\ protein\ \text{int}ake}$$

Analytical method: The chemical composition of samples of fish at the end of feeding trial and the feeds used were determined according to AOAC (1995). Moisture levels of samples were determined after drying the samples in an oven (80˚C) until a constant mass. Sample Crude protein level were measured using the Kjeldhal method (N% × 6.25), crude fat was measured using Soxhlet extraction with hexane, Ash content was determined using incineration at 550˚C for 24 h in the muffle furnace, and gross energy (GE) content of feeds and fish samples were calculated according to gross caloric values of crude protein (23.7 kJ/g), crude fat (39.5 kJ/g) and carbohydrate (17.2 kJ/g) reported by Guillaume et al. (1999). The mineral contents of the samples were determined by atomic absorption spectrophotometer according AOAC (2003).

Statistical analysis: Statistical analyzes of water quality parameters, feeding trial data and fish nutritional composition determined were performed with STATISCA 7.1 software. All data were expressed as means ± standard deviation. One-way ANOVA analysis of variance was used to compare the different values. Then, Duncan multiple range tests was used to compare differences among means. Differences were considered significant at $p < 0.05$.

RESULTS

Characteristics of feeds: Nutritional composition and cost of the three feeds produced for *Oreochromis niloticus* fingerlings by agro-ecological area are shown in Table 2. The three feeds produced were iso proteïque at 38% crude protein level with similar ($p > 0.05$) content of crude fiber. However, highest moisture level ($p < 0.05$) of feeds was recorded with feed G (8.60±0.30 %) followed by feed SG1 (7.90±0.18 %) when feed SG2 (6.30±0.14 %) recorded the lowest values. Formulated feeds SG1 and G presented the significant ($p < 0.05$) values of Ash (8.86±0.12 - 8.56±0.11 %), carbohydrates (33.23±0.26 - 32.99±1.22 %) and gross energy (18.00±0.85 - 18.18±0.16 kJ/g) compared to feed SG2. Inversely, feed SG2 (24.08±0.10 mg/kJ) recorded the highest level of protein/energy ratio value. Feed SG2 presented also, the high value of calcium (15.38 mg/g) and phosphor (18.99 mg/g) compared to the two other feeds.

Ponds water quality: Results of water quality parameters during the 60-days feeding trial period are shown in Table 3. The average values of the temperature, pH and ORP recorded in the ponds showed no significant difference ($p > 0.05$). The average values of theses parameters varied from 28.98 ± 0.17 and 29.33 ± 0.80 °C for the

Table 2. Nutritional composition and cost of the three feeds produced

Parameters*	Formulated Feeds		
	SG1	SG2	G
Moisture (%)	7.90±0.18^{b}	6.30±0.14^{a}	8.60±0.30^{c}
Crude protein (%)	38.28±0.20^{a}	38.85±0.13^{a}	38.56±0.04^{a}
Crude fiber (%)	5.55±0.42^{a}	5.45±0.09^{a}	5.70±0.26^{a}
Total fat (%)	6.18±0.09^{b}	5.48±0.09^{a}	5.59±0.29^{a}
Ash (%)	8.86±0.12^{a}	22.71±0.06^{b}	8.56±0.11^{a}
Carbohydrate (%)	33.23±0.26^{b}	21.72±0.06^{a}	32.99±1.22^{b}
Gross energy (kJ/g)	18.18±0.16^{b}	15.92±0.06^{a}	18.00±0.85^{b}
Protein/energy (mg/kJ)	21.05±0.04^{a}	24.08±0.10^{b}	21.42±0.78^{a}
Mineral composition			
Calcium (mg/g)	8.79	15.38	7.83
Phosphore (mg/g)	9.85	18.99	9.50
Cost** (USD/kg)	0.65	0.56	0.62

*^{a,b,c,}alphabetical letters on the same line show a significant difference among treatments at $p<0.05$
**1 USD = 565.64 FCFA based on August 2019 exchange data.

Table 3. Water quality parameters of experimental earthen ponds

Parameters*	Formulated feeds		
	Diet SG1	Diet SG2	Diet G
Temperature (oC)	29.33±0.80a	29.12±0.74a	28.98±0.17a
pH	8.31±1.21a	8.82±0.94a	8.31±0.43a
Dissolved oxygen (mg/L)	10.92±1.49b	9.03±0.76ab	8.59±0.91a
Conductivity (µs/cm)	44.75±11.50ab	35.25±9.95a	59.25±12.28b
TDS (mg/L)	23.25±5.44ab	17.25±5.00a	29.50±5.80b
ORP (mV)	88.50±48.30a	80.40±52.30a	82.65±39.64a
% dissolved oxygen	149.05±23.20b	117.83±9.65ab	110.40±12.51a
Salinity	0.00±0.01a	0.00±0.01a	0.00±0.01a

*a, b, c, alphabetical letters on the same line show a significant difference among treatments at the threshold of $\alpha = 0.05$

temperature, between 8.31 ± 1.21 and 8.82 ± 0.94 for pH and between 80.4 ± 52.30 and 88.5 ± 48 Mv for redox potential (ORP). Mean values for conductivity (59.25 ± 12.28µs/cm) and total dissolved solids (29.50 ± 5.8 mg/L) were significantly higher ($p<0.05$) in the ponds where fish were fed with feed G. Conversely, to the lowest ($p<0.05$) dissolved oxygen (8.59 ± 0.91 mg/L) content and percentage dissolved oxygen values (110.40 ± 12, 51) of the ponds fed with the same feed G.

Growth performance, feed utilization efficiency and survival rate

The evolutions of growth of juvenile *Oreochromis niloticus* fed 60 days with the three feeds formulated by agro-ecological area are presented in Figure 2. The three growth curves presented the similar growth trend however, fish fed feeds G and SG2 presented significantly ($p < 0.05$) higher growth rate than fish fed feed SG1. The results from growth and feed utilization parameters of juvenile *O. niloticus* at the end of feeding trial are shown in Table 4. Higher values ($p <0.05$) of fish final body weight, weight gain, daily weight gain, final body length, final biomass and specific growth rate values were found with fish fed feeds G and SG2 compared to the fish fed feed SG1. Highest ($p <0.05$) survival rate recorded with fish fed feeds SG1 (100%) and G (100%) when fish fed SG2 recorded 99.94% survival rate value. Feeds efficiency from fish fed were also affected by the type of feed used, significantly highest feed conversion ratio value was recorded with feed SG1 (2.06±0.23) and SG2 (1.75±0.19) with lowest values obtained with those of fish fed feed G (1.38±0.14). Conversely, fish fed feed G presented the higher ratio 1.89±0.20 of protein efficiency ratio than fish fed feeds SG1 (1.28±0.14) and SG2 (1.49±0.16).

Proximate composition of *O. niloticus* fingerlings after two months feeding

Nutritional compositions of *Oreochromis niloticus* fingerlings fed during 60 days with the three feeds pro-

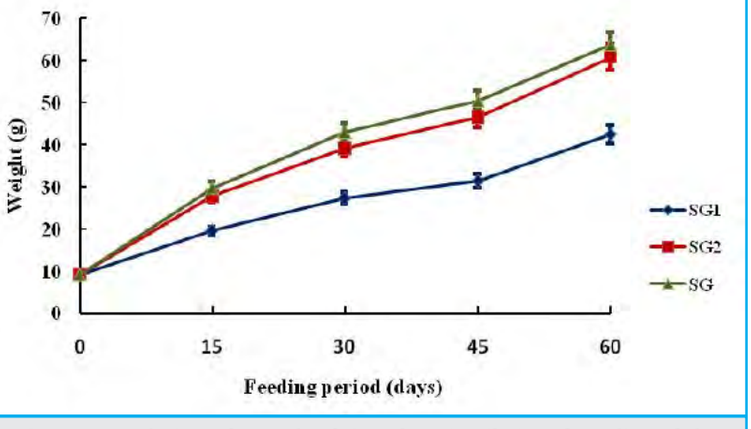

FIGURE 2. Evolution of growth of *Oreochromis niloticus* fingerlings fed 60 days with the three feeds formulated

Table 4. Mean values of growth and feed utilization parameters of juvenile O. niloticus fed with feeds formulated

Parameters*	Formulated feeds		
	SG1	SG2	G
Initial body weight (g)	9.07±1.54[a]	9.07±1.65[a]	9.07±1.45[a]
Final body weight (g)	42.43±3.65[a]	60.71±5.65[b]	63.58±5.65[b]
Weight gain (g)	33.36±3.65[a]	51.64±5.65[b]	54.51±5.65[b]
Mean daily weight gain (g/day)	0.56±0.06[a]	0.86±0.09[b]	0.91±0.09[b]
Initial body length (cm)	7.17±0.29[a]	7.17±0.29[a]	7.17±0.29[a]
Final body length (cm)	13.54±0.51[a]	15.12±0.58[b]	15.33±0.58[b]
Initial biomass (kg)	19.50±3.31[a]	16.33±2.97[a]	17.78±2.84[a]
Final biomass (kg)	91.22±7.85[a]	109.22±10.16[ab]	124.62±11.07[b]
Biomass gain (kg)	71.72±7.84[a]	92.90±10.16[b]	106.84±11.07[b]
Specific growth rate (%/day)	2.57±0.14[a]	3.16±0.16[b]	3.24±0.15[b]
Survival rate (%)	100±0.00[b]	99.94±0.00[a]	100±0.00[b]
Feed conversion ratio	2.06±0.23[b]	1.75±0.19[ab]	1.38±0.14[a]
Protein efficiency ratio	1.28±0.14[a]	1.49±0.16[a]	1.89±0.2[b]

*[a, b, c.] alphabetical letters on the same line show a significant difference among treatments at p<0.05

duced are shown in Table 5. No significant (p> 0.05) changes were found in crude lipid content (10.51±0.81 - 11.58±0.93 %) in carcass of fish fed at the different feeds. Conversely, fish carcass Ash, crude protein and gross energy content were affected by feed used. Highest carcass Ash values were recorded with feeds G (4.16±0.31 %) and SG1 (3.87±0.12 %) when highest values of fish carcass crude protein and gross energy were presented by fish fed feeds SG2 (16.66±1.04 %; 8.26±0.01 kJ/g) and G (17.10±1.04 %; 8.23±0.37 kJ/g).

DISCUSSION

Nutritional compositions of feeds showed that feeds formulated at 38% protein level with accessible (available and low cost) raw materials by agro-ecological area were influenced by the quality of the ingredients shoose and their levels of incorporation. The influences were translated by differences observed between the levels of moisture, total fat, Ash, carbohydrate and energy due to the fondamental difference between some ingredients such as the imported fish meal and local fish meal (Guillaume et al., 1999; Goddar et al., 2008). In fact, the higher levels of Ash, fat and carbohydrate in local fish meal compared to the imported high protein fish meal were due to the difference of fish specie, fish-processing (waste, flesh, carcass ...) and fish meals manufacture process (Hardy and Barows, 2002). Despite, the difference reported between nutritional qualities of feeds formulated, there were adapted to the nutritional require-

Table 5. Nutritional composition of carcass *of Oreochromis niloticus* fingerlings fed with three formulated feeds

Composition	Formulated feeds		
	SG1	SG2	G
Moisture (%)	70.86±0.55[b]	69.42±0.30[a]	69.12±0.80[a]
Ash (%)	3.87±0.12[b]	3.29±0.14[a]	4.16±0.31[b]
Crude protein (%)	14.74±0.03[a]	16.66±1.04[b]	17.10±1.04[b]
Crude lipid (%)	10.51±0.81[a]	10.92±0.02[a]	11.58±0.93[a]
Gross energy (kJ/g)	7.64±0.32[a]	8.26±0.01[b]	8.23±0.37[b]

[a, b, c.] alphabetical letters on the same line show a significant difference among treatments at p<0.05

ments of the *O. niloticus* fingerlings. In fact, 35-38% protein level, 25% carbohydrate level, 4-10% lipid level, less of 8% Ash and crude fibre levels are recommended for good growth of *Oreochromis niloticus* fingerlings (Lazard, 2007). However nutritional composition differences between the feeds formulated can influence the fish growth.

Moreover, the protein levels (38%) of feeds SG1, SG2 and G formulated for the breeding of *O. niloticus* fingerlings ranged between protein levels (30 – 57%) of imported high quality industrial commercial fish feeds (Koumi et al., 2015; 2016). Also, protein level of the three feeds formulated were higher than proteins levels of national industrial commercial fish feeds (28 – 30.15 % protein level), feeds sellers commercial fish feeds (16.20 – 24.90 % protein level) and farms made fish feeds (10.95 – 35.90 % protein level) used to feed all stages of tilapia *O. niloticus* included the fingerlings stage in the semi-intensive fish farms in Côte d'Ivoire (Koumi et al., 2015; 2016). The cost price of the three feeds formulated for *Oreochromis niloticus* fingerlings ranged between 0.56 and 0.65 USD/kg and were low cost compared to the prices (1.02 – 2.14 USD/kg) of most quality imported industrial commercial fish feed selled in Côte d'Ivoire (Koumi et al., 2015). Furthermore, difference in cost of raw materials used by feed, also influenced the cost price of feeds. So the use of low cost local fish meal in feed SG2 has resulted to the reduction to 0.06; 0.09 USD of this feed cost price compared to the price of feeds SG1 and G respectively due to the sheaper price of local fish meals compared to imported high quality fish meal. So quality and cost of the three feeds formulated and proposed offer the opportunity of fish farmers to made competitive local low cost fish feeds for *O. niloticus* fingerlings stage to resolve the inavailability of adapted quality fish feeds on the most Côte d'Ivoire fish farms. Despite the difference recorded between ponds water dissolved oxygen, % dissolved oxygen, conductivity and TDS values, temperature, dissolved oxygen, and pH values recorded were ranged between recommanded values for the good conditions of *O. niloticus* fingerlings breeding in earthen ponds (Tepe and Boyd, 2002; El-Sayed, 2006; Makori et al., 2017). Also, ponds water conductivity were above lethal values 3.8 to 10 µs/cm related by Stone et al (2013) and total dissolved solid (TDS) values (17.25±5.00 - 29.50±5.80 mg/L) recorded were less than 500 mg / l, reported as lethal value by Ibrahim and Ramzy (2013). Consequently, ORP values (21.6 ± 0.71 - 88.5 ± 48 mV) recorded in ponds related good oxydation conditions. Water physico-chemical parameters recorded had testified to the very good conditions of fingerlings breeding in earthen ponds with the formulated feeds.

Growth data analyses showed the better gorwth of *O. niloticus* fingerling with the feeds G and SG2 in spite of the difference on the nutritonal quality of the two feeds. In fact, feed SG2 presented highest levels of Ash (22.71±0.06 %) and protein/energy ratio (24.08±0.10 mg/kJ) values when feed G showed highest levels of carboghydrate (32.99±1.22 %) and gross energy (18.00±0.85 kJ/g) contents (Table 2). In addition, feed SG2 recorded higher level of calcium (15.38 mg/g) and phosphore (18.99 mg/g) content compared to the feed G (7.83 mg/g; 9.50 mg/g) although these levels were ranged between or above the recommended levels (6.5 – 9 mg/g) of calcium and phosphore in tilapia *O. niloticus* feeds. Results show that the mixes of ingredients of formulated feeds SG2 and G were better adapted for fingerlings *O. niloticus* growth. However, means daily fish weight gain recorded in this study oxcillated between 0.56±0.06 and 0.91±0.09 g/day were equal or greater than 0.35±0.05-0.86±0.20 g/day reported by Sumagaysay (2007) with *O. niloticus* of 2 to 83 g fed 4.4-10% of biomass of fish per day with the starter cruble and pellet feeds in intensive system. In addition, the specific growth rates (2.57±0.14- 3.24±0.15 %/day) and protein efficiency ratio (1.28±0.14-1.89±0.2) were similar of those recorded by Goddard et al. (2008) with fingerlings in outdoor cicular tanks in intensive fish farming system ranged between (2.7-3.1 %/day) and (1.67-1.92) respectively. When feed conversion ratios (1.38±0.14-2.06±0.23) recorded with feeds formulated presented in flour in this study were almost similar to those (1.3 -1.9) attained by El-Sayed (2013) using pelleted feeds in spite of the influences of feeds presentations forms on the feed conversion ratio in fish farm related by the same author. In fact best feed conversion ratio values were reported with extruded feeds, following by pelleted feeds when feed presented in flour form reported the lowest values in general (El-Sayed, 2013). Otherwise, variations observed in fish growth performance and feed utilization efficiency values with fish fed with feeds proposed by agro-ecological area were due to all differences between the three feeds such as ingredients shosed, and their incorporation levels, macro-and micronutriments compositions and the presence or not of the antinutritional substances (Workagegn et al., 2014). However, results demonstrated the opportunity to use the simple mixed feed formulated according to the recommended requirement, grounded in fine flour in the ponds to feed tilapia fingerlings in semi-intensive fish farm system. Results attest also the possibility to use local low cost fish meal in the formulation of adapted Tilapia *O. niloticus* fingerlings feeds despite the high quantity Ash induce by its use. Also, Abarike et al. (2016), reported good growth of tilapia with feeds contents up to 22.32% Ash and Goddard et al., (2008) reported best growth with of the feeds formulated at 38.9-39.8 % protein levels with local fish meal (16.1-24.4% of Ash). The absence of pathological

signs from fish fed and survival rate recorded (99.94 and 100%) during the feeding trial could be attributed to good management procedures during the study, i.e. proper handling of fish during sampling and storage of feeds which avoid mold growth, contamination with rodents.

At the end of feeding trial, differences were found in the moisture, ash, crude protein, and gross energy values of fish fed carcass. Similar results were also found by several authors (Goda et al., 2007; Agbo et al., 2011; Workagegn et al., 2014; Xiao et al., 2017). These differences were reported due to the nutritional value of feeds. Indeed, according to Elagba and Al-Sabahi (2011) and Navarro and al. (2012) the fish feeds influences the nutritional profile of fish. However, it is observed of lot of times, similar protein levels feeds used not influenced *O. niloticus* carcass protein levels (Koumi et al., 2009; 2011). But, in this study, for the fingerslings of *O. niloticus* at this fisrt stage of growth, the feeds G and SG2 which recorded the best growth were also recorded the high fish carcass protein levels. The carcass nutritional composition was must be influenced by the growth rate which can influence the level of flesh and bone of fish.

CONCLUSION

The local availability of raw materials used in the feed formulated had influenced the quality and the price costs of the feeds, but also the growth and quality of the carcass of fish fed in the context of the formulation of feeds adapted to the nutritionnal requirement of the fish. At the end of feeding trial, growth and survival results promote the formulation and the use of local quality feeds to improve the breeding of the tilapia *O. niloticus* in the semi-intensive fish farms. However, the presentation and the digestibility of these local feeds produced for fingerlings could be improve by the current production technologies such as extrusion.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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A multimodal biometric system for personal verification based on different level fusion of iris and face traits

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ABSTRACT

Recognizing users who access technological resources is one of the required processes to secure these resources. Based on previous studies, the most robust user recognition method is the biometric systems as these systems use unique behavioral or physical data to recognize individuals. There are two types of biometric systems: unimodal and multimodal biometric systems. Unimodal systems use only one biometric trait for user recognition, while multimodal systems use multiple traits. Experimental studies have shown that unimodal biometric systems have some problems related to performance and accuracy. In order to avoid the limitations of unimodal systems, multimodal biometric systems are deployed. In this paper, we propose a multimodal biometric system for human verification using a deep learning algorithm. The system depends on the face and iris identification model. The model uses an end-to-end convolutional neural network (CNN) for extracting features and identifying the user without using any image detection techniques. In the proposed model, two different approaches were tested to fuse the two biometric traits: feature-level fusion and score-level fusion. The experiments showed that the proposed model achieved an accuracy rate of 99.22% using the feature-level fusion approach, and using the score-level fusion approach resulted in an accuracy rate of 100%.

KEY WORDS: BIOMETRICS, CONVOLUTIONAL NEURAL NETWORK, DEEP LEARNING, MULTIMODAL BIOMETRIC SYSTEM, BIOMETRIC FUSION

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INTRODUCTION

With the growth and variety of technologies today, user recognition has become an essential process for accessing technological resources in order to allow only authorized people to access these resources. The use of biometrics data is the most powerful user recognition technique. Biometrics refers to the science of establishing the identity of a person based on physical traits (e.g. face, fingerprint, iris, palmprint) or behavioral traits (e.g. gait, signature, voice) of that person by using fully or semi-automated techniques (Jain, Ross and Nandakumar, 2011). Biometrics data are unique and cannot be forgotten, shared or guessed like passwords. Unlike ID cards, Biometrics data are impossible to be lost or stolen. Moreover, they are not easy to forge or duplicate since recent biometrics devices can distinguish a fake biometric from the original (Lumini and Nanni, 2017).

The system that uses biometric data to recognize users is called a biometric system. The biometric system consists of four modules: (1) the sensor module, which captures the data from the user through a suitable hardware device, (2) the feature extraction module, which extracts discriminatory features from the captured biometric data, (3) the matching module, which compares the extracted features from the biometric data with the saved features in the database to generates the similarity score between them, and (4) the decision module, which produces the decision based on the similarity score (Jain, Ross and Nandakumar, 2011). In general, any biometric recognition system operates in either identification or verification mode. The identification mode identifies who the individual is. The output of this mode is the identity of the user whose template has the largest matching score value. The verification mode checks if the claimed identity is correct or not. The output of this mode is accepting or rejecting the user. The aim of this mode is to prevent unauthorized users from accessing the system.

Biometric systems are classified into two types based on the number of traits that are acquired to recognize the user: unimodal and multimodal biometric systems. A unimodal biometric system uses a single biometric trait, such as a fingerprint or signature, to recognize a user. Despite the unimodal biometric systems are reliable, accurate and have advantages over traditional recognition techniques, the unimodal biometric systems have some limitations, such as (1) Noise in sensed data: it may occur as a result of imperfect acquisition biometrics by sensors. The noise in biometric data causes unsuccessful matching with the corresponding templates in the database. (2) Non-universality: the biometric system is considered as a universal system if all users possess a biometric trait for recognition. However, it is possible that some users do not possess the biometric trait because of a disability or other problems which can hinder the users from enrolling in the biometric system. (3) Vulnerability to spoofing attacks: the attackers can spoof biometric traits to take on the identity of individuals. (4) Intra-class variations: the input data from a user during recognition may be different from the enrollment image. This can be caused by improper interaction with the sensor or by some variation in a trait. (5) Inter-class similarity: large similarities may exist in the traits. For instance, some personal traits may be quite similar in identical twins (Oloyede and Hancke, 2016).

As for the multimodal biometric systems, it can recognize users by combining two or more biometrics traits. For example, combining the face with hand geometry (Jain, Ross and Nandakumar, 2011). The issues associated with unimodal biometric systems can be addressed using multimodal biometric systems. Multimodal biometric systems can prevent noisy data from negatively affecting the system decision. Biometric traits fusion solves the non-universality problem since the system can recognize a user by other biometrics if the user does not possess one particular biometric. More biometrics traits can provide a better classification of biometric data and address inter-class similarity and intra-class variations problems. Moreover, it is not easy to spoof several biometrics traits at the same time (Oloyede and Hancke, 2016). In multimodal biometric systems, the different traits are fused by using the available information in any of the four biometric system modules. The types of fusion are (1) sensor-level fusion, where the raw data from the sensors are fused to produce raw fused data, (2) feature-level fusion, where the features from each biometric trait are concatenated into one new feature vector using an algorithm, (3) score-level fusion, where the multiple matching scores from biometric traits are fused in one score using special techniques, and (4) decision-level fusion, where the final classification result is produced by an algorithm. Recently, much effort has been paid to multimodal systems because of their better recognition accuracy compared to unimodal systems (Jain, Ross and Nandakumar, 2011).

Many biometric studies have focused on using machine learning algorithms to recognize individuals (Chaudhary and Nath, 2016; Panasiuk, Szymkowski and Marcin, 2016; Bouzouina and Hamami, 2017; Hezil and Boukrouche, 2017; Veluchamy and Karlmarx, 2017). Machine learning algorithms need feature extraction techniques to extract features from raw biometric data and convert the raw data into a suitable format before classifying the data using machine learning algorithms (Lecun, Bengio and Hinton, 2015). Good results can be obtained by employing machine learning algorithms in biometric systems as pointed out by some researchers

(Chaudhary and Nath, 2016; Panasiuk, Szymkowski and Marcin, 2016; Bouzouina and Hamami, 2017). However, the feature extraction techniques require several pre-processing steps and some techniques may work in some biometric traits and not work effectively with different types of biometric traits or different datasets. Also, machine learning algorithms cannot handle biometric image transformations such as rotation and zooming (Lecun, Bengio and Hinton, 2015).

Recently, deep learning has provided state-of-the-art results for various systems (Lecun, Bengio and Hinton, 2015). Deep learning is a subfield of machine learning that is based on artificial neural networks and learns from deep layered architecture. In general, deep learning trains the machine to perform human-like tasks, such as classifying images and describing contents. The deep learning architecture contains tens or hundreds of successive layers that learn automatically from data (Chollet, 2018). It achieves high accuracy compared to other machine learning algorithms in areas such as computer vision, and natural language processing, among others (Guo *et al.*, 2016; Zhang *et al.*, 2018). The emergence of deep learning methods has solved some of the limitations of other ML techniques as deep learning has benefits over other machine learning algorithms. For instance, deep learning algorithms can solve problems from end-to-end without using features extraction techniques. The deep learning algorithms can automatically extract features from the raw data and classify them (Jürgen Schmidhuber, 2015). In addition, deep learning algorithms can handle image transformation properly. The usage of deep learning has been increased due to three main factors: the increase in the abilities of GPU units, the lower cost of computing hardware, and the improvements in the ML algorithms (Guo *et al.*, 2016). Deep learning has several types of algorithms such as the recurrent neural network (RNN), convolutional neural network (CNN) and deep belief network (DBN) (Lecun, Bengio and Hinton, 2015). CNN is one of the most popular algorithms which is used in image recognition and object detection. It receives an input of an image, which passes through different layers, and then it produces the output that could be the classification result (Guo *et al.*, 2016). The new research in biometric systems tends to use deep learning since such algorithms can achieve better performance and accuracy than other machine learning algorithms (Al-Waisy *et al.*, 2017, 2018; Zhang *et al.*, 2018).

This paper aims to develop a multimodal biometric system for human verification that combines iris and face traits. The iris and face were selected for individual recognition as the face is a natural and acceptable recognition trait and the iris is one of the most precise traits for individual recognition since it contains very unique information. The proposed verification system relies on a multimodal identification model, which is based on an end-to-end CNN deep learning model without deploying any image segmentation or detection techniques. To find out the best fusion approach with the CNN model, two fusion approaches were implemented: feature level fusion and score level fusion. To the best of our knowledge, this approach has not previously been investigated in the existing literature.This paper is organized as follows: Section 2 presents an overview of previous works related to multimodal biometric systems, Section 3 explains the used deep learning algorithm, Section 4 describes in detail the methodology of the proposed approach, Section 5 discusses the experimental results, and finally, Section 6 concludes the work conducted and indicates possible future work.

RELATED WORK

A considerable amount of studies has been done to develop multimodal biometric systems for recognizing individuals by using different techniques. This section provides a review of studies that adopted traditional machine learning approaches and deep learning approaches in multimodal biometric systems.

Machine Learning Approaches: Several biometric recognition studies have aimed to integrate different physical biometric traits. For example, (Chaudhary and Nath, 2016) proposed a multimodal biometric identification system to solve the problem of missing biometric traits. The system fused three biometrics traits (face, iris, and fingerprint) at the score level fusion. The fusion relied on support vector machines (SVMs). The experiments showed that using the three biometrics achieved a higher accuracy (99.8%) than using one or two traits. (Bouzouina and Hamami, 2017) proposed a multimodal verification system that combined the traits of face and iris at the feature level fusion. The system used different methods to extract features from the images, and it applied an SVM method to verify the user identity. The accuracy of the system was 98.8%.

Some physical traits, such as ear and palmprint, have texture patterns, which provide rich information. These information ease feature extraction and recognition. Therefore, (Hezil and Boukrouche, 2017) proposed a biometric system that combined the ear and palmprint traits at feature-level. For user identification, different texture descriptors were used, and three classification methods were tested. In another study, (Veluchamy and Karlmarx, 2017) developed a multimodal biometric identification system based on finger knuckle and finger vein traits, which were fused at feature-level. The system used a K-SVM algorithm, which is an integration of SVM and K-neural network (K-NN). The accuracy of the system was 96%.

There are studies that focused on recognizing the users by behavioral biometric systems, as these systems usually do not require specific devices and run in the background of applications. However, extracting features from behavioral biometric is difficult since it is hard to find a repeated pattern for the user. Thus, (Panasiuk, Szymkowski and Marcin, 2016) combined mouse movement with keystroke dynamics in order to develop a system that employs the K-NN classifier to identify the user. The accuracy of the system was 68.8%.

Deep Learning Approaches: (Ding, Member and Tao, 2015) proposed a deep learning framework for face recognition using multiple face images. The framework is composed of eight CNNs for feature extraction and a three-layer stacked auto-encoder (SAE) for feature level fusion. The CNNs were trained using two datasets: CASIA-WebFace and LFW. The accuracy rate of the framework was 99% and 76.53% when using LFW and CASIA-WebFace datasets respectively. Al-Waisy et al. published two studies (Al-Waisy et al., 2018) and (Al-Waisy et al., 2017), which proposed multimodal biometric systems for identifying users. In (Al-Waisy et al., 2018), the authors developed a system, called IrisConvNet, that combined both the right and left irises at ranking-level fusion. The system started by using an iris localization method to detect the iris region from the eye image. Then the detected region was entered into a CNN model. The experiment showed that the system achieved a 100% recognition rate. In (Al-Waisy et al., 2017), a biometric identification system was developed based on the face and left and right irises. For face identification, a face detection region method was used and then a deep belief network (DBN) was applied. For iris identification, IrisConvNet (Al-Waisy et al., 2018) was employed. The SDUMLA-HMT multimodal dataset was used for system evaluation. In the experiments, different matching scores fusion methods were used. The identification rate of the multimodal system was 100% and the run time of the system was around 3 seconds.

After reviewing the literature, it was noted that there is less usage of deep learning in multimodal biometric systems. The systems that have deployed deep learning in their approach (Al-Waisy *et al.*, 2018) and (Al-Waisy *et al.*, 2017) started by using region detection techniques before entering data into the deep learning model. When using a region detection technique, it is necessary to determine the appropriate technique for the trait, and sometimes these techniques take time (Jain, Ross and Nandakumar, 2011). The reviewed studies are summarized in Table 1.

Table 1. Summary of the related work

Ref #	Operation Mode	Traits	Fusion level	Algorithms Used	Dataset Used	Evaluation Result
(Chaudhary and Nath, 2016)	Identification	Face, Iris, Fingerprint	Score level	SVM	CASIA iris dataset, NIST face and fingerprint dataset	SVM for {Iris, Face, Finger} accuracy = 99.8%
(Bouzouina and Hamami, 2017)	Verification	Face, Iris	Feature level	SVM	CASIA-IrisV3-Interval iris dataset and ORL face dataset	Accuracy = 98.8%
(Hezil and Boukrouche, 2017)	Identification	Ear, Palmprint	Feature level	K-NN, SVM, CRC_RLS.	IIT Delhi-2 ear and IIT Delhi palmprint	Accuracy = 80.53 - 100%
(Veluchamy and Karlmarx, 2017)	Identification	Finger knuckle, Finger vein	Feature level	K-SVM = SVM+ANN	IIT Delhi finger knuckle dataset and SDUMLAHMT finger vein dataset	Accuracy = 96%
(Panasiuk, Szymkowski and Marcin, 2016)	Identification	Mouse movement, Keystroke dynamics	Feature level	K-neural network	The dataset was built from users' behaviors	Accuracy = 68.8%
(Ding, Member and Tao, 2015)	Identification	Multiple face images	Feature level	CNN	CASIA-WebFace dataset and LFW dataset	LFW accuracy = 99% CASIA-WebFace accuracy = 76.53%
(Al-Waisy et al., 2018)	Identification	Pair of irises	Rank level	CNN	SDUMLA-HMT, CASIA-Iris-V3 Interval and IITD iris dataset	Accuracy = 100%
(Al-Waisy et al., 2017)	Identification	Pair of irises, Face	Score/ Rank level	DBN, CNN	NIST, CASIA V1.0, MMU1 and SDUMLA-HMT	Accuracy = 99.91% - 100%.

In our study, we propose a verification multimodal biometric system, which combines face and iris images. The two traits are fused at two fusion levels: feature level fusion and score level fusion. The system applies an end-to-end CNN algorithm without using image detection or segmentation techniques, i.e., the CNN extracts features from the images and recognizes them.

MATERIAL AND METHODS

Convolutional Neural Network (CNN): The Convolutional Neural Networks (CNN) is one of the most famous deep learning algorithms and the most commonly used in image classification applications. In general, the CNN architecture contains three types of layers, which are convolutional layers, pooling layers, and fully connected layers. The CNN algorithm receives an input image that passes through the layers to identify features and recognize the image, and then it produces the classification result. The architecture of the CNN contains alternating convolutional layers and pooling layers, followed by a set of fully connected layers. The output of each layer in the CNN is the input of the following layer.

The input of the CNN is a 3D image (width × height × depth), the width and the height are the dimensions of the images. The depth is the number of input channels and it is three color channels Red, Green, and Blue (RGB).

The convolutional layers extract features from images. Each convolutional has matrices weights that are called filters or kernels which slide over the input image to detect particular information from the image. The filters of the first layers of the CNN detect colors and simple patterns. Then in the next layers, they gradually detect more complex patterns. To find features, each filter applies a convolution operation to output a feature map (Guo *et al.*, 2016). The feature map is defined as:

$$y = f(\Sigma\, W_i X_i), \tag{1}$$

Where W is the weight value from the filter, X is an input value from the image, and i is the pixel number. The function f is an activation function that produces a non-linear output. The used activation function here is Rectified Linear Unit (ReLU). ReLU replaces negative values with zero and it is defined as:

$$f = \max(0, z), \tag{2}$$

Where z is the output of the convolutional layer (Lecun, Bengio and Hinton, 2015).

In the pooling layer, the filter reduces the feature map size to minimize the complexity of CNN. There are different types of pooling and one of the most commonly used type is the max pooling which only takes the most important part of the feature map (Guo *et al.*, 2016).

The size of the resulted feature map from a convolutional layer or pooling layer depends on three values which are depth, stride and zero padding. The depth is the number of filters that are used for feature extraction. The stride is the number of pixels to move the filter across the image. Zero padding is added in some cases in order to control the size of the input images. The size of the feature map can be computed as:

$$\text{size} = ([W\text{-}F+2P]/S) + 1, \tag{3}$$

Where W is the input size, F is the filter size, P is the padding and S is the strides (Dumoulin and Visin, 2016).

The fully connected layers combine features in one-dimensional vector and give the classification result using the softmax classifier (Guo *et al.*, 2016). Softmax is a multiclass classification function and it determines the probability of belonging into each class in the dataset. The probability of belonging the data sample to the i class is computed as follows:

$$\text{Softmax}(i) = \frac{e^{y_i}}{\sum_{m=1}^{n} e^{y_m}}, \tag{4}$$

Where m represents the class, n is the number of the classes and y is computed as follows:

$$y = \sum_{l=0}^{k} w_l x_l, \tag{5}$$

Where x is the feature vector of the data sample and w is the weight vector. The outputted score vector from the softmax classifier can be represented as:

$$\text{Softmax output} = \{p1, p2, p3 \ldots pn\}, \tag{6}$$

Where the pi is the probability of belonging the data sample to the i class (Zeng *et al.*, 2014).

To prepare the CNN algorithm for training, the loss function and the optimizer should be specified. The loss function measures how much the predicted value differs from the actual value. The optimizer or optimization method is a mathematical function that is used to find the values of parameters such as weights and bias values that minimizes the loss value. (Shindjalova, Prodanova and Svechtarov, 2014). To train the CNN model, there are two types of the propagations; forward propagation and backpropagation. In forward propagation, the model takes the input of images and set the filters and the other parameters values randomly. The input is propagated forward through the model and use the random parameter values in order to compute the loss value which measures the distance between the actual output of the model and the expected output. After forward propagation, the model uses backpropagation to reduce the output loss value by an optimization algorithm. In backpropagation, the loss value from the forward propagation is fed back to modify the model weights and param-

eters values in order to reduce the loss value. Therefore, in backpropagation, the parameters are prepared for the next forward propagation. After a number of iterations of forward and backward passes, the model learning can be stopped (Chollet, 2018).

One of the challenges when creating a CNN model is tuning the hyperparameters. Hyperparameter tuning is selecting the optimal hyperparameter values for training the algorithm. Hyperparameters include all the training variables that are related to the model's structure or training algorithms (Chollet, 2018). The CNN hyperparameters include learning rate value, batch size, number of epochs, dropout layers, L1 and L2 regularization and batch normalization layers. In deep learning, there are some techniques that can reduce overfitting problems, for example, adding dropout layers, using L1 and L2 regularization and adding batch normalization layers. Moreover, data augmentation is a technique to reduce overfitting. Data augmentation is a technique which is used to increase the training data by generating more data samples from the existing training samples through various random image transformation techniques such as flipping, shifting and rotation (Lecun, Bengio and Hinton, 2015; Chollet, 2018).

In general, deep learning models are not trained from scratch; this is because deep learning requires a huge dataset and it is rare to have a huge dataset. Training deep learning is also a time-consuming process since training from scratch needs to try different parameters values (weights, number of layers, number of filters, etc.). Therefore, it is common to use a famous pre-trained model such as VGGNet, GoogleNet and AlexNet, which is previously trained on a huge dataset that is called ImageNet dataset (Birks *et al.*, 2016). ImageNet dataset (Krizhevsky, Sutskever and Hinton, 2012) is a dataset that is designed for image recognition research and contains more than 1.2 million images and 1000 classes.

VGGNet: VGGNet is a CNN model and it is developed by Visual Geometry Group at Oxford University. VGGNet is a very deep learning model that has two versions VGGNet-16 and VGGNet-19. The VGGNet-16 contains 16 weighted layers and the VGGNet-19 contains 19 weighted layers. VGGNet achieved an accuracy of 92.3% and 7.3% error rate on ImageNet dataset. Figure 1 shows the structure of the VGG16 model. It takes an RGB image input of size 224 ×224 pixels. The image is passed through five convolutional blocks which have convolutional and pooling layers. VGGNet has a classification block that uses three fully connected layers; the third fully connected layer has 1000 nodes (representing the 1000 classes in ImageNet). A softmax classifier is used in the last fully connected to give the classification result (Simonyan and Zisserman, 2014).

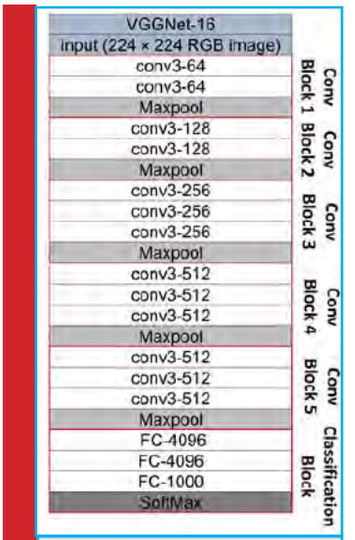

FIGURE 1. VGGNet-16 structure

In Figure 1, the parameters of the convolutional layer are represented as "conv(filter size)-(number of filters)," the pooling layers are represented as "Maxpool," and the fully connected layers are represented as "FC-(number of nodes)" (Simonyan and Zisserman, 2014).

Proposed Method

Figure 2 shows a flowchart of the proposed system. The system utilizes a multimodal model to identify the user from iris and face images. The system captures iris and face images of the claimed user. Then, the user identity

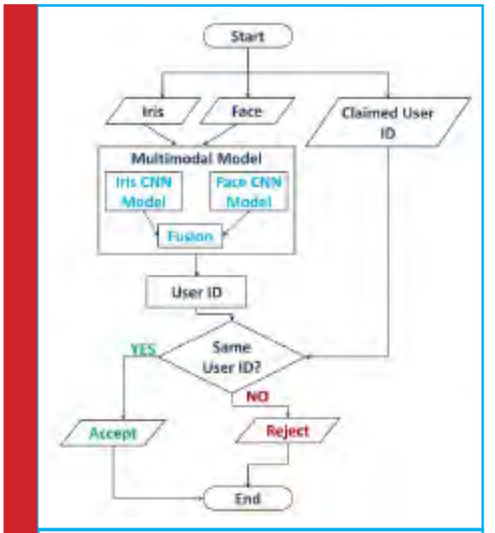

FIGURE 2. Flowchart of the proposed system

is recognized by using the multimodal model, which is composed of two fused CNNs, for face and iris recognition. Finally, the system compares the claimed user ID with the recognized user ID. If they are the same, the system will accept the user, otherwise, it will reject the user. The following subsections present more details about the proposed multimodal model.

The Multimodal Model

Before building the multimodal model, which is based on iris and face data, a unimodal model for each trait was created separately by the CNN algorithm. Following that, the multimodal model for iris and face was built. Therefore, the process of building the model is as follows: Training and testing the iris CNN model. Training and testing the face CNN model.Training and testing the multimodal model for iris and face.We started with training and testing the model of each trait separately to check the effectiveness of each unimodal model before fusing the two unimodal models to be one multimodal model. In our study, we fused the two traits using two different fusion approaches (feature level fusion and score level fusion) to investigate which approach performs better for the proposed system. The feature level fusion is selected because the fusion is done before matching module and usually fusion before matching module is effective since the data in this module have rich information about the features (Vinay Kumar and Srikantaswamy, 2015). The score level fusion is selected as it usually produces good performance and contains sufficient information to recognize biometric data. In addition, it can enable multimodal biometric systems to operate in less constrained conditions (Fernandes and Bala, 2016).

Figure 3 and Figure 4 presents the created multimodal CNN models that used the feature level fusion approach and score level fusion approach, respectively. As it is shown in the two approaches, face and iris images are retrieved from the datasets and then a simple pre-processing step is done. After that, each individual biometric trait is fed into a fused CCN model that outputs classification result which is the user identity. The following parts explain each part of the model.

CNN Models

To create the iris CNN model and face CNN model, we did not train the models from scratch. We used the pre-trained model VGG16 (Simonyan and Zisserman, 2014). One of the necessary modifications that was replacing the last fully connected layer in VGG16 with a new fully connected layer that has a number of nodes equal to the number of classes in the used dataset. To build our CNNs models using VGG16, the first four blocks in VGG16 weights were frozen since the base layers' filters search

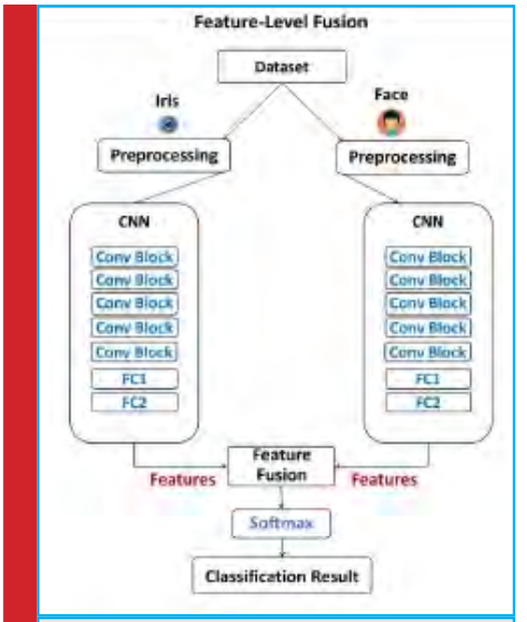

FIGURE 3. Multimodal Biometric CNN models using feature-level fusion approach, Conv Block represents a block of convolutional layers and pooling layers and FC represents a fully connected layer

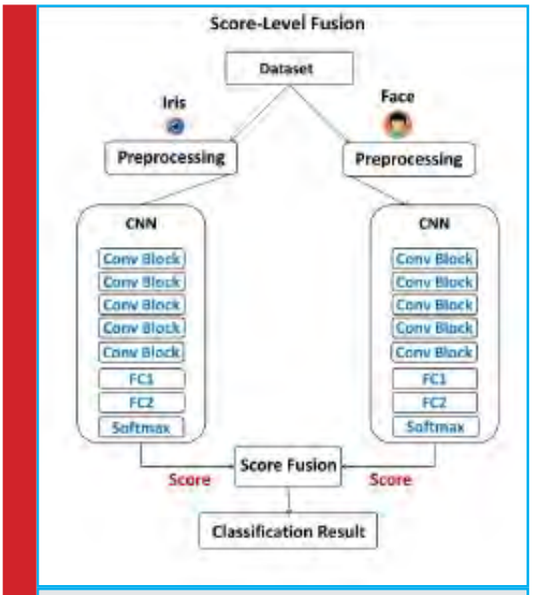

FIGURE 4. Multimodal Biometric CNN models using score-level fusion approach, Conv Block represents a block of convolutional layers and pooling layers and FC represents a fully connected layer

for generic features from the images, for example, lines and angles. Only the top layers (the fifth block) were trained because its filters search for more specialized features such as complex objects.

In each CNN model, hyperparameter tuning was performed in order to find the hyperparameter values that result in the best performance.

Data Pre-Processing

Two pre-processing techniques were applied: resizing the images and data augmentation. Each image was resized to 224 × 224 pixels to be suitable for the VGG16 model. Data augmentation is used to increase the training data. The augmentation techniques that used to increase the number of the iris images were rotation, shearing, zooming, width shifting and height shifting. And to increase the faces images rotation, shearing, zooming, width shifting, height shifting, and horizontal flipping were used.

Fusion Approaches

Feature-level fusion: In this fusion approach, the model learned how to recognize the combined features during the training phase. The output of the second fully connected layers of the face and iris CNN models are fused. So, the features vectors that resulted from the second fully connected layer of the two CNN models become one vector of features which can be defined as:

$$\mathbf{X} = \mathbf{xr} \mid \mathbf{xf} \tag{7}$$

Where *xr* is the extracted features from the iris image and *xf* is the extracted features from the face image.

Then, the resulted vector is entered into the softmax classifier, which classifies the image based on the similarity score and then recognizes the person's identity.

Score-level fusion: In this approach, in each CNN model of iris and face, the output of the second fully connected layer is entered into the softmax classifier to calculate the similarity scores. Then, the scores of each person (class) are fused using the Arithmetic Mean rule fusion method. The Arithmetic Mean rule combines the scores by adding the scores for the two traits and then dividing the result by the number of traits (2 traits). The following equation presents the Arithmetic Mean rule is calculated:

$$S = \sum_{m=1}^{J} S_t / j \tag{8}$$

Where S_t is the score vector of the trait t and j is the number of traits (Alsaade, 2008).

The final score vector is normalized, and the output of the model is the identity of the person who has the highest fused score value.

RESULTS AND DISCUSSION

Experimental Setup

Google Colaboratory (Colab) was used to implement the system. Colab is a tool for machine learning research and it allows the user to run the code in a hosted CPU or GPU (*Colaboratory*, 2018). Keras Python library (Chollet, 2015) was used for implementation. Three datasets were used in our study: SDUMLA-HMT, IT Delhi and FERET. IT Delhi and FERET were used for initial experiments on iris and face models respectively. SDUMLA-HMT dataset was selected to evaluate the performance of the proposed multimodal system. SDUMLA-HMT is a multimodal biometrics dataset, which includes multiple images of different biometric data (e.g. face, finger vein, gait and iris) from 106 individuals (Yin, Liu and Sun, 2011). IT Delhi iris dataset contains data of 244 individuals and it includes multiple images of iris trait (Kumar and Passi, 2010). FERET dataset contains multiple images of faces of 1199 individuals (Phillips *et al.*, 2000).

We tried initially in our experiments to divide the dataset into different percentages, such as [(80:10:10), (60:20:20), (90:15:5), and (70:20:10)] and test the behavior of the system using these percentages. The experiments showed that the best results were achieved using the (60:20:20). Therefore, we divided the dataset into 60% for training, 20 % for validation, and 20% for testing the model.

The dataset images are arranged in three folders for training, validation and testing. Each of the three folders includes folders for all classes, and each class folder contains images. The training set was used to train and fit the deep learning model using the forward and backward passes through the model. The validation set was used to evaluate the given model during the training process, and it is used only the forward pass. The testing set was used to evaluate the final model fit through only the forward pass.

Evaluation Metrics

The main focus is to assess the correctness and the performance of the proposed multimodal system with respect to accuracy and loss value. The following are the metrics that have been used to evaluate the proposed models and explore the hyperparameters effects:

Accuracy:The accuracy is the ratio of correctly classified images to the total number of images (Jain, Ross and Nandakumar, 2011).

$$\text{Accuracy} = \frac{\text{Number of correctly classified images}}{\text{Total Number of Images}} \tag{8}$$

Loss value:The distance between the targeted output of the model and the predicted output. The following equation computes the loss value of the model:

$$\text{Loss Value} = -\frac{1}{N}\sum_i \sum_j t_{i,j} \log(p_{i,j}), \quad (9)$$

Where N is the number of samples, i is a sample, j is the class, $t_{i,j}$ is 1 if sample i is in class j and 0 otherwise, and $p_{i,j}$ is the predicted probability that sample i is in class j (*Categorical crossentropy*).

Iris Model Experiments

A large number of experiments were conducted on the iris model to find the final optimal design of the model. During the training phase, the training was carried out on different learning rate values between 0.00001 to 0.001. The model also was trained using batch sizes of 16, 32 and 64, and different epoch numbers ranging from 20 to 50. The dropout rate was set to values between 0% and 50%. The values used to set L2 regularization were 0.0001 and 0.00001. Moreover, Adam and RMSprop optimization methods were also tried. The final structure of the iris CNN model was the same as VGG16 model, but with some modifications. More layers were added to VGG16. Two batch normalization layers were added after each fully connected layer. One dropout layer was also added before the classifier. The dropout rate was set to 0.3. L2 regularization was added to the two fully connected layers and set to 0.00001. As for the number of epochs, 30 was selected, as this is where the validation accuracy stopped increasing. The model was trained for a batch size of 32 and a learning rate of 0.00001. Adam optimizer (Shindjalova, Prodanova and Svechtarov, 2014) was employed since it obtained the best results among the other optimizers. For calculating the loss value, categorical cross entropy loss function was employed as it is used for multiclass classification tasks. As a result of testing the model using IT Delhi dataset, an accuracy value of 99.55% was achieved along with a loss value of 0.0869 and an error rate of 0.45%.

Face Model Experiments

After conducting a lot of experiments on the face model, the final optimal face CNN model was established. The model was trained using a learning rate of 0.000001 - 0.001, 20 - 50 epochs and a batch size of 16-128. We also tried to drop out of 0% - 50% the neurons. L1 and L2 regularization and regularization parameter values between 0.00001 and 0.001 were also tried. Additionally, several optimization algorithms were tried, such as Adgrad, Adam, Nadam, RMSprop, and SGD.The final model was similar to VGG16 model and some additional layers were added. Three batch normalization layers were added after each convolutional layer in the fifth block of the model. Two batch normalization layers were also added after each fully connected layer. Moreover, a dropout layer was added before the classifier, which its rate was set to 0.3. It was found that L2 with the value of 0.00001 was the best value, as it obtained a lower loss value. The employed learning rate value was set to 0.00001 and the selected batch size was 32. The best rate of accuracy was achieved at the 45th epoch. Adam optimization function (Shindjalova, Prodanova and Svechtarov, 2014) and the categorical cross entropy loss function were employed.The results of model testing using FERET attained an accuracy rate of 98.90%, a loss value of 0.1347 and an error rate of 1.1%.

MULTIMODAL MODEL EXPERIMENTS

Initial Experiments Prior to Building the Multimodal System

As a preparation step for constructing the multimodal system, we tested the proposed iris and face unimodels using the iris and face datasets that are included in the multimodal dataset (SDUMLA-HMT). This has been done for the sake of comparison between the proposed multimodal and unimodal systems on the same dataset. In addition, we wanted to make sure that the iris and face models are effective when using the multimodal data before fusing them together for creating the multimodal system. When using the SDUMLA-HMT-iris dataset on the iris model, the model achieved an accuracy of 98.58%, a loss value of 0.1779 and an error rate of 1.42%. The face model obtained an accuracy value of 98.72%, a loss value of 0.0993 and an error rate of 1.28%. It can be noticed from the obtained results that the proposed iris and face unimodals perform well on the SDUMLA-HMT-iris and SDUMLA-HMT-face datasets.

Multimodal Biometric System Fusion Approach

The multimodal model was created by fusing together the two previous unimodal models (iris and face). The following sections illustrate the experiments of the two applied fusion approaches, which are feature and score level fusions.

Feature-level fusion

For training this model, various epoch values, learning rates, batch sizes and dropout values were considered in the attempt to optimize the model.

The best model was obtained after tuning the learning rate parameter to 0.00001, batch size of 64 and 20 epochs, and adding one dropout layer with a rate of 0.3 before the classifier. Regarding the used optimization method and loss function, Adam and categorical cross entropy were employed.

The proposed model with feature level approach achieved an accuracy rate of 99.22%, a loss value of 0.2417 and an error rate of 0.72%.

Table 2. Evaluation results using the four CNN biometric models

Biometric Model	Iris	Face	Multimodal Biometric	
			Feature Level Fusion	Score Level Fusion
Accuracy	98.58%	98.72%	99.22%	100%

Table 3. Comparison with a previous study

Method	Iris identification model		Face identification model		Multimodal identification model	
	Deep Learning Method	Accuracy	Deep Learning Method	Accuracy	Running	Accuracy
Proposed Model	CNN	98.58%	CNN	98.72%	Feature-level: 0.022 second	Feature-level: 99.22%
					Score-level: 0.021 second	Score-level: 100%
(Al-Waisy et al., 2017)	CNN	100%	DBN	85.34%	Score-level: 3 seconds	Score-level: 100%

Score-level fusion

This approach combines the classification scores of the iris and face models by using the average.

As a result of testing the model using the score level approach, the accuracy value of 100% was achieved along with a loss value of 0.1707 and an error rate of 0%.

Table 2 summarizes the evaluation results of the conducted experiments. It is clear from the table that the multimodal biometric model with two types of fusion achieved higher accuracy when compared with the unimodal models as expected. Thus, it is apparent that multimodal biometrics is an effective method to improve the accuracy of person recognition. The accuracy of the multimodal model that used the score level fusion was better than the multimodal model that used feature level fusion. This is probably because the performance of the softmax classifier was better when receiving the average of the two scores from the iris and face models than when receiving a vector of fused features from the iris and face models.

Comparison with a Previous Study

For the sake of comparison and to clarify the impact of the proposed models, the proposed models were compared against other models from a previous study (Al-Waisy et al., 2017) that used SDUMLA-HMT dataset to train a multimodal biometric system for identifying the iris and face traits. Table 3 presents the comparison results of the proposed models and the previous model in (Al-Waisy et al., 2017). As it is clear from Table 3, the iris identification model in the previous study (Al-Waisy et al., 2017) got an accuracy of 100%. The reason behind the high accuracy of this iris identification model is that the authors treated the iris identification model as a multimodal system, which is based on the traits of both right and left irises. Another reason for the high accuracy rate could be because of the multiple pre-processing steps that were applied to the iris images in order to detect specific parts of the images before inserting the images into the deep learning model. The pre-processing of the images consumed time and therefore increased the running time of the model. Additionally, it can be seen from the table that, the accuracy of the face identification model which the multimodal system depended on was 85.34%, and the accuracy of our proposed face identification model is 98.72%. So, the CNN algorithm that utilized in our proposed face identification model can achieve better accuracy than the DBN algorithm in (Al-Waisy et al., 2017). Moreover, the multimodal identification model in the previous study achieved a higher accuracy (100%) than our model (99.22%), when the feature level fusion approach is adopted. On the other hand, the accuracy rate of the proposed model is 100% with the score level fusion. Moreover, the authors in (Al-Waisy et al., 2017) used matching score fusion methods that combine scores at the score level (e.g. sum, weighted sum and max) and rank level (e.g. highest rank). Combining the scores using these methods can make the effect of the iris model, which obtained high accuracy rate, on the system outweighs the effect of the face model (Alsaade, 2008). So, the multimodal model got a very high accuracy, even though the face identification model got much less accuracy.

CONCLUSION

This paper proposes a multimodal biometric system for human verification using a CNN deep learning algorithm. The proposed system uses a combination of face and iris traits. Two CNN models are designed; one is used for

iris recognition, while the other is for face recognition. Following this step, the multimodal model is created to verify a user's identity. The performance of the models was assessed using SDUMLA-HMT dataset. Different hyperparameters combinations were explored in order to find the optimal hyperparameters for the deep learning algorithm. The results of the experiments showed an accuracy of 98.58% for the iris CNN model, while the face CNN model obtained an accuracy of 98.72%. The multimodal identification model obtained an accuracy rate of 99.22% and 100% when fusing the traits at the feature level and the score level, respectively. Finally, the proposed multimodal model was utilized for the user verification system. With regard to future work, we plan to extend our experiments to test the behavior of the proposed model when dealing with different multimodal datasets and explore the effect of using deep learning algorithms on recognizing other biometric traits such as hand geometry, signature, and DNA.

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Genetic Polymorphism Studies in MTHFR Gene with Acute Myeloid Leukemia in the Saudi Population

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ABSTRACT

Acute myeloid leukemia (AML) is connected with the leukemia cells, highly malignant, which invades the bone marrow and results in normal hematopoiesis. AML is the most commonly seen in adults as acute leukemia. The current study aims to investigate the possible association between C677T and A1298C polymorphisms in the *MTHFR* gene in AML patients in the Saudi population. In this case-control study, 100 AML cases and 100 normal healthy controls were adopted based on the inclusion and exclusion criteria of the subjects. For each patient, 2 mL of the peripheral blood was collected in an EDTA vacutainer and genomic DNA was extracted using the specialized kits. Polymerase chain reaction was performed for the C677T and A1298C variants using the specific primers in both AML cases and controls. The risk of AML through molecular analysis of cases and controls were analyzed through statistical analysis. The significant difference was found with the age in AML cases and controls (p=.02), but not with the gender (p>.05). No significant association was occurred either with allele or genotype frequencies in C677T (T vs C: OR-0.75 (95% CI:0.38-1.46); p=.39); CT vs CC: OR-0.72 (95% CI:0.35-1.46); p=.37) and A1298C polymorphisms (C vs A: OR-1.03 (95% CI:0.60-1.76); p=.89); AC vs AA: OR-1.04 (95% CI:0.57-1.89); p=.88). The results of this case-control study suggested for the first time in the Saudi population that the C677T and A1298C polymorphisms were not associated and may not constitute a shared genetic risk factor for AML patients in the Saudis.

KEY WORDS: ACUTE MYELOID LEUKEMIA, MTHFR, C677T AND A1298C

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INTRODUCTION

Leukemias are defined as group of heterogeneous malignancies which are normally categorized through acquiring somatic mutations such as chromosomal translocations, inversions and deletions, (Liu et al., 2016). Acute myeloid leukemia (AML) is one type of leukemia, which remains as one of the leading causes of global disease in both children and adolescents, (Zampini et al., 2018). AML, one of the common blood cancers, which is highly prevalent, is organized as an aberrant developmental hierarchy maintained through functionally distinct in leukemia stem cells, (Fujita et al., 2018, Wingelhofer et al., 2018). AML is known to be a heterogeneous group of disease characterized by high degree of heterogeneity with respect to chromosomal abnormalities and genetic/non-genetic variants, which translate to marked alterations in treatment response and survival, (Park et al., 2018).

Cytogenetic analysis is closely associated with specific cyto morphological subtypes well-defined by French-American-British criteria, consists of translocations between t (8; 21), t (15; 17), and t (16; 16) /inv (16), (Rose et al., 2017). As per the classification of world health organization (WHO), there were about more than 20% of AML blasts, (Bosshard et al., 2018). Almost 80% of AML patient receives complete remission and among them 50% of them relapse at a later time. The cytogenetic and molecular genetic changes play a major role in the pathogenesis of AML and also have an impact on prognosis of AML patients, (Both et al., 2017).

The precise molecular mechanisms of AML are unknown. Apart from this both genetic and environmental factor plays a major role in the development of AML. (Rashed et al., 2018) Genetic polymorphism has been identified with AML disease and role of polymorphism can affect the protein function, promoter activity, and mRNA stability and splice variants.(Seedhouse et al., 2004) Case-control, epidemiological and meta-analysis studies have connection with AML and methyltetrahydrofolate reductase (*MTHFR*) gene. (Qin et al., 2014) (Lien et al., 2017) MTHFR is a key enzyme in the encoded protein in folate metabolism converts 5,10-methylenetetrahydrofolate to 5,10-methylenetetrahydrofolate, a co-substrate for homocysteine methylation to methionine, (Smolkin and Perrotta, 2016).

Modifications in quantitative and qualitative in folate metabolism are enhancing the risk factors for leukemia, (Hussain et al., 2012). The common functional polymorphisms in *MTHFR* gene; C677T and A1298C could affect the activity of the enzyme, (He et al., 2014). The rs1801133 (C677T) polymorphism appears at exon 4 and modifies amino acid substitution of alanine-valine at codon 222. The rs1801131 (A1298C) polymorphism present in exon 7 and varies the amino acid substitution of glutamine-alanine at codon 429. (Jiang et al., 2014) Limited studies have been contributed to perform the meta-analysis studies with C677T and A1298C polymorphisms in AML. (Qin et al., 2014) From Saudi Arabia, no genetic studies have been documented till now and based on prior studies; AML subjects were genotyped with C677T and A1298C polymorphisms in the *MTHFR* gene. Here, the present study aimed to investigate the relationship between C677T and A1298C polymorphisms in *MTHFR* gene and susceptibility to acute myeloid leukemia in the Saudi population.

MATERIAL AND METHODS

AML subjects: In this study, 100 AML cases and 100 healthy controls were recruited from the Department of Hematology and Oncology in Riyadh regional hospital. AML diagnosis was confirmed through (i) bone marrow examination, (ii) full blood count and with (iii) flow cytometry. Apart from this pathology tests, cytogenetic analysis, such as chromosomal report and fluorescent in situ hybridization was also performed to reconfirm the results. During January 2016-November 2017, the blood samples were collected for this study. The inclusion criteria for AML cases were based on the following norms such as (I) Saudi nationality, (II) adolescent male and female subjects, (III) disease diagnosed through histopathological/cytogenetic confirmation and (IV) written and signed consent inform. The exclusion criteria were (I) Non-Saudi, (II) Patient diagnosed with another type of cancers and (III) Unsigned consent form. Age matched controls (N=100) were selected in Saudi nationality without effecting any type of specific cancers. From Riyadh regional hospitals, ethical grant was received through the Ministry of Health Affairs along with the signed inform consent form from 200 participants elaborated in this study as per the Declaration of Helsinki. From all the participated subjects, 2 mL of the peripheral blood was collected in an EDTA vacutainer and stored in the freezer for the further molecular analysis.

Molecular analysis: Two hundred genomic DNA was extracted from blood samples using with the genomic DNA purification kit (Sigma-Aldrich) as per the companies' protocol. Isolated and purified genomic DNA were confirmed through 1% agarose gel electrophoresis and cleansed DNA samples was stored at -40C in the freezer. The rs1801133 (C677T) and rs1801131 (A1298C) polymorphisms were genotyped using polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) analysis. Complete details of the primers, restriction enzymes and PCR sizes are shown in Table 1. The 50-microliter PCR master mix consisted of

Table 1. Involvement of primer sequences in this study

SNP	rs number	Primer sequences	Amino acid substitution	PCR product	Enzyme
C677T	rs1801133	F: TCACCGGATCATGGCCAGCA R: TTCCTTACTGGTCCTCACATCTC	Ala222Val	198bp	HinfI
A1298C	rs1801131	F: GAACTCCCTGAAAAGCTAAAGC R: GTTGGGCTCAAATATACGGTGG	Glu429Ala	145bp	MboII

4 µL of genomic DNA (40-60 ng/µL) and 30 µL of PCR mix containing 10X buffer, $MgCl_2$, dNTPs, and 10x Taq DNA polymerase. The 10 pmoles of 2 µL of forward and reverse primers were added to the master mix followed by the addition of 12 µL of distilled water. PCR reaction was standardized for the final volume of 50 µL. C677T and A1298C primers were adopted from earlier studies, (Khan et al., 2015, Tanyildiz et al., 2016). PCR conditions for C677T and A1298C polymorphisms were as follows. Initial denaturation and denaturation were carried out for 5 mins at 94°C and 94°C for 30 secs, followed by 35 cycles. The annealing temperatures for C677T were 56°C and 58°C secs for A1298C polymorphisms respectively. Extension and final extension were found to be 72°C for 45 sacks and 5 mins respectively.

The PCR products were digested for 16 hours at 37°C with both the restriction enzymes as *HinfI* and *MboII*. The digested products of *HinfI* enzyme when electrophoresed through 2% agarose gel indicated the normal homozygote (CC) as 198bp. Mutant homozygote (TT) will expose two bands of 175 bp and 23 bp, whereas the heterozygous (CT) genotype will inferred from three bands of 198 bp, 175 bp, and 23 bp. The A1298C polymorphism abolishes a *MboII* restriction site and digestion results in 100 and 45bp fragment in the presence of the 1298C allele; and 75,45 and 25bp fragments in the absence of the 1298A alleles. Both the fragments from the AC fragments as 100,75,45 and 25 bp respectively.

Statistical analysis: Clinical data were statistically analyzed using Openepi software.(Khan et al., 2015) To compare the observed and expected genotype frequencies, Hardy-Weinberg Equilibrium (HWE) was performed. Genotype differences between cases and controls were executed with the odds ratios, upper and lower limits of the 95% confidence intervals (95% CI) for C677T and A1298C polymorphisms. The overall values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Characteristics of the subjects: One hundred AML cases and 100 healthy controls were recruited in this study. Data on clinical traits between the cases and controls such as age and gender were recorded and documented in Table 2. The age range of AML cases were in between 19-82 years [mean 38.9 (15.1)] and in controls, it was 18-63 years' old [mean 39.91(2.06)]. There was a significant difference between the cases and controls in the age (p=.02). The AML patients comprised of 61 males and 39 females and in the control subjects there were 54 males and 46 females.

HWE tests and genetic analysis: Genotypic distribution between C677T and A1298C polymorphisms were in accordance with HWE (p<.05). Both the allelic and genotypic distributions between AML cases and controls in C677T and A1298C polymorphisms were shown in the Table 3. The results showed in Table 3 both the allelic and genotypic association in C677T (T vs C: OR-0.75 (95% CI:0.38-1.46); p=.39); CT vs CC: OR-0.72 (95% CI:0.35-1.46); p=.37) and A1298C polymorphisms (C vs A: OR-1.03 (95% CI:0.60-1.76); p=.89); AC vs AA: OR-1.04 (95% CI:0.57-1.89); p=.88) were not significant between AML cases and controls. The dominant, co-dominant and recessive models also failed to show the significant associations in both the polymorphisms (Table 3).

The current study has designed as case-control to explore the association between C677T and A1298C genetic polymorphisms in the *MTHFR* gene and its effect

Table 2. Anthropometric details of the patients involved in this study

	AML cases (n=100)	Controls (n=100)	p-Value
Age (Years)	38.9±15.1	39.9±12.06	0.02
Minimum and maximum ages	19-82	18-63	-
Males	61 (61%)	54 (54%)	-
Females	39 (39%)	46 (46%)	-
N/A= Not analyzed/ Not applicable			

Table 3. Genotype and allele frequency distribution between AML cases and controls with C677T and A1298C variants in the MTHFR gene

MTHFR (rs1801133)	AML Cases (n=100)	Controls (*n*=100)	Odds Ratio	(95% CI)	*p* value[a]
Genotype and allele	N (%)	N (%)			
CC	83 (83%)	78 (78%)			
CT	17 (17%)	22 (22%)	0.72	0.35-1.46	0.37
TT	00 (00%)*	00 (00%)*	1.00	0.02- 99.99	0.99a
CT+TT vs CC	17 (17%)*	22 (22%)*	0.73	0.36-1.47	0.37a
CT vs CC+TT	17 (17%)*	22 (22%)*	0.73	0.36-1.47	0.37a
TT vs CC+CT	00 (00%)*	00 (00%)*	1.00	0.02- 99.99	0.99a
C	183 (91.5%)	178 (0.89)			
T	17 (8.5%)	22 (0.11)	0.75	0.38-1.46	0.39
MTHFR (rs1801131)					
AA	67 (67%)	68 (68%)	Reference		
AC	33 (33%)	32 (32%)	1.04	0.57-1.89	0.88
CC	00 (00%)	00 (00%)	1.00	0.01-51.12	0.99a
AC+CC vs AA	33 (33%)*	32 (32%)*	1.04	0.58-1.88	0.88a
AC vs AA+CC	33 (33%)*	32 (32%)*	1.04	0.58-1.88	0.88a
CC vs AA+AC	00 (00%)*	00 (00%)*	1.00	0.01-50.88	0.99a
A	167 (83.5%)	168 (84%)	Reference		
C	33 (16.5%)	32 (16%)	1.03	0.60-1.76	0.89
* & a *p value* after Yates Continuity Correction.					

on AML disease in the Saudi population. The current findings showed non-significant association between the cases and controls. To the best of my knowledge, this is initial study implemented with the association of C677T (rs1801133) and A1298C (rs1801131) polymorphisms in the *MTHFR* gene in AML disease risk in the Saudi Arabia. C677T and A1298C polymorphisms in *MTHFR* gene is well-known genetic polymorphisms that have an effect on human diseases such as PIH, GDM and PCOS, (Wu et al., 2013, Khan et al., 2015, Carlus et al., 2016). AML is confirmed as biological and clinically heterogeneous cancer of bone marrow, characterized through the rapid growth of abnormal myeloid cells.(Passaro et al., 2017) AML is known to be genetic and molecular heterogeneous disorder characterized by uncontrolled proliferation and blocked maturation of abnormal myeloid precursors.(Zhang et al., 2018b).

The disease has been classified based on histologic, cytogenetic and molecular genetic characteristics. AML is known to have genetic and molecular changes that alter normal hematopoietic growth and differentiations results in the accumulation of large numbers of abnormal, immature myeloid cells in the bone marrow and cytogenetic- molecular morphologies becomes the cornerstones of the therapeutic plainings, (Bacher et al., 2010). Patients diagnosed with AML and CCAAT/ enhancer-binding protein alpha (*CEBPA*) mutations confirm the deficiency of leukopenia, which leads to infections. Fever and weight loss are common symptoms associated with *CEBPA* variants, (Ho et al., 2009, Mannelli et al., 2017).

Recent advanced molecular techniques such as second generation advanced technique, micro array and the detection of molecular markers and their characterization has been aided with AML, (Bacher et al., 2010).The relation with AML and C677T/A1298C polymorphisms has been documented with multiple molecular studies. The enzyme 5,10 methylenetetrahydrofolate reductase plays a crucial role by irreversibly reducing 5,10 methylenetetrahydrofolates to 5 methyltetrahydrofolate, the predominant circulatory form of folate, (Bănescu and Trifa, 2015). A couple of the genetic polymorphisms involved in this study plays a role in reducing the MTHFR enzymatic activity from 40-70% in homozygous or either heterozygous subjects, affecting the folate metabolism, (Yaliwal and Desai, 2012). Low levels of folic acid may

lead to elevated uracil incorporation into DNA and this reduced DNA repairs the capacity which then leads to altered DNA methylation, which may promote leukemogenesis, (Duthie et al., 2000). Through this mechanism, the connection was bonded between AML and *MTHFR* genetic polymorphisms, (Kaur and Kaur, 2016, Jin et al., 2018, Rai, 2016, Rai, 2018, Zhang et al., 2018a).

Till now only single meta-analysis study has been documented with negative association in AML.(He et al., 2014) Maximum studies carried out with AML and *MTHFR* gene showed the negative association with the combination of the C677T and A1298C variants, (Skibola et al., 1999, Deligezer et al., 2003, Chen et al., 2006, da Costa Ramos et al., 2006, Bolufer et al., 2007, Moon et al., 2007, Barbosa et al., 2008, Lightfoot et al., 2010, Vahid et al., 2010, Hussain et al., 2012, Huang et al., 2015, Liu et al., 2016., Lien et al., 2017). The individual combination of C677T, (Moon et al., 2007, Skibola et al., 1999) and A1298C, (Vahid et al., 2010, Zheng et al., 2013) variants showed the positive associations with AML. There are limited genetic studies on next-generation and exome sequencing studies have been documented with AML through the worldwide, (Ley et al., 2008, Koh et al., 2014, Ilyas et al., 2015, Heo et al., 2017, Zhang et al., 2018b).

Ley et al., (2008) had conducted an initial study to perform next-generation sequencing in AML patients whereas, Heo et al., (2017) performed whole exome sequencing analysis in 36 Korean patients and identified 11 novel mutations; among them five of them were previously documented. Zhang et al (Zhang et al., 2018b) performed the whole exome sequencing studies in pediatric AML children. The results concluded from this study confirm the novel insights into the genetic basis of treatment failure in AML children.

The strength of the current study was the incorporation of 100 AML cases and 100 healthy controls. This study has certain limitations such as skipping the body mass index, family history and other clinical details. I have skipped the cytogenetic analysis and FISH data of AML patients and healthy controls. I did not validate the genotyping results through Sanger sequencing. Although the purpose of recruiting the patients from the hospital is to ensure the complete geographical coverage of the Kingdom of Saudi Arabia, this study results may not reflect the trend of the entire Saudi population.

To the best of my knowledge, this is the first genetic study investigated the association of the C677T and A1298C genetic polymorphisms with AML risk in Saudi Arabia. These results confirm the negative association; therefore, the *MTHFR* gene polymorphisms may not be associated with susceptibility to AML in the Saudi population. However, earlier global results along with meta-analysis studies confirms the negative association. Further studies would be required in different ethnic populations, especially in Arabic countries to facilitate a meta-analysis-based investigation in the future. I strongly recommend employing next-generation sequencing, exome sequencing-based examination in a larger cohort of AML cases with elaborated clinical information of the patients.

Conflict of Interest

There is no conflict of Interest towards this manuscript

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Role of Genetic Variants in Immunoregulatory and Oxidative Stress Genes with Predisposition to Pre-eclampsia: A possibility for Predicting the High Risks in Synergetic Reaction

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ABSTRACT

The multifactorial basis of pre-eclampsia (PE), also known as pregnancy-induced hypertension, involves a combination of genetic risk factors that cause disease development in women during pregnancy. The prevalence of PE varies in different ethnicities from 8 to 20%. The precise etiology and pathophysiology remain unclear, and immunoregulatory pathway genes Forkhead Box P3 (*FOXP3*) and Cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) and oxidative markers angiotensin converting enzyme (*ACE*) and endothelial nitric oxide synthase (*eNOS*) have been suggested as risk factors for PE development. This review article describes the possible synergic interactions between polymorphic variants in *FOXP3*, *CTLA4*, *eNOS*, and *ACE*, which contribute to immunoregulatory and oxidative stress in women with PE. We screened for studies describing the combinations of all four variants. Previous studies showed that all these genetic markers lower the expression and production of surface molecules, which may enhance the risk and susceptibility to PE. Based on previous studies and meta-analysis, we recommend that genetic screening of a large sample size must be carried out to confirm the roles of these variants in PE.

KEY WORDS: PRE-ECLAMPSIA, FOXP3, CTLA-4, ENOS, ACE, POLYMORPHISM

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INTRODUCTION

Pre-eclampsia (PE) is a pregnancy-specific disorder characterized by hypertension and proteinuria that appears in the 20th week of gestation. This multifactorial pregnancy-related disorder is associated with maternal morbidity and mortality (Steegers et al., 2010, Jahan, et al., 2013, 2014 and Al-Jameil et al., 2014). Globally, PE affects up to 20% of pregnant women. The incidence of PE is associated with genetic and environmental factors. The pathophysiological relationship between genetics and PE remains unknown (Khan et al., 2015 Berhe et al., 2018).

Various factors play important roles in the predisposition to pregnancy-associated conditions including obesity, dyslipidemia, oxidative stress, and possibly maternal immune responses to fetal antigens (Bianco et al., 1998; Hubel, 1998; Zenclussen, 2013). PE, also known as pregnancy-induced hypertension (PIH), disproportionately occurs in the first pregnancy (Kamineni 2015). Genetic evidence from family-based studies revealed that PE is common among the daughters' and sisters of women who had PE (Buurma et al., 2013). PE is typically diagnosed during late pregnancy based on proteinuria, edema, and increased vasoconstriction leading to maternal hypertension and decreased uteroplacental blood flow. Numerous studies confirmed that PE is an immune-mediated disorder. Normal pregnancy involves maternal immune tolerance in the fetus. Immunological factors appear to greatly contribute to the predisposition to PE, particularly in primipara women. Forkhead Box P3 (*FOXP3*) and Cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) are immunoregulatory genes, while angiotensin converting enzyme (*ACE*) and endothelial nitric oxide synthase (*eNOS*) are oxidative stress genes. A relationship between immunoregulatory and oxidative stress genes has been demonstrated in PE, (Bianco et al., 1998; Hubel, 1998; Zenclussen, 2013, Pendeloski et al., 2011,Ye et al., 2017).

This review focuses on the combined effects of immune and oxidative components on PE development (Metz et al., 2012). Markers of oxidative stress are pro-oxidant enzymes with endogenously high activity (genetically determined) related to PE. The stimulation and regulation of the immune system is tightly regulated. Additionally, oxidative stress appears to be a central component of both placental and endothelial dysfunction, (Aouache et al., 2018).

Immunoregulatory genes associated with PE

The characteristics of immunopathology for prevalent complex diseases are linked with various immune responses, such as genetic regulation. The initiation, maintenance, and progression of PE have been evaluated by examining combination of genetic factors such as genetic polymorphisms (Mullighan et al., 1999). Numerous genetic polymorphisms and genes were shown to be associated with immunoregulatory pathways; among them, *FOXP3* and *CTLA-4* were shown to be related to PE.

Genetic association with *FOXP3* in women diagnosed with PE

FOXP3 is an immunoregulatory gene that plays an important role in pregnant women and specifically in PE as suggested previously. Regulatory T cells (Tregs) have important functions in the immune response and in genes. *FOXP3* is important in the development of Treg cells (Gholami et al., 2018) and contains 12 exons coding for 431 amino acids with a molecular weight of 47.25 kDa. *FOXP3* is located on chromosome at Xp11.23. In mouse models, inactivation in *FOXP3* results in a lack of Tregs and notable organ-specific autoimmunity (Metz et al., 2012). A few genetic variants (-924A/G (rs2232365); -3279C/A (rs3761548) are associated with PE in the global population.

Recently, Hosseini-Teshnizi et al (2019) performed a meta-analysis of the rs2232365 and rs3761548 polymorphisms in pregnant women with PE and infertile women with recurrent pregnancy loss and concluded as *FOXP3* variants affect pregnant women, including those with PE. The -3279C>A polymorphism is a well-known marker of PE used in many studies in different populations. The CC genotype is associated with low FOXP3 levels, while the AA genotype is associated with overexpression of FOXP3. Thus, genotypes at single-nucleotide polymorphism (SNP) position -3279 may play a decisive role in determining risk or protection for such clinically important conditions like PE given the high maternal/fetal morbidity and mortality of this condition (Jahan et al., 2013).

However, Nourouzian et al (2016) confirmed the negative association between -3279C>A polymorphism and PE risk. The CC genotype (C allele) of the SNP at -3279 has been suggested to play a role in other pregnancy-related complications, such as unexplained recurrent spontaneous abortion (Wu et al., 2011). In individuals with the FOXP3 -3279 CC genotype, due to reduced expression of $FOXP3^{+}$ normal functioning of Treg cells assessed by direct contact with the responder cells showed greatly hampered, as a consequence of this certain degree of inflammatory response of mother against fetal antigens can be expected positively contributing to premature delivery. From this perspective, the characteristics of FOXP3 regulatory molecules are important for identifying high-risk mothers. Chen et al (2015) showed that FOXP3 expression levels in the placental tissue of patients with PE are lower than those in normal pregnant women. This indicates that decreased *FOXP3*

expression decreases immunosuppressive functions and causes a maternal-fetal imbalance to induce PE.

Role of *CTLA-4* in women with PE

CTLA-4 is a member of the immunoglobulin family and transmits inhibitory signals to T cells. *CTLA-4* is a CD28 homologue expressed on the surface of T-lymphocytes. Cytotoxic T-lymphocyte antigen is considered as an important immunoregulatory molecule expressed constitutively in Treg cells. While these regulatory molecules must be expressed in conventional T cells following activation, CTLA affects CD4 T-cell activation (Frauwirth & Thompson, 2002; Rasti & Nasiri, 2016). CTLA-4 dysregulation appears to play a vital role by affecting normal fetal tolerance through exacerbated activation of T-cells on the fetal antigen (Kaufman et al., 1999). Gene mapping analysis revealed that *CTLA*-4 is on chromosome 2q33 and consists of at least 100 polymorphic sites (Karabon et al., 2009). *CTLA-4* plays a role in maintaining pregnancy and fetal-maternal tolerance through its expression in placental fibroblast and decidua cells. Polymorphic variants of *CTLA-4* are associated with reduced expression are likely involved in pregnancy-related disorders (Bonyadi et al. 2017).

Further quantitative variations in *CTLA-4* expression affected by genetic variants (rs231775) may be associated with pregnancy-related disorders such as PE. Some studies reported that the +49A>G rs231775 SNP causes recurrent spontaneous abortions. However, Dehaghani et al 2005) suggested that a heterozygous *CTLA-4* A49G allele is a predisposing factor for severe preeclampsia in Iranian women. Zhou et al (2016) found no association with the +49 A/G (rs231775) polymorphism in *CTLA-4* in PE in a Chinese population. Pendeloski et al (Pendeloski et al., 2011) found a similar pattern of a negative association in Brazilian women. Finally, in a Finish population, Jaaskelainen et al (2008) found a significant genetic association with the +49A/G polymorphism in women with PE.

Synergistic effect with combined *FOXP3* and *CTLA-4* variants in identifying genetic risk of PE

Because of the important regulatory function of FOXP3 and requirement for its continuous synthesis in Tregs cells to ensure normal functioning, any SNP either in the promoter or exon regions may greatly impact gene expression. The -3279 variant may alter FOXP3 levels. Reduced expression of this regulatory molecule, as observed in the CC genotype, likely leads to impaired functioning of FOXP3 regulatory molecules, such mutations in combination with CTLA the -4 GG or G allele in heterozygotes, which may confer a high risk of a maternal inflammatory response against the fetus. The G allele in CTLA-4 reduces the regulatory ability of Treg cells because of the significantly decreased CTLA-4 surface levels. A combination of SNPs in these two loci likely to contribute to protection against the mother's response to fetal antigens. This is possible in a *FOXP3* genotype with normal activity which does not affect the normal expression of FOXP3. In combination with CTLA-4 A49G genotype, which results in a normal level of surface expression of CTLA-4 molecule with relatively reduced clonal expansion. This hypothesis is important, as there are no published reports on the synergistic effects of genes involved in predisposition to important pregnancy-associated disorders. However, in recurrent spontaneous abortion, a similar synergistic effect was observed for SNPs in the FOXP3 and CTLA genes which differ from that proposed in the present study (Fan et al., 2018).

Oxidative stress markers: An imbalance between oxidants and antioxidants leads to redox signaling disruption and molecular damage, a condition known as oxidative stress (Aouache et al., 2018). PE is a pregnancy-related disorder considered to have multifactorial origins including the involvement of genetic and environmental factors. Oxidative stress marker (ACE and eNOS) variants have been suggested to significantly affect the development of PE (Hubel, 1998).

Risk of PE in women with *eNOS* genetic markers: Enhanced nitric oxide synthase (eNOS) and PE are both related to hypertension. eNOS is synthesized in endothelial cells by nitric oxide synthase to regulate blood flow and vasomotor tone. eNOS also increases the blood volume to enhance cardiac output and reduce blood pressure. The eNOS gene is on chromosome 7q35-36 in humans. This gene has been widely suggested to be involved in PE development. Three important variants have been reported to increase the risk of PE (-786T/C; 4b/4a; and G894T). A mutation in the promoter region at position 786 causes a T to C substitution. A variable number of 27-base pair tandem repeats in intron 4 (4b/a) and G-T mutation were found at nucleotide position 894 in the eNOS gene. Position 298 is prone to amino acid substitution from Aspirin to glutamine (rs1799983) (Dai, et al. 2013; Ma et al., 2016).

The eNOS enzyme synthesizes NO from L-arginine and functions as a vasodilator molecule crucial for regulating endothelial function and consequently maintaining homeostasis. An SNP in the eNOS gene at G894T results in low NO production, thereby causing endothelial dysfunction and contributing to pregnancy-related hypertension (Rahimi et al. 2013; Zeng et al., 2016). Few reports have also suggested that the polymorphism G894T reduces eNOS activity and decreases the plasma level of NO. Rahimi et al (2013) investigated the influence of eNOS 4a/4b and its synergistic potential with eNOS G894T polymorphisms on affecting PE risk and

confirmed a negative association; the T allele was not associated with PE. However, a concomitant risk was observed for eNOS and T allele did not significantly increase the risk of severe PE. Another study by Zeng et al (2016) confirmed the positive association between the recessive model and PE. Qi et al (2013) found that the inveterate TT genotype is associated with the eNOS G894T polymorphism and an increased risk of PE.

Ma et al (2016) performed a meta-analysis of 11,700 subjects (4028 cases vs 7672 controls) and observed that the G894T polymorphism in eNOS negatively affected PE/PIH. The samples were selected from 36 case-control studies of African, American, Asian, European, and Latin American populations. In Asians, the T allele was associated with a higher risk of PE compared to the G allele. A dominant model association was observed with PE in Latin American and African populations. However, in both Americans and Europeans, no association was detected. Another meta-analysis study showed that the -786C/T and 4b/a variants greatly contribute to the risk of PE, while the G894T polymorphism showed no association with PE (Dai et al., 2013).

Genetic role of insertion and deletion polymorphism in ACE gene in women with PE

ACE plays a major role in the renin-angiotensin system cascade by converting angiotensin-I to angiotensin-II to affect blood pressure. The 287-base pair Alu sequence affects the presence or absence of the intron 16 sequence in *ACE* (Khan et al 2014). During pregnancy, the circulating and intrarenal renin angiotensin aldosterone system control the salt-water balance to maintain maternal blood pressure and adequate placental perfusion both in the mother and fetus (Cristina et al., 2019).

Gene mapping analysis revealed that the *ACE* gene is located at 17q23 and consists of 26 exons and 25 introns (Khan et al., 2014). Genetic studies have been carried out to evaluate global population and confirmed the significant and non-significant associations of *ACE* I/D gene polymorphisms in women with PE (Cristina et al., 2019; Jahan et al., 2014; Ma et al., 2016; Miao & Gong, 2015; Qi et al., 2013). Four studies conducted in different regions in India revealed negative (Aggarwal, Jain, & Jha, 2010; Aggarwal, Dimri, Tandon, & Agarwal, 2011; Kaur, Jain, Khuller, Gupta, & Sherawat, 2005) and positive associations between the *ACE* gene and PE (Jahan et al., 2014). Meta-analysis studies also showed positive (Zhong, Wang, Zhu, & Zhao, 2012) and negative associations (Shaik, Sultana, Bammidi, Sampathirao, & Jamil, 2011).

Combination of synergistic effect with *ACE* and *eNOS* variants: While ace and eNOS have been evaluated individually, studies are needed to examine their synergistic effects, which may be useful for predicting the risk of PE and gestational hypertension. The synergistic effect may involve a combination of two D alleles with *ACE* I/D polymorphism and low nitric oxide-producing eNOS gene likely enhances the risk of PIH. The ACE enzyme in the renin-angiotensin-aldosterone system converts Ang I to Ang II. Ang II is a central molecule in this system and is responsible for increasing blood pressure via more than one mechanism (Khan et al., 2014) such as by (i) vasoconstriction, (ii) inducing NADPH oxidase, an enzyme involved in generating free radicals of oxygen, and (iii) producing reactive oxygen species which may interact with nitric oxide and converts it into peroxynitrite, thereby reducing the bioavailability of nitric oxide. The synergistic interaction between ACE and eNOS and their effects on PIH require further detailed analysis. Understanding such synergistic interactions conferred by genotypes at two loci in the same patient can enable gynecologists to manage hypertension and associated conditions in mothers with PE and gestational hypertension.

CONCLUSION

A relationship may exist between the genetic polymorphisms documented in immunoregulatory genes and oxidative markers related to PE. Global populations showed genetic associations with *FOXP3*, *CTLA-4*, *eNOS*, and *ACE* polymorphisms in women with PE, which may be related to ethnicity. Thus, similar polymorphisms should be examined in similar populations with larger sample sizes and the results should be compared with those of previous studies in different ethnicities. Genetic screening studies including large sample sizes are needed.

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Correlation of English Language proficiency with Multidisciplinary Examination Score Achieved by Indonesian First Grade Medical Students

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ABSTRACT

Problem-based learning (PBL) has been implemented to replace classical teaching method with college system in Undergraduate Program of Medical Faculty, Padjadjaran University, Indonesia. In PBL, students are encouraged to be independent to find answers for the problems they faced during discussions based on references that are mostly in English. Since adequate English ability is believed to play pivotal role in helping the students during the learning process, we investigated whether the English proficiency is correlated with academic achievement. A cross-sectional analytical research was conducted to analyze the correlation between Test of English as a Foreign Language TOEFL score and the multidisciplinary examination (MDE) score of the 1st Reproductive System among 194 first grade medical students of Padjadjaran University. Pearson correlation test revealed a significant correlation between TOEFL score and MDE score (r=0.49, IK 95% (0.37; 0.59), p < 0.001). This study revealed that English proficiency is correlated with the student academic achievement.Subjects: Medical education, English proficiency, Problem-based learning, higher education

KEY WORDS: TOEFL SCORE; ENGLISH PROFICIENCY; PBL; ACADEMIC ACHIEVEMENT; INDONESIA

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INTRODUCTION

English is recognized as an international language and also dominantly used in various fields, such as science, medicine, international business, technology, and education. International educational field also used English as the main language, including in medical education (Boulet *et al.*, 2001). Problem-based learning (PBL) is a learning method that encourages the students to learn actively through problems embedded in cases (Barrows, 1996). In PBL implementation, medical students discuss the trigger cases in small groups. Through that process, medical students are expected to be able to understand the learning objectives based on the problems presented in the cases. Problem-based learning has been applied as the main learning method in Undergraduate Program of Medicine, Faculty of Medicine, Universitas Padjadjaran, in 2001, in which English was used as the delivery language. Even though English is not used as daily language in Indonesia, most of learning materials, such as study guide, course materials, laboratory modules, and clinical skill modules, are written in English. Moreover, all student evaluation methods, including multiple-choice questions (MCQ) in the form of multidisciplinary examination (MDE), are also written in English, (Srikrai *et al.*, 2016 Clyne and Sharifian, 2008; McKay, 2018).

Test of English as a Foreign Language (TOEFL) is one of the measurement tools to assess English ability for people who are not using English as the main or mother language (Alderson and Hamp-Lyons, 1996). It tests listening, reading, writing, and speaking ability (Alderson, 2009). There are three types of TOEFL: paper-based TOEFL (PBT), computer-based TOEFL (CBT), an Internet-based TOEFL (IBT). In order to assess the English ability of the students, Faculty of Medicine, Universitas Padjadjaran, hold the TOEFL-ITP (Institutional Testing Program) at the first grade with passing grade score of 550 for promotion to second grade. The TOEFL ITP series was the TOEFL PBT implemented by universities to their students to assess english-language skill with a convenient, affordable and reliable assessment.Since study elaborating the correlation between academic achievement of Medical students and their English ability in Indonesia is still scarce, this study aims to investigate the correlation between TOEFL-ITP score of first grade Medical students of Padjadjaran University and their MDE results.

MATERIAL AND METHODS

Ethical statement: This study was approved by Ethical Committee of Universitas Padjadjaran No. 6482/UN6. C1/DL/2017.Study design: This study is a cross-sectional analytical research using secondary data. The data used were institutional TOEFL PBT score of first grade students and MDE results of Reproductive System I, one of learning modules delivered in first grade in the academic year 2015/2016. The TOEFL ITP was held by Language center, Faculty of Literacy, Universitas Padjadjaran.

Materials and/or Subjects: The population of this research was the first grade students of Undergraduate Program of Medicine, Faculty of Medicine, Universitas Padjadjaran, academic year 2015/2016.

The variables were analyzed in this study were the TOEFL ITP score and MDE score of the 1st Reproductive System of first grade medical students. The data were obtained from Academic Assessment Unit, Faculty of Medicine, Padjadjaran University.

Statistics: This study used total sampling technique. The size of the minimum sample was estimated using the sample size formula for the correlation test. Statistical analysis was performed using Pearson correlation testing, and processed using Graphpad Prism software version 7.

RESULTS AND DISCUSSION

The number of students who met inclusion criteria and were included in this study was 194 subjects (Suplementary data 1). The average TOEFL score for those students was 517.1±66.7. Seventy-two subjects (37.1%) passed the cut-off TOEFL score for promotion to second grade (TOEFL PBT score 550), with the average score was 584±29. The average MDE score of the 1st Reproductive System among subjects was 64.6±9.2. Of all subjects, 160 students (85.2%) passed the minimum MDE passing grade score of 56.Correlation analysis using Pearson test showed positive correlation between TOEFL score and MDE score of Reproductive System I (r=0.49, IK 95% (0.37; 0.59), p<0.001) (Figure 1). These data also revealed that the higher the TOEFL score, the higher the MDE

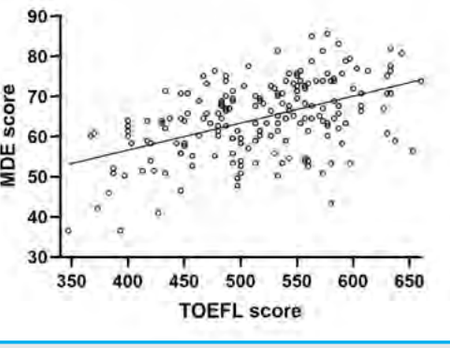

FIGURE 1. Correlation between TOEFL score and the 1st Reproductive System MDE score. Data were analyzed using Pearson correlation test (r=0.49, IK 95% (0.37; 0.59), p<0.001).

score of the 1st Reproductive System could be achieved by the students.

Our study showed significantly positive correlation between TOEFL score and MDE results of the 1st Reproductive System. It implied that English ability, which can be measured by TOEFL, is one of the factors that could influence students' academic performance (Johnson, 1988). Our findings is consistent with the study conducted in 2016 that revealed the ability to understand English-based text can influence the academic performance of the students who do not use English as the main language, but currently were educated with the learning materials written and delivered in English (Srikrai *et al.*, 2016). That study also identified two challenges that might be faced by the students in that situation; difficulty in understanding the meaning of the English-written text and understanding the learning material itself. Therefore, low English ability might result in low academic performance.

A study conducted in 2011 suggested that the students who undergo education in English but do not use English as the main language, will have a complex cognitive process on understanding the learning materials. Conceptual cognitive process is influenced by the students' skills to understand English in the learning materials. The better student's English proficiency, the better the understanding of learning materials (Bernardo, Jennifer and Gaerlan, 2012) more non-native speakers of English are learning English so that they can learn in English. In this paper, we review studies (mostly involving Filipino-English bilinguals. For the learning materials that involves memory, language consistency used during learning process and examination is one of the important factors that influence the academic result. Furthermore, high English language skill for the students who do not use English as the main language becomes an advantage during the learning process as there could be more variation in learning materials available.The results of our study is in line with a research conducted by Martirosyan *et al.* in 2015 which showed a positive correlation between TOEFL score and academic performance of foreign students who undergo education in the English-using university level. This study found that students with TOEFL score of 500 or more had higher Cumulative GPA as compared with students with TOEFL score less than 500 (Martirosyan, Hwang and Wanjohi, 2015).

In addition, that study also proposed several factors other than English ability that could affect academic performance, such as confidence, motivation, and positive attitude in the education using foreign language (Martirosyan, Hwang and Wanjohi, 2015). This phenomenon might also apply in our current study as several students with TOEFL score higher than 550 (the passing grade score for TOEFL) could not pass the minimum passing grade score for the 1st Reproductive System MDE (56). Reversely, several students with TOEFL score less than 550 could achieve MDE score higher than 56. In addition to the factors already mentioned, the students' ability to understand the English-written questions during MDE could also affect the students' academic performance (Srikrai *et al.*, 2016).

CONCLUSION

In this study, we showed positive correlation between TOEFL score and the results of multidisciplinary examination. One of the main influencing factors which might influence students' academic achievement is the students' ability to understand the learning materials written in English. The result of this research could be used as a reference by Faculty of Medicine to improve their students' English skill starting from the first grade to help them understand medical sciences through enjoyable learning process.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Supplementary data

Supplement 1. Data files are available from: Open data repository address https://osf.io/yxwf3/.

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Impact of Endemic Calciphilous Flora of the Central Russian Upland on the Nitrogen Regime of Carbonate Soils and Sub-Soils

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ABSTRACT

Unique plant communities that are complementary to the cretaceous substrate tend to be formed on the cretaceous outcrops of southern European Russia. They are characterized by a wide spread of calciphilous species of higher plants, including endemic ones. Under these conditions, stable plant aggregations are created by the species that are able to populate substrates being toxic to most plants. The study was conducted in order to examine the nitrogen status of soils and sub-soils under endemic calciphilous species: *Matthiola fragrans* Bunge, *Hyssopus cretaceus* Dubjan. and *Andorsace koso-poljanskii* Ovcz. The research tasks included a comparative analysis of the dynamic changes in the content of organic matter carbon, total nitrogen, easily hydrolysable nitrogen, nitrate nitrogen, and nitrification capacity of soils and sub-soils in the course of vital processes of the species under study. It has been found that during the ten-year period of the experiment for *M. fragrans* Bunge, *H. cretaceus* Dubjan significantly increases the number of particles of <1 mm

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in size. Over the 10–year period, their share has increased by 186.6 and 131.4% in relative terms respectively. There are two parallel on-going processes observed: increasing the number of fine particles and changes in the content of different forms of nitrogen in the substrate. We have noted positive dynamics in the accumulation of easily hydrolysable nitrogen and nitrate nitrogen under the species that can develop at the early stages of cretaceous outcrops overgrowth. It is most noticeable under *M. fragrans* Bunge, *H. cretaceus* Dubjan. We have found that *A. koso-poljanskii* Ovcz. can have a preserving effect on the substrate, which becomes obvious through its more stable nitrification capacity in time.

KEY WORDS: ENDEMIC PLANT, CARBONATE SOILS, TOTAL NITROGEN, TOTAL CARBON, SOIL BIOLOGICAL ACTIVITY

INTRODUCTION

Landscape systems with a wide spread of soil-forming rock exposures are typical for different regions of the world. They are formed due to particular terrain features, climatic conditions, and natural erosion processes, as well as under anthropogenic influence, and have distinctive vegetation. Carbonate outcrops hold a unique position among them (Scholle et.al. 1983, Cowling and Hilton-Taylor 1994, Robinson and Hermanutz 2015). The composition and productivity specific of plant associations is largely due to the diversity of habitat conditions, including lithological composition of rocks and pedodiversity (Lisetskii 2012, Lisetskii et al. 2016, Gusev et al. 2017). Distinctive plant communities with widespread calciphilous species of higher plants are formed on substrates made of chalk and marl. Some species that are able to populate carbonate substrates, while producing toxic effect on most plants, form stable plant groups and effectively absorb macronutrients and trace elements, which includes consortive relations with soil microorganisms (Lousley 1969, Maschinski 2004, Baskauf and Burke 2009, Kurkina et al. 2015, Abe et al. 2018, Dorofeeva et al. 2018).

The role of the anthropogenic factor became apparent 450,000 years ago and resulted in changing grassy steppes (Lisetskii 1992). For forest steppe, the human influence has also contributed to the reduced forest area over the last 300 years (Ukrainskij et al. 2017) and it is currently translated into one-way changes in the regional climate (Lisetskii 2007). In recent decades, the share of arable land has decreased, more fallow lands have appeared, and the rate of erosion has decreased. Ravine systems with outcrops of carbonate parent rocks (residual chalk deposits and marl) are widespread in the geographical coverage of the landscapes of the southern part of European Russia (Gorbunov and Bykovskaya 2012, Dumacheva et al. 2015). It is noteworthy that an important feature of carbonate outcrops is that they are formed on a mobile substrate, which is due to the constant influence of water run-off and weathering processes. The substrate mobility makes it difficult for plant matter and fine-grained soil to be accumulated, which in its turn causes a lack of humus. Chalk substrates are characterized by low average daily temperature fluctuations and lower average annual surface temperature in comparison with zonal soils. The flora of such territories is characterized by a high degree of endemism and uniqueness (Golitsyn 1956, Abramova 1973, Khadeeva et al. 2011).

Earlier (Degtyar and Chernyavskih 2006) they estimated the chemical content (active form P, K, Ca, Mg, S) of communities with prevailing rare and endemic species in the substrate surrounding rhizosphere and in the aboveground vegetation mass for the south the Central Russian Upland. It is established that endemic highly specialized calciphilous species – *M. fragrans* Bunge, *H. cretaceus* Dubjan, *Scorophularia cretacea* Fisch. ex Spreng have the strongest environment-transforming role on any substrate that has not yet been affected by biogenic processes. The process of further soil formation is associated with the development of communities where prevail *A. koso-poljanskii* Ovcz. (Degtyar and Chernyavskih 2006).

It becomes important to study the nitrogen status in soils and sub-soils, as well as its dynamics in relation to specific carbonate substrates. It is of interest to have studies on nitrogen-related nutrients consumption and on increased bioresource territory potential by preserving the existing populations and possible introduction of new species (Altay et.al. 2016, Rodríguez-Celma et.al. 2016).This study was conducted to examine the nitrogen status in the substrates occupied by three calciphilous species: *M. fragrans* Bunge, *H. cretaceus* Dubjan. and *A. koso-poljanskii* Ovcz. The scope of the study included a comparative analysis of the dynamic changes in the content of organic matter carbon, total nitrogen, easily hydrolysable nitrogen, nitrate nitrogen, and substrate nitrification capacity in the course of vital processes of three calciphilous species within the ten-year experiment.

MATERIAL AND METHODS

Study area: The research covered the southwestern macro slope of the Central Russian Upland, where landscapes with carbonate soils and cretaceous outcrops are widely spread within the Belgorod region (Russian Federation). This region has a high level of agricultural and

industrial development and it is characterized by apparent and soil erosion. Against this background, the region has a high diversity of vegetation and a high degree of endemism, which is related to the extra-glacial territory position during the last glaciation. The climatic conditions of the region are diverse. With the sunshine duration of about 1800 hours, the amount of solar radiation is 4000 MJ/m^2 on the average. The mean annual temperature ranges from 5.4°C to 6.7 °C. The average summer temperature increases from 18.4°C to 19.6 °C towards the southeast. The average winter temperature drops from -6.5°C in the south to -8.0°C in the north. The snow cover period lasts from 100 (SE) to 120 days (S). The average annual rainfall ranges from 530–550 mm in the northwest to 465–490 mm in the southeast. In some years, the amount of precipitation can increase up to 700–800 mm, and other reduced to 300 mm. The vegetation period accounts for about 70% of the precipitation.The studies were aimed at the local populations *M. fragrans* Bunge, *H. cretaceus* Dubjan, *A. koso-poljanskii* Ovcz., that were identified in the model area with cretaceous outcrops near Alekseevka, Belgorod region (50°38'50''N, 37°49'35"E). For this area, the average duration of the period with Σt > 10°C is 157 days, the average annual rainfall is 486 mm, the average annual air temperature is + 6.2°C. The absolute maximum temperature is + 43°C, the absolute minimum is – 38°C.

Methods: Stationary sample plots with an area of four m^2 were used in six-fold repetition on the sites with a share of the studied species of over 90 % in 2003. The initial data on the content levels of nitrogen and carbon were obtained in 2003–2004. In 2014, we assessed the changes, which had occurred over a ten-year period. Observations, records, and preparation activities were arranged as follows: We took substrate samples using a sampler from the 0–20 cm horizon at 10 points on the registered site in the following three months: April, July, and October. Mixed samples of soils and sub-soils were taken from each of the six plots for three options. After the preparation of mixed samples, the soil was brought to the air-dried condition. By using the method of round-cell sieve sifting d=1 mm, fine-grained soil (mechanical substrate separates of < 1 mm in size) and the skeletal part (separates >1 mm) were isolated. The fine-grained soil and the skeletal part were ground down and analysed separately. The repetition for the determination of all biological indicators is 2–fold and the total one is 12–fold. For each fraction, we determined the following: total carbon according to A. Ansteta as modified by V.V. Ponomareva and T.A. Nikolaeva, easily hydrolysable nitrogen according to Cornfield, total nitrogen – according to Kjeldahl, and nitrate nitrogen – using the ionometric technique (Arinushkina 1970). To determine substrates nitrification capacity, the samples were composted in a thermostat for 7 days with the soil moisture of 60% and at a temperature of 28 °C.

RESULTS AND DISCUSSION

The regional distribution of the studied species is related to incomplete replacement of the past flora, which was widespread in Eurasia by current zonal communities. Three species (*M. fragrans* Bunge, *H. cretaceus* Dubjan, *A. koso-poljanskii* Ovcz.) are limited to landscapes with extra-zonal conditions and represent an obsolete element since they do not belong to the current zonal type of the regional flora. These species are listed in the Red Book of the Russian Federation (Red book... 2008).

M. fragrans Bunge (*Brassicaceae*) species. This redivive is a rare type of cretaceous outcrops. A pioneer species appearing on an exposed carbonate substrate.*H. cretaceus* Dubjan (*Lamiaceae*) species. This half-shrub is an obligate calcicolous plant and an endemic species of the cretaceous outcrops in the southern part of the Russian plain. A pioneer species, which inhabits the cretaceous substrate immediately after exposure. It belongs to upland xerophytes. It forms specific communities — cretaceous hyssop crops, where it acts as an edificatory. The associations have a protective cover of 15–25%.

A. koso-poljanskii Ovcz (*Primulaceae*) species. This is an herbaceous polycarpous and endemic plant with large loose tussocks from dense basal rosettes of the Central Russian Upland. It belongs to the Alpine mountain flora, which was formed in the glacial era. It is distributed on soils with well-defined soil horizons, with the number of substrate particles <1 mm in size of more than 60%. The projective cover of the species can reach maximum 70–90%.Due to the active manifestation of soil erosion, these species move across various relief features as the dominants of the communities.The main biological processes of substrate changing under the studied species develop in two main directions:

1. In the rhizosphere of *M. fragrans* Bunge and *H. cretaceus* Dubjan species, which have a core root system, the basic soil formation processes actively develop in chalk cracks, where particles of < 1 mm in size are accumulated. Accumulation of plant residue and root secretions, their humification, and nitrogen and carbon accumulation occur around the root systems to the depth of their penetration. 2. Local soil formation under *A. koso-poljanskii* Ovcz. It belongs to Alpine meadow polsters by type, when the biological pedogenesis processes occur on the soil surface. A substrate, which form polsters inside, mainly consists of dead leaves decomposing on the soil surface.In comparison with the environment, the stable in-chalk temperature conditions contribute to high biological activity within the root system

distribution area. As part of the vital plant processes, the rhizosphere accumulates amino acids, sugars, and high molecular and slightly degradable organic matter residues in the form of fiber, lignin, etc. There are changes occurring in the soil physical properties. First of all, the proportion of small particles and gravel in the substrate changes.

It has been established that over a 10–year period the number of particles of < 1 mm in size is significantly increases under *M. fragrans* Bunge, *H. cretaceus* Dubjan. During the 10–year period, their proportion has increased by 186.6 and 131.4% in relative terms respectively (Table 1). No significant differences were detected in the fraction of small particles under *A. koso-poljanskii* Ovcz. Communities with the predominant *A. koso-poljanskii* mainly occupy the upper part of the slope on the dense substrates that have already undergone pedogenesis processes largely (low stone crushing degree, well-formed soil water-bearing structure, and well-defined soil horizons). The physical properties of the soil remain relatively stable.

The involvement of organic matter in the mineral substrate is accompanied by increased substrate biological activity in the rhizosphere due to microorganisms, which are included in the nitrogen transformation cycles. There are two parallel on-going processes observed: increasing the number of fine particles in the substrate and changes in the content of nitrogen of related different forms.The accumulation of total nitrogen (N_{total}) and total carbon (C_{total}) and their ratio (C_{total}:N_{total}) are the most important indicators of the overall direction of nitrogen status dynamics processes.The most significant changes in the content of N_{total}, C_{total} occurred under the pioneering species of *M. fragrans* Bunge, *H. cretaceus* Dubjan which initially populate carbonate substrates. As compared to the initial state, a significant increase in carbon and nitrogen was reported both in small and large particles. The C_{total} content in the particles <1 mm increased by 27–31%, and in particles of > 1 mm in size by 16–17%. The number N_{total} respectively 40–50% in particles of < 1 mm in size and 32–34% particles of > 1 mm in size. No significant difference between the total nitrogen and the total carbon was found in the particles of < 1 mm in size under *A. koso-poljanskii* Ovcz., but it was found that these elements had increased in the skeletal part (Table 2).

The C_{total} and N_{total} content in the substrate dominated by *A. koso-poljanskii* Ovcz. is much higher than their content in the substrate under the other two calciphilous species. The ratio of their content in the substrate particles of < 1 mm in size and particles of > 1 mm in size is very narrow and it is within 1.3–1.4. This can be explained by the washing action of water coming from the surface. Because of this process, a water-soluble (fulvic acids) part of the plant polster humus enters the deeper horizons and is absorbed by the skeletal part of the chalk as a porous structure and it is accumulated in the upper part of the soil. The same consistent pattern is evident for the total nitrogen. As a result, the substrate becomes more homogeneous with respect to the chemical properties as compared to the substrate under *M. fragrans* Bunge, *H. cretaceus* Dubjan.

The C_{total}:N_{total} ratio in the substrate particles of < 1 mm in size and particles of > 1 mm in size under *A.*

Table 1. Change in the proportion in substrate particles of < 1 mm (%) in size under individual of calciphilous species

Plant species	Year of research	
	2004	2014
Matthiola fragrans Bunge	11.2±0.6	32,1±1.6
Hyssopus cretaceus Dubjan.	12.4±0.6	28.7±1.8
Andorsace koso-poljanskii Ovcz.	64.1±3.7	65.7±2.4

Table 2. Changes in total carbon and total nitrogen in individual mechanical elements of soil under the local populations of calciphilous species of plants

Plant species	Content Ctotal in the substrate, %		Content Ntotal in the substrate, %		Ratio Ctotal: Ntotal	
	1	2	1	2	1	2
Mechanical elements <1 mm						
Matthiola fragrans Bunge	0.950±0.062	1.207±0.024	0.133±0.006	0.186±0.009	7.2±0.2	6.5±0.4
Hyssopus cretaceus Dubjan.	1.092±0.071	1.434±0.035	0.144±0.008	0.220±0.014	7.6±0.9	6.6±0.3
Andorsace roso-poljanskii Ovcz.	3.754±0.169	4.018±0.204	0.444±0.018	0.454±0.013	8.5±0.5	8.9±0.1
Mechanical elements >1 mm						
Matthiola fragrans Bunge	0.214±0.005	0.249±0.010	0.064±0.005	0.085±0.008	3.4±0.3	3.0±0.1
Hyssopus cretaceus Dubjan.	0.054±0.002	0.063±0.008	0.049±0.013	0.066±0.003	1.2±0.3	1.0±0.1
Andorsace roso-poljanskii Ovcz.	2.764±0.193	3.580±0.181	0.337±0.025	0.352±0.020	8.3±0.9	10.2±0.6
Note: 1 – research 2003-2004; 2 – research 2014						

Table 3. The change in the content of easily hydrolyzed nitrate nitrogen and the nitrification capacity in individual mechanical elements of soil under the local populations of calciphilous species of plants for 10 years

Plant species	Content N l.g. in the substrate, mg kg^{-1}		Content $N\text{-}NO_3$ in the substrate, mg kg^{-1}		Nitrification capacity, mg kg^{-1}	
	1	2	1	2	1	2
Mechanical elements <1 mm						
Matthiola fragrans Bunge	57.4±3.9	90.0±3.9	12.9±0.7	17.3±1.2	15.4±1.0	20.9±1.3
Hyssopus cretaceus Dubjan.	63.9±2.3	78.2±2.2	5.6±0.5	8.6±0.4	8.6±0.6	11.2±1.1
Andorsace koso-poljanskii Ovcz.	226.8±	227.8±6.1	8.0±0.6	8.3±0.2	17.6±0.6	19.2±0.4
Mechanical elements >1 mm						
Matthiola fragrans Bunge	7.3±0.8	12.2±0.8	6.2±0.2	9.8±0.6	3.4±0.3	12.5±0.3
Hyssopus cretaceus Dubjan.	4.4±0.4	8.1±0.4	2.7±0.9	5.1±0.2	0.4±0.1	5.6±0.7
Andorsace koso-poljanskii Ovcz.	224.7±5.1	223.6±5.1	4.5±0.3	4.6±0.4	15.5±0.5	17.0±0.2
Note: 1 – research 2003-2004; 2 – research 2014						

koso-poljanskii Ovcz. was not significantly different from each other and it was within 8.3–8.5 in 2003–2004 and 8.9–10.6 in 2014. Under the other species, this ratio was significantly lower, especially particles of > 1 mm in size, which may indicate that there was a more intensive organic, matter mineralization process in the rhizosphere of the pioneer *M. fragrans* Bunge, *H. cretaceus* Dubjan. as compared to *A. koso-poljanskii* Ovcz.

It is known that the plant growth and development is more dependent on the content of available forms of soil nitrogen rather than on its total reserves (Arinushkina, 1970). Easily hydrolysable nitrogen is a potential reserve for ground accumulation of the most important forms of soil nitrogen – nitrate and ammonium. The growth rate of roots and the content of related auxins in the plants, which receive nitrogen in the form of nitrates, are higher.Table 3 contains data on the accumulation dynamics for easily hydrolysable and nitrate nitrogen in the soil under certain types of calciphilous species and on changes in nitrification capacity for 10 years of the experiment. We established a positive accumulation dynamics for the easily hydrolysable nitrogen and nitrate nitrogen under species, which develop at the early stages of cretaceous outcrops overgrowing, both in substrate particles of < 1 mm in size and in a larger fraction of > 1 mm in size. These processes run most effectively under *M. fragrans* Bunge, *H. cretaceus* Dubjan. The content of N l.g. in substrate particles of < 1 mm in size increased for 10 years by 22-56%, with the highest intensity being under *M. fragrans* Bunge, and the accumulation under *H. cretaceus* Dubjan was 67–84% more intense in the substrate particles of > 1 mm in size. No statistically significant trend was established for the accumulation of N l.g. over a 10–year period under *A. koso-poljanskii* Ovcz.The content of easily hydrolysable nitrogen was consistently high under *A. koso-poljanskii* Ovcz. both in substrate particles of < 1 mm in size, and in particles of > 1 mm in size and had no significant differences.A trend similar to easily hydrolysable nitrogen was established for the content of nitrate nitrogen. Accumulation of nitrates both in substrate particles of < 1 mm in size and in a larger fraction of > 1 mm in size for 10 years was more significant under *M. fragrans* Bunge and *H. cretaceus* Dubjan. and made up to 34–52%. In particles of > 1 mm in size, the increase under these two species was 25–34%. The content of *A. koso-poljanskii* Ovcz. nitrates was close, and no mathematically proven dynamics was established for a 10–year period.

A change in the nitrification activity is an indirect indicator of the biological activity dynamics. It has increased under the pioneering species over a 10–year period by 1.3 times in particles of < 1 mm in size under *M. fragrans* Bunge, *H. cretaceus* Dubjan. Particles of > 1 mm in size showed differences that are more significant. If the substrate nitrification activity under *M. fragrans* Bunge is increased 3.6 times, it will become 13.8 times higher under *H. cretaceus* Dubjan, which indicates to a sharp increase both in biological activity and in intensity of substrate destruction by root systems. The substrate nitrification capacity under *A. koso-poljanskii* Ovcz. was steadily high.The results of the assessments of the nitrate content in the substrate and its nitrification capacity give important findings on higher biological activity of the pioneer species, which develop in the early stages of carbonate substrate overgrowth as compared to species participating in successions later.

CONCLUSION

The endemic species of *Matthiola fragrans* Bunge and *Hyssopus cretaceus* Dubjan, which inhabit cretaceous outcrops the southern of the Central Russian Upland in

the early self-growth stages, actively change the substrate towards increasing the content of total nitrogen, total carbon, and easily hydrolysable nitrogen. These species increase the biological activity of both substrate fine part (particles of < 1 mm in size) and the skeletal part (particles of > 1 mm in size). The nitrification capacity of the skeletal part of the substrate (particles of > 1 mm in size) under pioneering species *M. fragrans* Bunge and *H. cretaceus* Dubjan becomes higher with increasing the related fractions of < 1 mm in size, which can be diagnosed using average strength correlation (r=0.634±0.121). However, a similar trend was not identified (r=0.234±0.321) under *Andorsace koso-poljanskii* Ovcz. growing on a fresh chalk substrate for 10 years. The experiment results have showed that there is a negative correlation (r=0.463±0.234) between the substrate carbon accumulation and the substrate nitrate content. We have found that *A. koso-poljanskii* Ovcz. can have a preserving effect on the substrate, which becomes obvious through its more stable nitrification capacity in time. It has been diagnosed using a wider ratio of the total carbon to the total nitrogen both in particles of < 1 mm in size (C_{total}:N_{total}=8.5±0.5÷8.9±0.1), and in particles of > 1 mm in size (C_{total}:N_{total}=8.3±0.9÷10.2±0.6).

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Role of Bacteriocin in Tackling the Global Problem of Multi-Drug Resistance: An Updated Review

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ABSTRACT

Bacterial resistance to antimicrobials has reached an unacceptable level which threatens the very existence of man and animal alike if the situation is not corrected in the near future. The development of novel antibiotics is a slow time consuming process which leaves us with inadequate means to control microbial infections. It is imperative that we adopt alternative therapeutic strategies to ensure removal of resistant micro-organisms from our living space. Bacteriocins are powerful bactericidal peptides produced and secreted by a varied group of micro- organisms including yeast, protozoa and of course bacteria and they cause death and removal of non bacteriocin producing pathogenic bacteria. These bacteriocin treatments offer more benefits over antibiotic therapies in present time as they are natural bioactive peptides having no side effects. This paper is a review of MDR (Multiple rug Resistance) related issues which have become a global problem and on the possible role of bacteriocins as an effective option for fighting against MDR disease causing bacteria. The potential of Bacteriocins as an alternate or adjuvant to antibiotics needs to be studied and made available to the medical community. Recent trends suggest that if an effective alternate to antibiotics is not found quickly then the very existence of mankind could come under threat .The safety profile of bacteriocins is much superior to antibiotics. This is another important reason to study bacteriocins and tap their therapeutic potential to combat drug resistant bacterial infections.

KEY WORDS: ALTERNATIVE THERAPEUTIC STRATEGY, ANTIMICROBIALS, BACTERIOCINS, GLOBAL PROBLEM, MULTI-DRUG RESISTANCE

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INTRODUCTION

Microorganism impervious to antibiotic treatment have become wide spread which is a global phenomena needs to be controlled before it becomes a unmanageable deadly threat to the health and wellbeing of human life. The phenomena of antibiotic resistance can be attributed to inappropriate, self medication and excessive prescription of antibiotics over the past several years all over the world according to Carlet et al (2012). Developmentof reasonable alternatives to antibiotics are in need to get universal public health out of danger (Carlet et al., 2012 Oldfield and Feng, 2014 and WHO, 2015). Antimicrobials used in food industries, bio-preservatives or antibacterial peptides like Nisins, pediocin, mersacidin, mutacin and lactacin have proved to be active against Vancomycin Resistant Enterococci and Methecillin Resistant Staphylococcus aureus strains, have instance of potential therapeutic strategy to kill bacterial infections and multidrug-resistant bacteria (Papagianni and Anastasiadou, 2009, Nishie et al., 2012, Bodaszewska-Lubas et al., 2012, Lohans and Vederas 2012, Laxminarayan et al., 2016, Santos et al., 2017, Delpech 2017, Mathur, et al., 2018).

The bacteriocins are very small ribosomally synthesized peptide molecules secreted by archea, gram-positive and some of gram-negative bacteria (Klaenhammer et al., 1988 and Zheng, et al., 2015) as well as synthesized by ribosomes with antimicrobial properties against various groups of microorganisms (Chikindas, et a., 2017). The activity of bacteriocins is influenced by temperature, pH, and composition of culture medium (Guinane et al., 2015 and Turgis, et al., 2016). The bacteriocins were first described in 1925, however their production, functions and applications in medical field has been explored in recent times (Chikindas, et al., 2017). Recently, it has been defined that bacteriocins are secreted not only by bacteria but also by others like yeast and mould, virus, eukaryotic cells like sperm cell, cancer cell and also from protozons (Drider et al., 2016, Jiang, et al., 2016, Chikindas et al., 2017, Mills et al., 2017, Diep, et al., 2018, Lopetuso, et al., 2019).

Bacteriocin has already found commercial application in food preservation and dairy farming therefore it is not far-fetched to assume that Bacteriocins will soon be available for the benefit of humans also this may be as a therapeutic agent against MDR bacteria. They are being investigated as a potential alternative or adjuvant in combination with antibiotics to combat disease causing pathogens. The fact that they have an excellent safety and resistant profile is an added incentive towards studying them. They are considered as therapeutic complements despite therapeutic alternative to chemical antibiotics as they have high stability and very low toxicity.

Advantages of Bacteriocin Treatment over Antibiotic Therapy

Antibiotics are a group of pharmaceuticals that play a vital role in keeping both humans and animals disease free thus having an important influence on the quality of life of an individual (Stepanauskas, 2006). Antibiotic resistance is a growing threat to the efficacy of these agents and has serious consequences in terms of morbidity and mortality of those undergoing treatment (Fair, et al., 2014). Due to the development and dispersal of antibiotic resistance and their several side effects, treatment with antimicrobial peptides is a needed requirement (Chen et al., 2012). Bacteriocins are bacterial extracellular ribosomal integrated peptides or proteins having antibacterial action against closely related microbial species (Tashakor et al., 2017, Castro et al., 2011, Opsata et al., 2010, Morisset and Frère2002). Bacteriocins influence the immune system and inhibit competitive strains by directly influencing the niche competition among commensals (Kommineni, et al., 2015). The bactofencin A or bacteriocin 21 produced by Enterococcus faecalis can eradicate multidrug resistant bacteria and contribute to regulation of niche competition among intestinal bacteria (Kommineni, et al., 2015). Likewise, LAB bacteriocins play role against Staphylococcus aureus (Umu, et al., 2017), some vancomycin resistant enterococci (Kommineni, et al., 2015), Salmonella enteritidis (Umu, et al., 2017), Clostridium difficile (Rea, et al., 2013) and Listeria monocytogenes (Umu, et al., 2017). Additional investigations are required to test the therapeutic potential of above outcomes.The antibacterial properties of bacteriocins are exploited by applications in food technological research. In particular, bacteriocins are used as food preservatives (Oldak, et al., 2017) and preservation of dairy products (Linares, et al., 2017) with categorization such as partially purified bacteriocins, crude-fermented dairy bacteriocins and protective cultures bacteriocins (Henning, et al., 2015, Anacarso, et al., 2017, Chikindas, et al., 2017, Ahmad, et al., 2017, Hammami, et al., 2019).

Mode of Action of Bacteriocin

Most of the bacteriocins can inhibit growth of pathogens in order to defend their producer and play a role in signalling peptides (Hegarty, et al., 2016). They can act as pore-forming agents or membrane perturbers (Etayash et al 2015) or interfere with the cell division processes. The bacteriocins possess antiviral, spermicidal (Chikindas, et a., 2017), anticancer properties (Kaur et al 2015) and capable of enhancing the positive effects of probiotic bacteria as seen in the Bifidobacterium strain (Weinstock, et al., 2016 and Hegarty, et al., 2016).

Different viewpoints distinguish bacteriocins from antimicrobial drugs: (i) Bacteriocins are synthesized on

the ribosomal surface in bacterial cells, while antibiotics are bacterial secondary metabolite; (ii) Antibiotic producers are easily affected by antimicrobial agents whereas the producers of bacteriocin are not susceptible to antimicrobial agents; (iii) Bacteriocins can get fixed to the target bacterial cell surface anywhere because target bacterial cell surface doesn't possess any specific receptors; iv) Bacteriocins get attached to the target cell wall surface and form pore in the outer membrane surface because of ionic imbalances (Morisset and Frère, 2002) another side chemical antimicrobials are responsible for disruption of cell wall (bacterial) synthesis, formation of genomic protein and replication processes (Svetoch et al., 2011, Cotter et al., 2013, da Silva Sabo et al., 2014, Woraprayote et al., 2016, and Perez et al., 2018).

Emergence of Bacterial Resistance to Antibiotic

The resistant bacterial microorganisms are acquired by human being through consumption of animal meat. These bacteria show changes at the gene level which plays a role in them acquiring resistance to antibiotics. Consequently the consumer of such meat becomes insensitive to the action of antibiotics involved (Ventola, 2015, Holmes, et al., 2016, Chakchouk-mtibaa, et al., 2017, Vijayakumar and Muriana, 2017, Costa, et al., 2019).

Bacterial resistance emerges and propagates due to the reasons listed below:

1. Excessive consumption of antibiotics: Over utilization of antibiotics leads to multiple drug resistance which invariably selects the resistant species of normal flora. The excessive utility of those medicines might be due to lack of information or overzealous and sometimes even profit driven treatment for various viral and bacterial infections (Nitsch-Osuch et al., 2016).
2. Overprescribing of antibiotics: Apart from excessive consumption, the number of inappropriate prescriptions of antimicrobial agents is also shocking. It has been found that there are errors of about 30 - 50 % in terms of choice and duration for which the antibiotics need to be consumed (Ventola, 2015). It has been observed that the sub therapeutic doses of antibiotics promote bacterial phenotypical variations and resistance to bacterial infections builds up (Viswanathan, 2014).
3. Antibiotic use in agronomy as well as animal husbandry: It was observed that in 2011 in the US, the use of antimicrobials as a growth enhancer in livestock was approximately 13,000 tons. That year more than 42,000,000 tons meat was produced which was an average of at least 320 mg of the bactericidal per kilogram of meat (Aarestrup, 2015).

Research Regarding Bacteriocin's Effect on Multi Drug Resistance Bacteria

Researchers from Howard University Washington DC have shown that probiotic isolate from yogurt has strong antimicrobial activity. Among the multiple *Lactobacillus* isolated and studied one particular isolate *Lactobacillus parafarraginis*, was reported to be sensitive against fourteen multi drug resistant bacteria (Allen-McFarlane et al., 2019).

Recently, the bacteriocins from *Vibrio, Aeromonas, Pseudoal-teromonas* and *Alteromonas* were sourced from the ocean and were noted to have high bactericidal activity. They provide defence system against multidrug-resistant bacteria by establishing bacteriocins. The system is found to be reasonable alternatives to antibiotics for high biodiversity of the ecosystem (Desriac et al., 2010).

On the contrary, Sachsenrödder et al. (2014) postulated that the administration of probiotics (bacteriocins from *Enterococcus faecium*) to diarrhoea causing virus in pig gut would be therapeutic but they failed to prove the same through actual results (Sachsenrödder et al., 2014). Previous study also showed that pyocin, a bacteriocin produced by *Pseudomonas aeruginosa* were not able to treat pulmonary disease caused by pseudomonas in patients (Ghoul et al., 2015) Bacteriocin isolated from *Bacillus subtilis* that have been used to increase shelf life of food items have showed activity against only a very few gram positive bacteria like *S. epidermidis*. They failed to show activity against any drug-resistant bacteria (Sharma et al., 2018) Studies have been also conducted on AS 48 and nisin, a bacteriocin from *Lactococcus lactis* with AS48 being effectively bactericidal against *Staphylococcus* in cereal drink only when those are combined with phenol compounds (Antonio, et al., 2019).

Researchers from Leuven Belgium have studied the mode of action of LipA bacteriocin which helps to kill multidrug-resistant *Pseudomonas aeruginosa*. Their work has shown that the bacteriocin is both effective and very specific in targeting the pathogenic organism (Martín-Escolano, et al., 2019).Perales-Adán J, et al (2018) had shown the bactericidal action of bacteriocins (AS-48 and nisin) against drug-resistant *Staphylococcus aureus* present in goat milk cheese both individually and in combination. The combined action of these bacteriocins is even more effective because of synergy (Perales-Adán, et al. 2018). Scientists from Pakistan have studied the BAC-IB-17 bacteriocin produced by *Bacillus subtilis* and wasfound it to be effective against MRSA. This bacteriocin was highly thermo stable and therefore would retain its activity in a range of extreme environments (Ansari, et al., 2018).

Indian researchers from CSIR- Institute of Microbial Technology, Chandigarh, have shown that the bacteri-

ocin 'Sonorensin' is effective against antibiotic-resistant *staphylococcus aureus* biofilms and other bacteria of gram- positive and gram- negative types (Chopra, et al., 2015).

Five antimicrobial peptides are designed by Indian Institute of Science, Bangalore scientists.The peptide Ω76 which is among the five peptides was effective against carbapenem and tigecycline-resistant *Acinetobacter baumannii* in mice. The peptides are nephrotoxic lead to side effects in patients which may be treated with conventional antibiotics (Nagarajan, et al., 2019). A scientists' team from China studied antimicrobial peptide Cec4 for its structure and mode of action.The peptide is effective against the drug-resistant nosocomial infections caused by *A.baumanii* (Peng, et al., 2019).

A team of scientists at MIT has discovered a peptide from the venom of a South American wasp. They successfully developed and refined several variants of this peptide and tested their efficacy in mice infected with Antibiotic-resistant *Pseudomonas areuginosa*. Among all the peptides tested one peptide was seen to eradicate the infection- an encouraging and interesting result (Marcelo, et al. 2018). The bactericidal actions of bacteriocins were studied VidhyaPrakash et al. which were produced by *L. fermentum* and *L. casei* 335 showed effective against antibiotic-resistant Escherichia coli and also drug resistant *Salmonella typhi* bacteria. This study showed that both the pathogenic bacteria were inhibited by bacteriocin action (Prakash, et al. 2018). Also in our research we have found a bacteriocin producing lactobacillus which is very much effective against Methicillin resistant *Staphylococcus aureus* (Bonhi and Imran, 2019).

MDR Bacteria and Antibiotic Resistance – The Global Scenario

According to report published by UN in April 2019, there may be loss of lives every year numbering up to 1,00,00,000 caused by drug resistant diseases by 2050. The causality may be similar to disastrous economic loss during 2008-09. Antimicrobial resistance may lead to 24 million into extreme poverty by 2030.Drug resistant infections lead to death of minimum of 7,00,000 individuals every year. Tubercle bacilli multidrug-resistant infection lead to death of 2,30,000 additionally. Sexually transmitted infections, Urinary tract infections and common respiratory tract infections are untreatable in present context. The treatments meant for life saving became risky and modern food systems are increasingly uncertain (Chaib, et al. 2019).

The prevalence of carbapenems degrading enzymes New-DelhiMetallo-beta - lactamase-1 and *Klebsiella pneumonia* carbapenemase-2 are matter of concern to the researchers for treatment of infectious diseases due to inadequacy of antibiotics (Liu et al., 2016). The "One Health" approach of UN recommends countries to redress antimicrobial resistance and to ensure efficacy for essential medicines by framing strategic plan for deployment activities like finance and arranging awareness programs for prudent use of antimicrobials.It insists to invest in research and development for new technologies to combat antimicrobial resistance and growth of critically essential antimicrobials in agriculture (Chaib, et al. 2019).

MDR Bacteria and Antibiotic Resistance – The Indian Scenario

The problem of Antibiotic resistance has assumed serious proportions in India too. Findings from a study published by ICMR show the presence of antibiotic resistant bacteria in the digestive system of 2 out of every 3 individuals tested thus confirming the high prevalence and spread of Antibiotic resistance in Indian population. The resistance was more for frequently used antibiotics like cephalosporins (60 %) and flouroquinolones (40%). This study is a wakeup call for the future because a similar resistance for higher end antibiotics would be disastrous. Of even more concern is the threat of Drug resistant tuberculosis. The Central TB division is found with declaration furnished in 2018 by Ministry, Health and Family Welfare.As per the declaration, there were approximately 2.8 million new cases of TB every year and 1,47,000 new cases of drug resistant TB. There were 87,000 new cases of HIV-TB every year and deaths due to TB excluding HIV was 4, 23,000 whichis alarm for immediate effective action. The Indian government has resolved to eliminate TB by 2025.Consequently, Indian Council of Medical Research (ICMR) launched nation's first wide-scale trial for two newly invented vaccines of tuberculosis (TB) on 15/7/ 2019 to facilitate prevention in spread of Drug resistant TB (Mascarenhas, 2019). NDM-1(New-Delhi Metallo-beta - lactamase-1) is a gene produced by bacterial microorganisms which makes the concerned bacterium multi drug resistant. This gene produces an enzyme which makes the antibiotics resistant. This Gene has originated in India and was first detected in a Swedish patient who had visited India for a surgery in the year 2008. There after this resistant bacteria (Klebsiellapnemoniae) having MDR-1 gene had spread all over India and across 70 different countries all over the world. The Indian government had taken various measures to tackle this situation and also focused on improving sanitation and providing clean and healthy water. These measures in public health will definitely help in reducing microbial resistance (Aggarwal, 2019).

The Indian government had also taken strict action in August 2019 to combat the menace of MDR. Notable among them is the ban on colistin, one of the last resort

antibiotics for human infectious diseases. This ban is not only on use of colistin as a growth promoter but also on its manufacture, sale and distribution because the consumption of such food product whether meat or poultry would expose the Indian population on the risk of MDR (Ghafur, 2019).

Future Perspectives

Bacteriocins are already being used commercially for food preservation and as a probiotic but their use as a therapeutic agent against human diseases still in the development stage and a reason for much excitement in the scientific and medical community alike. A massive and sustained effort is required to achieve this goal to combat the threat of MDR in shortest possible period of time. Scientists from different parts of the world are trying to isolate a broad spectrum anti - pathogenic bacteriocin which could be as effective as antibiotic therapy and have a lot of untapped potential as therapeutic agents. A cocktail of bacteriocins with differing spectrum of action could be as effective as a broad spectrum antibiotic. Bacteriocins could be combined with an antibiotic without compromising on the efficacy but reducing the unwanted side effects of antibiotic therapy. This would also help prevent development of both antibiotic resistance and bacteriocin resistant microbes. The pairs or group of bacteriocins showing augmented bactericidal action when working together as compared to when they act alone is another property being looked for (Antonio et al., 2019). The future prospects for bacteriocins as therapeutic agents have lots of potential as shown promising activity against biofilms (Kim et al., 2019). Experts have estimated that by the year 2060 more than 20 new classes of antibiotics would be needed to cope-up with the challenge posed by antibiotic resistance (Li et al., 2018). Only A few antimicrobial peptides are approved so far by FDA and EMEA (Cattoir et al., 2019). The phama industry is reluctant to support research activities of bacteriocins due to high production cost. With commercialization of bacteriocin based products and improved low cost production techniques coupled with increase in demand with time it is estimated that the cost to the consumer will go down significantly.

CONCLUSION

The human population is heading towards disaster unless antibiotics with broader antimicrobial activity are developed soon. There may be a number of unpublished antibiotics which are in preclinical stage of development. Approval process for newly developed drugs that include bacteriocins and antimicrobial peptides by medical control councils has been slow due to several safety and clinical tests which involve analysis of resistance to antimicrobial activity, allergies and effect on immune system of hosts. Bacteriocins can be used for prevention of infections, to fight against antibiotic resistance and treatment owing to their diversity and abundance. Bacteriocins are an interesting option being studied for future use as a therapeutic option against multi drug resistant bacteria. Much more research is required towards development of novel effective bacteriocins which successfully target complex bacterial systems such as cell membranes.

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Usnic acid inhibits cell proliferation via downregulation of proliferating cell nuclear antigen (PCNA) expression in gastric carcinoma AGS cells

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ABSTRACT

Usnic acid is a secondary metabolite obtained from various species of lichen. Previous studies have shown various biological activities of usnic acid such as anti-oxidant, anti-microbial, anti-viral, anti-protozoal, anti-inflammatory and anti-proliferative activities in different models. Its anti-proliferative activities in gastric cancer cells are still unexplored. Herein, we have investigated the effects of usnic acid on cell proliferation and viability and associated molecular alterations using MTT {3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} assay, RT-PCR (Reverse-Transcriptase-Polymerase Chain Reaction), and western blotting assay in human gastric carcinoma AGS cells. The treatment of usnic acid (2.5-25 µM) dose-dependently reduced cell proliferation. The mRNA expression of tumor suppressor gene PTEN (phosphatase tensin homolog) in the usnic acid-treated AGS cells was increased, which may play a role in the inhibition of cell proliferation and induction of cell death. We also observed a decrease in the expression of proliferating cell nuclear antigen (PCNA) that regulate cell proliferation by playing an important role in DNA replication. The expression of cyclin-dependent kinase inhibitor p21 which may play a role in cell cycle and proliferation inhibition was found uninfluenced with usnic acid treatment. Thus, collectively these results for the first time revealed that usnic acid inhibits the cell proliferation of AGS cells through downregulating the expression of PCNA and can be further evaluated *in vivo* models for its therapeutic potentials.

KEY WORDS: CELL PROLIFERATION, CANCER, PCNA, TUMOR-SUPPRESSOR GENES

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INTRODUCTION

Gastric cancer is the fifth most commonly diagnosed and third leading cause of cancer-related death throughout the world. The recent reports have revealed that gastric cancer incidence is twofold higher in men than women. Despite significant advancement in diagnostic and surgical oncology in recent decades and the use of various adjuvant therapy, gastric cancer remain a major cause of mortality due to metastasis. Various natural agents having the capacity to inhibit the cancer cell proliferation or carcinogenesis or tumorigenesis or reverse/arrest the progression of malignant cells have been used for cancer management (Bray et al 2018, Zhang et al., 2018). Usnic acid, a dibenzofuran derivative, is obtained from a number of lichen species, has shown its potential in various biological activities including anti-inflammatory and anti-proliferative activities (Ingolfsdottir, 2002). Previous studies have demonstrated that usnic acid has the potential to inhibit tumor cell proliferation, angiogenesis, invasion and migration in various types of tumor cells (Singh et al., 2013, Geng et al., 2018).

However, the mechanism of usnic acid-induced anti-proliferative and cells death-inducing properties in gastric cancer cells is yet to be explored. Uncontrolled cell proliferation and division are the primary characteristics of cancer cells (Farber, 1995). Cell proliferation plays a key role in initiation, promotion or progression during cancer development. Abnormal regulations of cell division can lead to over proliferation and accumulation of abnormal cells. A number of key genes and proteins that regulate cell proliferation and the process of cell division have been identified (Valdespino-Gomez et al., 2015). It is now clear that mutations in key genes affect the action of regulating proteins and enzymes and lead to unregulated cell proliferation which is seen in cancer. Different types of cancers have different mutational signatures. However, the study of multiple types of tumor has revealed that certain genes are mutated in cancer cells more often than others. Other cancer-related mutations inactivate the genes that suppress cell proliferation or those that signal to program cell death. These genes are known as tumor suppressor genes that normally function as a brake on cell proliferation and are involved in the protection of cells from a single event or multiple events that lead to cancer (Wang et al., 2018b).

In the present study, we have explored the effect of usnic acid on gastric carcinoma AGS cells. Our study revealed that usnic acid inhibits cell proliferation via modulating the expression of tumor suppressor genes. The up-regulation of PTEN and the down-regulation of PCNA expression clearly indicates the relevance of this study in highlighting the usnic acid as a probable cancer targeting agent in gastric cancer cells. However, further validation of usnic acid in *in vivo* model to develop as a potential anti-cancer agent is obligatory.

MATERIAL AND METHODS

Cell Culture and Reagents: Gastric carcinoma AGS cells (ATCC, USA) were grown in Ham's F-12 nutrient mixture medium (Gibco BRL, Grand Island, NY) supplemented with 1% antibiotic-antimycotic solution (HiMedia) and 10% Fetal Bovine Serum (Gibco Life Technology) in a humidified 37°C incubator. (+)- Usnic acid and MTT dye were procured from Sigma Aldrich, USA, and SRL, India respectively. Procurement of anti-PCNA, anti-p21 (CIP) and anti-GAPDH was done from Cell Signaling Technology, USA.

Cell Viability Assay: Cell viability of gastric carcinoma AGS cells was examined through MTT assay as described earlier (Mohan et al., 2016). Briefly, AGS cells (8,000/well) were seeded in a flat bottom 96 well plate and after overnight incubation treated with 0, 2.5, 5, 10, 15 and 25 µM usnic acid for 72 h. Further, cells were processed for MTT assay.

Semi-quantitative RT-PCR Assay: Cells (5×10^5/plate) were seeded in 100 mm plates and after 24 h incubation treated with 0, 10, 15 and 25 µM usnic acid for 48 h. Once the treatment period was over, cells harvested for RNA isolation followed by cDNA synthesis, and RT-PCR as detailed earlier (Jaiswal et al., 2018). Briefly, Total RNA isolation was done by Trizol Methods, then 5 µg RNA was used for cDNA synthesis. The final volume of the PCR reaction mixture was 25 µl with 3 µg of cDNA, forward and reverse primers (20 pM) and all required components. PCR tube was kept in a thermal cycler (Eppendorf, India) for 25 cycles. Agarose gel (1%) with (1µg/ml) ethidium bromide separate the amplified DNA and visualized in the GelDoc system. The following primer sequence was used: for PTEN forward primer- ACC AGG ACC AGA GGA AAC CT, reverse primer- GCT AGC CTC TGG ATT TGA CG, for GAPDH forward primer- AAG GCT GAG AAC GGG AAG CTT GTC ATC AAT, reverse primer- TTC CCG TTC AGC TCA GGG ATG ACC TTG CCC.

Immunoblotting: Cells were seeded and treated as described in the previous section. After 48 h incubation, cells were lysed to prepare whole cells lysate as described (Kumar et al., 2019). The protein concentration was estimated by the Bradford method. Further, the protein was resolved in 10% denaturing SDS-PAGE gel and transferred on PVDF membrane. Skimmed milk (3%) in blocking buffer was used to block the membrane. Subsequently, the membrane was incubated in specific antibody (diluted in skimmed milk) followed by appropri-

ate HRP-linked secondary antibody and the bands were detected by ECL and capture on X-ray film. The band density was analyzed by scanning the film with high-resolution scanner and Image J program (NIH, Bethesda, MD).

Statistical Analysis: For statistical significance, Graph-Pad Prism-5 program was used. Student's t-test was applied to detect the degree of significance between control and treated samples. *P*-value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Effect of usnic acid on cell viability of AGS cells: With the aim to evaluate the effects of usnic acid on cell proliferation and viability, gastric carcinoma AGS cells were exposed to increasing concentrations of usnic acid ranging from 2.5 to 25 µM for 72 h. Treatment with 2.5 µM usnic acid resulted in no marked decrease in cell viability however at (5-25) µM concentrations showed (6-65%) decrease in cell viability after 72 h [Fig. 1].

These results indicated that usnic acid caused a concentration-dependent reduction in cell viability of AGS cells.

Gene and protein expression modulation by usnic acid treatment in AGS cells: To access the effect of usnic acid on the expression of tumor suppressor gene and proliferation-inducing proteins, AGS cells were treated with (10-25 µM) of usnic acid for 48 h and processed for semi-quantitative PCR and western blot analysis. The result of semi-quantitative PCR revealed that usnic acid induces the expression of PTEN at 48 h time point [Fig. 2]. Similarly, the immunoblotting analysis revealed that usnic acid decreased the expression of PCNA which has an important role in DNA replication and hence cell proliferation [Fig. 3 (A)]. However, at a lower concentration of usnic acid the expression of p21 has no effect but at higher concentration (25 µM), its expression was decreased [Fig. 3(B)].

In various cases of gastric cancer, the tumor suppressor protein PTEN (phosphatase Tensin Homolog) has been detected functionally inactive and shown to be closely associated with the development, progression, and prognosis of the disease (Xu et al., 2014, Lu et al., 2016). PTEN can induce apoptosis and suppress cell proliferation by antagonizing the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Our results have shown that usnic acid induces the gene expression of PTEN. Which might be helpful in controlling the cell proliferation and inducing cell death of AGS cells.

On the other hand, PCNA, a DNA clamp that is essential for DNA replication and has been closely associated with cell proliferation (Wang et al., 2018a). It has an active role in cyclin-dependent kinase regulations during cell division (Strzalka and Ziemienowicz, 2011). Its

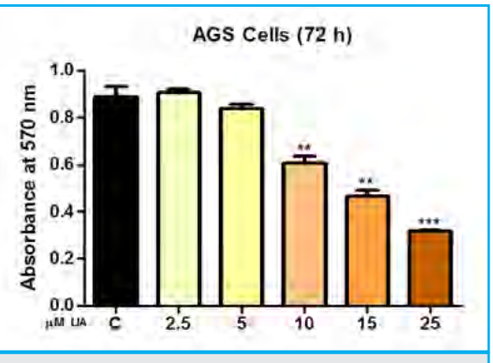

FIGURE 1. Effect of usnic acid on cell viability of AGS cells. AGS cells were seeded and treated with 2.5 5, 10, 15 and 25 µM of usnic acid. After 72 h of incubation cells were processed for MTT assay as described in Material and Methods. Results are representative of three independent experiments. The data are presented as mean of triplicate samples for each treatment. Data points are mean of s.e.m. of three experiments. ** *P < 0.005*, *** *P < 0.001*. The *P*-value is determined by comparing each treatment with control group.

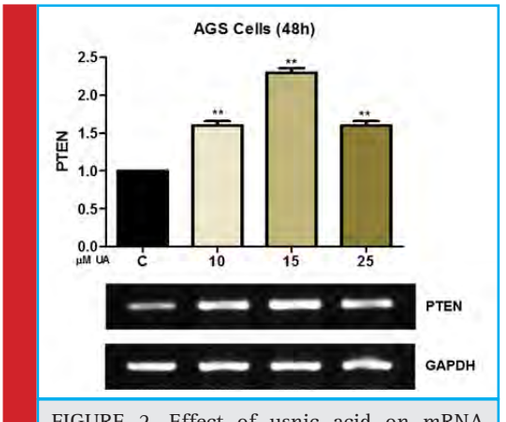

FIGURE 2. Effect of usnic acid on mRNA expression of PTEN in human gastric carcinoma AGS cells. Cells were treated with DMSO, 10, 15 and 25 µM of usnic acid for 48 h and processed for RNA isolation and semi-quantitative PCR as detailed in Material and Methods. Gel image of PTEN and GAPDH. Bar graph is the representative of observed densitometry data. Data points are mean of s.e.m. of three experiments. ** *P < 0.005*. The *P*-value is determined by comparing each treatment with control group.

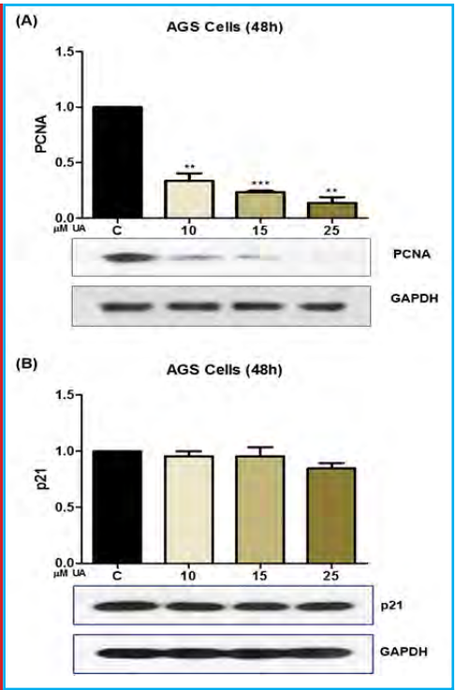

FIGURE 3. Effect of usnic acid on expression of PCNA and p21 proteins in human gastric carcinoma AGS cells. Cells were treated with DMSO, 10, 15 and 25 µM of usnic acid for 48 h and processed immunoblotting as detailed in Material and Methods. Band image of (A) PCNA and (B) p21. Membrane was stripped and re-probed with anti-GAPDH for loading control. Bar graph is the representative of observed densitometry data. Data points are mean of s.e.m. of three experiments. ** $P < 0.005$, *** $P < 0.001$. The P-value is determined by comparing each treatment with control group.

expression is associated with metastasis and malignancies (Peng et al., 2019). Hence, the expression of PCNA can be regarded as a marker of proliferating cells. Our result has shown that usnic acid dose-dependently decreases the expression PCNA in gastric carcinoma AGS cells. However, the expression of cyclin-dependent kinase inhibitor p21 (Cip) relatively ineffective with usnic acid treatments. These results indicated that usnic acid targets the PCNA expression and hence cell proliferation without modulating the expression of other cell cycle inhibitors at least p21.

CONCLUSION

Our results for the first time shows that usnic acid negatively modulates the cancer cell viability that can play important role in cancer prevention and therapeutics. The down-regulated expression of PCNA and up-regulated expression of PTEN is associated with inhibition of cell proliferation and induction of cell death in gastric carcinoma AGS cells. However, the cell death induction appears poorly associated with the expression of cyclin-dependent kinase inhibitor (p21).

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Use of *Bougainvillea glabra* Plants in Minimizing Vehicular Pollution in Jazan Area of Saudi Arabia

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ABSTRACT

The research aimed at comparing the physiological and biochemical parameters in Bougainvillea glabra grown at a polluted site with the control site in addition to studying its Air Pollution Tolerance Index (APTI) and Anticipated Performance Index (API). The polluted site was selected from the main road in Addarb Jazan, while as, the control site was ensured free from any traffic movement. Parameters like Dust fall, Photosynthetic Pigments, Protein Content, Soluble sugar Content, Free amino Acid Content were studied in addition to the parameters necessary for the calculation of air pollution tolerance index like Total Chlorophyll, pH, Ascorbic acid content and Relative Water Content. Anticipated Performance Index was also calculated. There was an increase in chlorophyll, free amino acid content, soluble sugar content and pH of leaf extract and while as the protein content, ascorbic acid content and the relative water content showed a decrease in polluted plants as compared to the control. Our research finds that despite the polluted plant being under some biochemical stress, it shows a high tolerance to the vehicular pollution depicted by a high air pollution tolerance and anticipated performance index. Hence this plant species can be recommended for inclusion in the urban greenbelt development plan and city landscaping.

KEY WORDS: PHYTOMONITORING, GREEN BELT, AIR POLLUTION TOLERANCE INDEX, APTI

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INTRODUCTION

Air pollution is one of the major challenges that the world is facing today. Air pollution is any variation in any atmospheric element than the value which would have existed without human interference. Vehicular pollution amounts to about 2/3rd of air pollution in the urban cities. The main pollutants released by the vehicles include Carbon monoxide, Nitric Oxide, Sulphur Dioxides, Hydrocarbons lead (Pb) etc in addition to the suspended particulate matter (SPM) which has deleterious effects on the human health and ecological balance, (Ravindra, et al., 2001, Desai et al., 2018, Orlinksi et al., 2019).

The World Health Organization estimates that air pollution causes over a million premature infant deaths around the world.Saudi Arabia has seen maximum economic and infrastructure development inrecent decades.To accomplish the demands of population growth, infrastructure andvehicles have seen an unprecedented increase. Vehicular pollution is the major contributor of air pollution in Saudi Arabia, because of majority of population use cars, (Ahmad et al., 2016).To check the different plant species about the values of their tolerance to air pollutants is necessary. So that the identification of plants species as sensitive, and tolerant is of vital significance because the sensitive species can serve as indicators and the tolerant ones can be used as sinks to monitor the vehicular pollution in urban cities (Singh et al., 1991, Ogunrotimi et al., 2017, Jain et al., 2019).

World-Wide significant work has been done to identify the plants suitable for phytomonitoring the vehicular pollution (Pathak et al,. 2015Khalid et al., 2019), but nothing significant has been done to see the phytomonitoring capacity of Saudi Arabian flora. Given this, the research was aimed to study the air pollution tolerance Index of *Bougainvillea glabra* plants to suggest the use of this plant for urban landscaping.

MATERIALS AND METHODS

Plant description:*Bougainvillea glabra*is a genus of thorny ornamental vines, bushes, and trees.It is an evergreen vine and diligently creeps up on fences. The flowershave found use as teas to alleviate mild respiratory disorders. The leaves of some types of bougainvillea plants have been studied to help with diabetes, blood pressure as well as HDL-LDL balancing (Adebayo et al., 2009, Gaurav et al., 2010).

Site Description: Two sites were selected in Addarb, Jazan, Saudi Arabia. The site whichserved as control had almost zero traffic movement while as the other had a heavy traffic movement. Both sites were from the same climatic condition (desert conditions with virtually no rainfall).

Cars/Minutes: Traffic volume was monitored using a mobile camera. Hourly traffic count was calculated by analyzing the video footage taken during the field observation. Hourly traffic count was expressed in number of vehicles per minute (Kadiyali, 1996).

Dust fall: The leaves of *Bougainvillea glabra* plants were washed carefully to completely remove dust particles from the leaf surfaces. The plants growing near traffic signals were selected and the leaves growing above 2 feet from the ground were selected. After washing, the plant species were marked with a ribbon tied to it. On the seventh day, the dustiest leaves from the same plant species were collected in the zipper pouches in ice boxes for sampling.Leaves were brought to the Laboratory and were washed with water and filtered on pre-weighed Whatman's filter paper No 1. The filter paper was then oven dried at 60 ^{0}C and later weighed to calculate the dust fall. (Joshi, 1990).

Pigment concentrations: Hiscox and Israelstam's (1979) method was used to estimate the pigment concentration in the samples. The method involves the estimation of plant pigments without maceration. Leaves were washed with distilled water (DDW) and chopped. 100 mg of chopped leaf material were taken in vials in triplicates and 10 ml of Dimethyl sulphoxide was added to each vial which was heated in oven at 65˚ C. After 30 min, the vials were taken out and the OD of the solution was measured at 480, 510, 663 and 645 nm. The Chlorphyll a, b Toral chlorophyll content and the Carotenoid content was calculated according to these formula:

$$\text{Chlorophyll } a \text{ (mg g}^{-1}\text{ fw)} = \frac{12.3\,(A_{663}) - 0.86\,(A_{645})}{d \times 1000 \times W} \times V$$

$$\text{Chlorophyll } b \text{ (mg g}^{-1}\text{ fw)} = \frac{19.3\,(A_{645}) - 3.60\,(A_{663})}{d \times 1000 \times W} \times V$$

$$\text{Total chlorophyll (mg g}^{-1}\text{ fw)} = \frac{20.02\,(A_{645}) + 8.02\,(A_{663})}{d \times 1000 \times W} \times V$$

$$\text{Carotenoids (mg g}^{-1}\text{ fw)} = \frac{7.6\,(A_{480}) - 1.49\,(A_{510})}{d \times 1000 \times W} \times V$$

Where d= distance traveled by the light path, W = weight of the leaf material taken, V = volume of the extract and A = Absorbance

Soluble Protein: Bradford (1976) protocol was used for protein estimation.Procedure:0.2 g of fresh and clean and chopped leaf material was homogenized in 2 ml 0.1 M/pH 7.2 phosphate buffer with the help of a pre-cooled

mortar and pestle. Homogenate was transferred to the pre-cooled centrifuge tube and the centrifugation was done at 5000 rpm for 10 min. 1.0 ml of supernatant was added to equal amount of chilled 10 % Trichloroacetic acid (TCA) in a microfuge. It was centrifuged for 10 min at 3300 rpm and the supernatant obtained was thrown while as the remaining pellet washed with acetone. 1 ml of 0.1 N NaOH was used to dissolve the pellet. 0.5 ml Bradford's reagent was added to 0.1 ml aliquot and was subjected to vortex. The tubes were left to let the color develop for 10 min. OD was taken at 595 nm on spectrophotometer.BSA was used as the standard to calculate protein concentrations and the protein content was expressed as mg g^{-1} FW.

Soluble Sugar Content Soluble sugar content in the leaf samples was estimated by the method of Dey (1990).Procedure:0.1 g of fresh leaf sample was taken to which 10 ml ethanol was added and the mixture was incubated at 60° C for one hour. Final volume was made up to 25 ml with ethanol. From this 1 ml aliquot was taken and 1 ml of phenol was added to it and mixed thoroughly. 5 ml of sulphuric acid was added to the reaction mixture, which was then cooled in air. Optical density was measured at 485 nm on uv-vis spectrophotometer (Model DU 640 B, Beckman, USA).The corresponding concentration of sugar was determined against the standard curve of sugar prepared by glucose ($C_6H_{12}O_6$) solution. The amount of sugar was expressed as mg g^{-1} fw.

Free Amino Acid Content The free amino acid content was estimated by the method of Lee and Takahashi (1966).Extraction:A 0.5 g of fresh leaf material was kept overnight in 5.0 ml of absolute ethanol and ground with the help of mortar and pestle and transferred to the centrifuge tubes. It was then centrifuged at 5500 rpm for 10 min at 4° C. After that supernatant was taken in a test tube and alcohol was evaporated by incubating the tubes at 100° C for 1h in a water bath. The pellet obtained was dissolved in 10 ml of 0.5 M citrate buffer (pH 5.6).

Estimation: To a 0.5 ml of aliquot, 1.2 ml of 55 % glycerol and 0.5 ml ninhydrin solution were added. The vials were kept in water bath for 20 min at 100° C and after the appearance of blue colour; the volume was made upto 6 mL. Optical density was measured at 570 nm on uv-vis spectrophotometer (λ BIO 20, Perkin Elmer, Germany). The concentration of amino acid was determined against the standard curve prepared by using glycine solution of different concentrations and the amino acid content was expressed in mg g^{-1} FW.

Ascorbic Acid: Ascorbic acid content was determined by the method ofSadasivam and Manickam (1996). For ascorbic acid content determination, a homogeneous mixtureof 1 g of leaves was prepared by addition of 25 mL of 4%oxalic acid, and was dehydrogenated by addition of a fewdrops of bromine water to form dehydroascorbic acid. Fordehydroascorbic acid, 1 mL of 2,4-dinitrophenyl hydrazine(DNPH) was added which led to the formation of compoundosazone, which was further dissolved in 7 mL of 80% sulfuricacid. The absorbance of the solution was measured at 540 nmthrough a spectrophotometer.

Leaf pH: To get leaf extract pH, about 4 g of fresh leaf was homogenized in 40 mL deionized water and centrifuged at 2,500 rpm for 3 min. Extract pH was measured using pH meter.

Relative water content: Fresh weight was obtained by weighing the leaves. The leaf samples were then immersed in water over night and blotted dry and then weighed to get the turgid weight. The leaves were then dried overnight in a hot air oven at 70°C and reweighed to obtain the dry weight. RWC was determined and calculated by the method as described by Singh et al (1991).

$$RWC= [(FW-DW)/ (TW-DW)] \times 100.$$

Where: FW-Fresh weight,DW-Dryweight and TW-Turgid weight.

APTI Determination: The air pollution tolerance index (APTI) was computed by using the equation of Singh et al (1991)APTI = [AA (TCh + pH) + RWC ÷10 Where AA = Ascorbic acid content (mg/g), TCh = total chlorophyll (mg/g), pH = pH of leaf extract, and RWC = relative water content of leaf (%).

RESULTS AND DISCUSSION

We noticed that there was an average of 28 small vehicles/minute and 0.53 large vehicles/minute plying near our polluted site.While as on the Control site, there was no vehicular movement.

2. **Dust fall**: We recorded a foliar dust fall of 0.14 and 2.18 near Control and polluted sites, respectively. A greater dust retaining capacity is indicative of a plant's use in phytomonitoring of polluted sites.

We also noticed an increase of chlorophyll pigments in polluted plants in comparison to the control while as there was a decrease in the Carotenoid content of the polluted plants in comparison to the control. Previous studies have revealed that chlorophyll content in plant species varies with the pollution status of that area i.e. higher the pollution level in the form of vehicular exhausts, lower the chlorophyll content. It also varies with the tolerance as well as sensitivity of the plant species i.e. higher the sensitive nature of the plant species, lower the chlorophyll content. The shading effects due to deposition of suspended particulate matter on the leaf

FIGURE 1. Traffic movement in polluted site/minute

FIGURE 2. Dust fall near Control and Polluted plants

surface might also be responsible for the decrease in the concentration of carotenoids in the polluted area

In the present study, we found a decrease in soluble Protein content in polluted plants in comparison to the control plants. Our results are in line with the findings of Rai et al (2016), who attributed the reduction in protein content of plants at the polluted site to the enhanced rate of protein denaturation as well as the breaking of the already existing proteins to amino acid because of proteolysis under pollution, which is also supported by the findings of Panda and Rai (2015) and Saha and Padhy (2011). Many other researchers have reported a decrease in the total protein content owing to the presence of SO_2 and NO_2 pollutants (Panda and Rai 2015; Rai and Panda 2015). Agarwal and Deepak (2003) reported that SO_2 pollution resulted in decrease of protein levels in two wheat cultivarsand attributed this decrease to increased proteolysis and reduced protein synthesis.

Soluble sugar is an important component of all living things and also an important source of energy for living things. It is produced by plants during photosynthesis and it is broken down during respiration (Tripathi

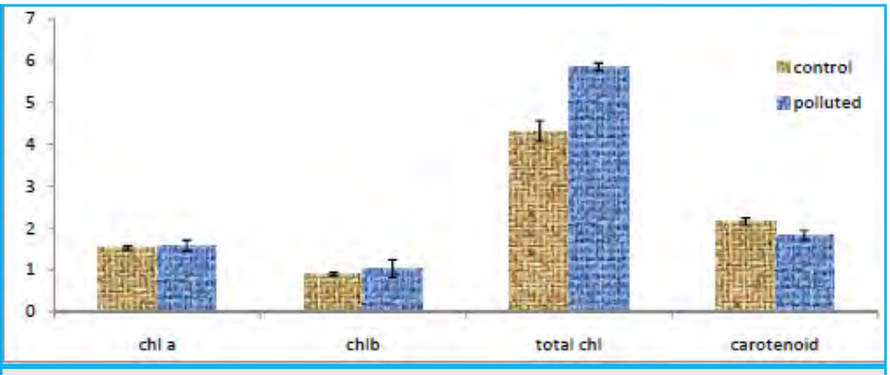

FIGURE 3. Pigment content (mgg^{-1}FW) of *Bougainvillia glabra* in control and polluted plants

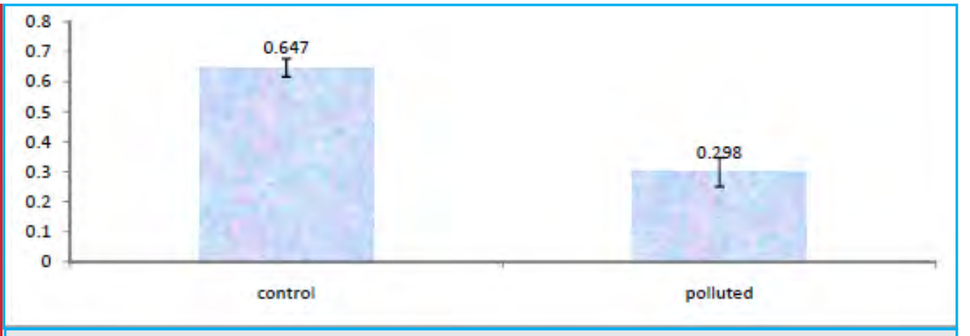

FIGURE 4. Soluble Protein Content (mgg^{-1} FW) in Control and Polluted plants

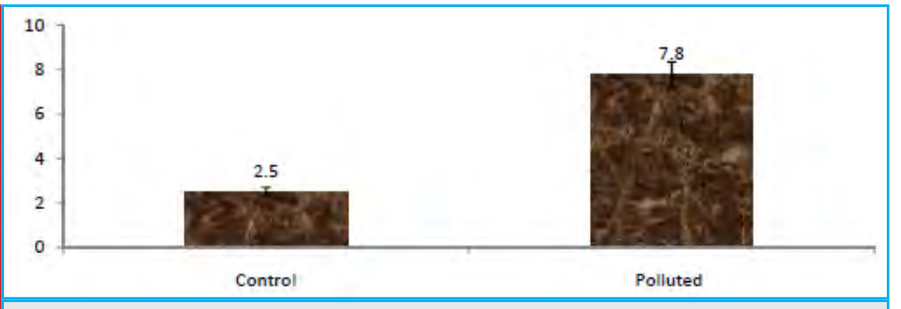

FIGURE 5. Soluble Sugar Content (mgg^{-1} FW) of Control and Polluted plants

& Gautam, 2007). In our study, we noticed an increase in the soluble sugar Content.Accumulation of different active ions, sugars and amino acids like proline has an important role in osmotic adjustment in plant cells (Königshofer and Löppert, 2015). These Osmotic adjustments are responsible for maintaining turgor pressure, controlling the cell expansion and photosynthesis and for maintaining the water flow during the shortage of water.

In our study, we saw an increased free amino acid content in the Polluted plant leaves as compared to control. The reason for this increase may be because of the accumulation of proline, which is increased when the plant is in stress. Also the increased proteolysis under stress results in the increase of free amino acid content. The decreased protein content together with increased free amino acid content in our polluted plants supports this theory.

We noticed a decrease in Ascorbic Acid Content in polluted plants in comparison to the control plants. Ascorbic acid has an importantrole incell wallsynthesis, plant defense in addition to the cell division(Conklin, 2001).It also serves as a reducing agent in addition to being an important part of photosynthetic carbon fixa-

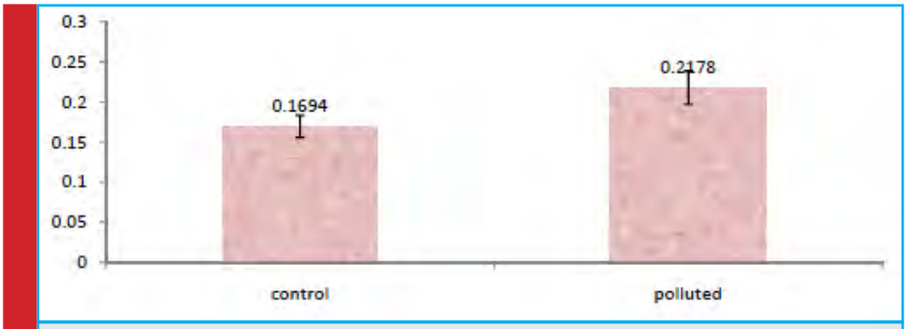

FIGURE 6. Free amino acid Content (mgg^{-1} FW) in Control and polluted plants

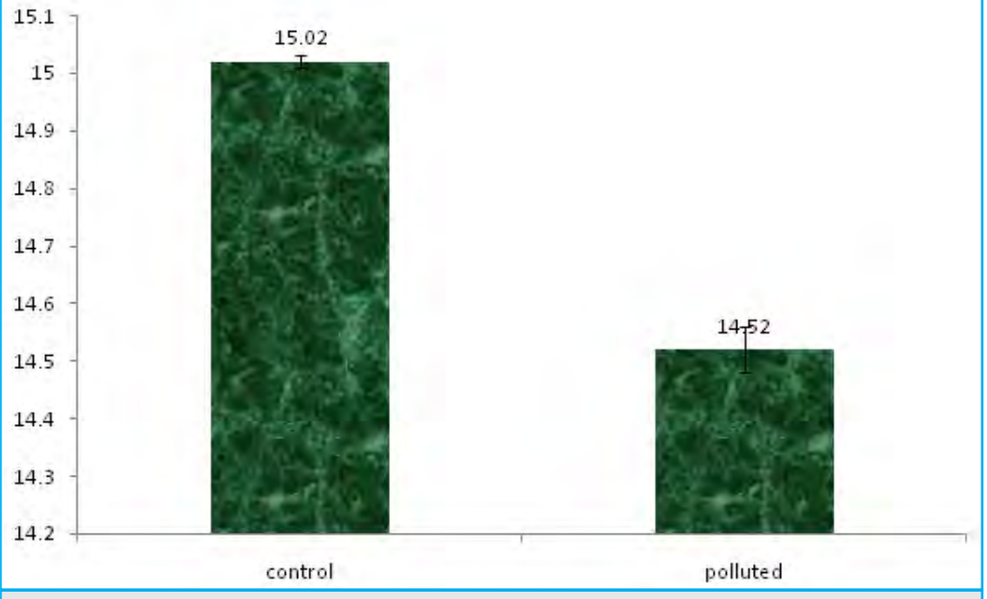

FIGURE 7. Ascorbic Content (mgg^{-1}) in Control and treated plants

tion (Pasqualini, 2001).The high amount ofAscorbic acid is favorable for pollution tolerance in plants (Keller and Schwager, 1977; Lee et al., 1984).

The leaf extract pH was more in polluted plants than in the control. Leaf extract pH plays a significant role in regulating SO_2 sensitivity of plant. Irerhievwie et al (2014) opined that an elevated level of leaf extract pH in plants subjected to pollution may increase their tolerance level to air pollutants.

We found a low relative Content in Control plants with comparison to the polluted plants. Relative Water Content (RWC) of a leaf is the water present in it relative to its full turgidity. Relative water content is related to protoplasmic permeability of cells (Aggarwal et al., 1997). High water content within plants serves as an indicator of drought resistance in plants (Dedio, 1975) and helps to maintain its physiological balance under stress conditions such as exposure to air pollution when the transpiration rates are usually high.

The air pollution tolerance index of our polluted plants was 26.95 which according to the literature is a strong index (Prajapatiand Tripathi, 2008).The APTI values offer a credible method to screen the flora of any location for their tolerance and susceptibility to vehic-

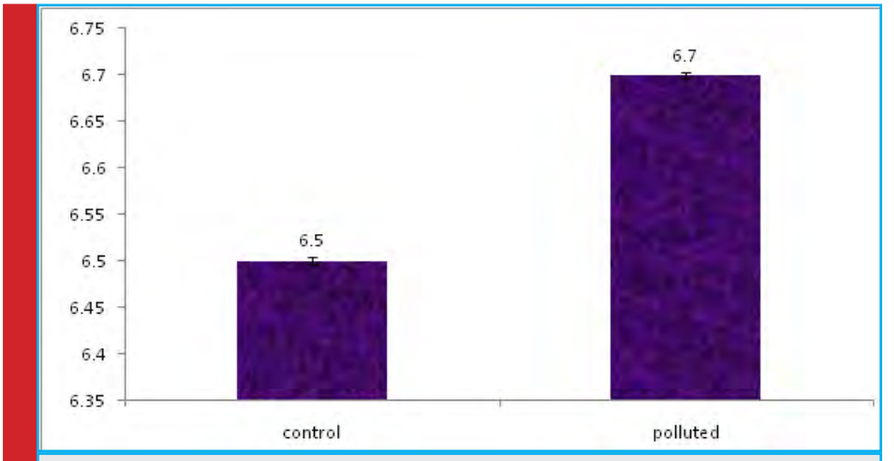

FIGURE 8. pH in Control and poluted plants

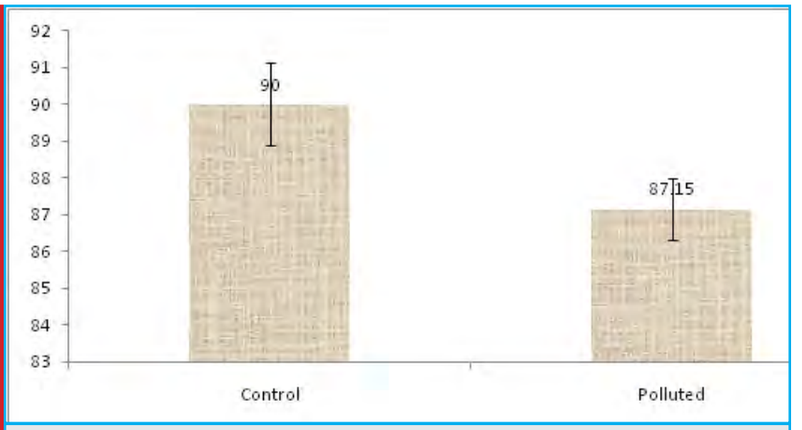

FIGURE 9. Relative Water Content (%) In Control and polluted plants

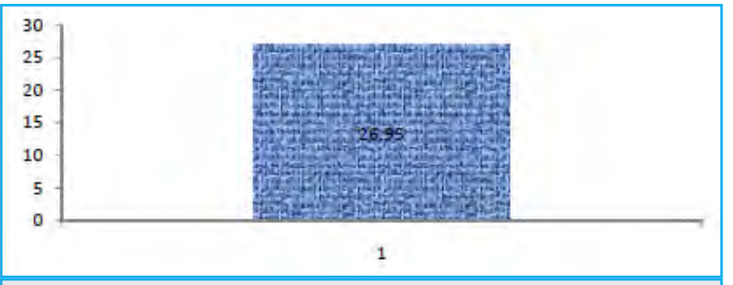

FIGURE 10. Air Pollution Tolerance Index (APTI) of *Bougainvillea glabra* plants

ular pollution (Singh et al., 1991). Given the excellent APTI of *Bougainillea glabra*, this part can be categorized as a tolerant plant to air pollution.

Anticipated Performance Index

Bougainvillea glabra plant scored 87.5% which is excellent in API grading system according to the criteria of Prajapatiand Tripathi (2008). The plant is highly tolerant to the vehicular pollution and can be expected to perform well as a phytomonitoring agent. It has a dense plant canopy and is evergreen and can afford protection frompollution stress. The economic and aesthetic value of this tree is well known and it may be recommended for extensive planting on urban roads for aesthetic and pollution-remediation purposes.

The research aimed at the study of phytomonitoring potential of *Bougainvillea glabra* for vehicular Pollution. The physiological parameters and biochemical parameters studied showed that despite the plant being subjected under the stress posed by the vehicular pollu-

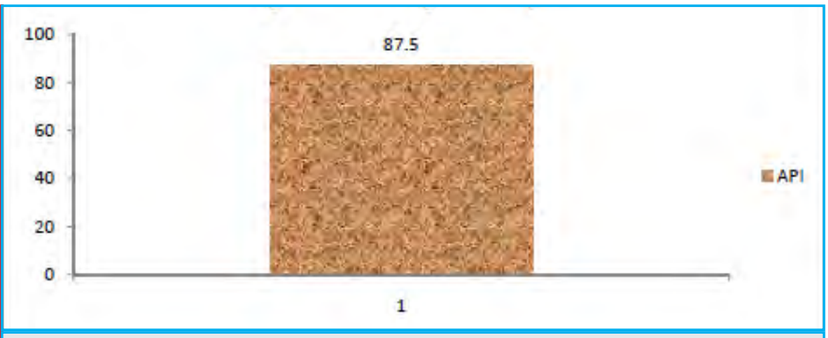

FIGURE 11. Anticipated Performance Index (API) % of Bougainvillea glabra plants

tion depicted by the reduced Carotenoid content and the protein content, the plants showed tolerance against the stress as depicted by its greater air pollution tolerance index and excellent anticipated performance Index. This plant can be suggested as a model plant to be grown on the roads to reduce the particular matter owing to its high dust trapping capacity in addition to its tolerance as shown by its high APTI and API values.

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Characterization studies on starch extracted from the stem of pineapple plant (*Ananas comosus*) at different growth stages

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ABSTRACT

Starch is the most common carbohydrate in human diets and is contained in large amounts in potatoes, wheat, corn, rice, and cassava. Starch granules are highly variable in their structure, and each has unique structure depending upon their botanical source. In this study, starch was isolated from the stem of pineapple plant at different growth stages-3 months (before flowering), six months (before flowering), nine months (before flowering), 12 months (after flowering), 15 months (after flowering), 18 months (after fruiting). X-ray diffraction (XRD) studies and scanning electron microscopic (SEM) analysis were carried out on these samples as part of characterization studies. Results indicate that maximum starch yield obtained from plant stem at nine months age. X-ray diffraction data revealed this plant stem possess A-type crystals and is same in all growth stages. Size of the granules slightly increased with growth stages and have irregular polygonal in shape. Characterization studies on starch will help assess their specific use in both food and non-food industries.

KEY WORDS: PINEAPPLE PLANT, SEM, STARCH, STEM, XRD

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INTRODUCTION

The pineapple (*Ananas comosus*) is a tropical plant and most economically significant plant in the Bromeliaceae family. Starch is the most abundant biomolecule on earth after cellulose and the major carbohydrate reserve in plants. Demand for native starches increased globally as it can minimize the use of chemically modified starches. Native starches have many applications in the food industry, pharmaceutical industry, paper making industry, cosmetics industry, etc. X-ray diffraction is a valuable tool for the structural elucidation of starch granules and is used to study the crystalline properties of starches. Starch crystallinity is due to the formation and packing of double helix between the chains of amylopectin molecules (Lawal, 2004, Diop et al., 2011). Scanning electron microscopy (SEM) is a frequently used technique for granular characterization of starches and can be used for studying the granule morphology more accurately than light microscopy (Chmelik et al., 2001,Lindeboom et al., 2004). Surface characteristics of granules have many practical applications as it influences the enzyme actions.The enzyme action on starch granules largely determines the quality of starch-based food products, (Kowsik and Mazumder, 2018).

Viscosity, one of the main physical properties of starches, is influenced mainly by their granule morphology (Kumar and Khatkar, 2017). The objective of the study is to characterize the starch from the pineapple plant stem at various growth stages. This study helps to check whether there is any difference in the morphological and crystalline structure of the starch granules with age. The results could serve as a reference for identifying starch from the stem at a particular growth stage to choose in the specific starch industry.

MATERIAL AND METHODS

Extraction of starch: The stem was collected from the experimental plantation at different growth stages – three months, six months, nine months (before flowering), twelve months, fifteen months (after flowering) and eighteen months (after fruiting). It was then washed with water and mild acid to remove soil, and other debris and starch were extracted. The stem was then ground in a mixer grinder with distilled water and filtered through double-layered cheesecloth. The steps were repeated for several times until the milkiness of the slurry disappeared or became minimal, centrifuged and discarded the supernatant. The residues obtained were washed with 60% alcohol, 0.1N NaOH, and distilled water. The retained residues were dried at 40 °C, powdered and passed through a standard sieve (75µm), collected and stored in desiccators.

FIGURE 1. Pineapple plants before flowering

FIGURE 2. Pineapple plants after flowering

Scanning electron microscopic analysis: The surface and structure of starch samples were characterized using a scanning electron microscope (Carl-ZEISS Gemini SEM 300), using a secondary electron detector with

FIGURE 3. Pineapple plants after fruiting

Table 1. Starch yield at different growth stages of the pineapple plant

Sl. No.	Age of the plant	% of starch obtained
1	3months (Before flowering)	3.93 ± 0.52
2	6months (Before flowering)	8.4 ± 0.68
3	9months (Before flowering)	16.03 ± 0.84
4	12months (After flowering)	11.56 ± 0.53
5	15months (After flowering)	11.58 ± 0.44
6	18months (After fruiting)	11.08 ± 0.77

2.00 kV of acceleration (Central Sophisticated Instrumentation Facility (CSIF), University of Calicut, Kerala, India).

X-ray diffraction: X-ray diffraction studies on stem starch were carried out by an X-ray diffractometer (Model-XRD- Rigaku Miniflex 600).

Statistical analysis: Microsoft Office Excel 2007 and OriginPro 8.0 were used to analyse the experimental data.

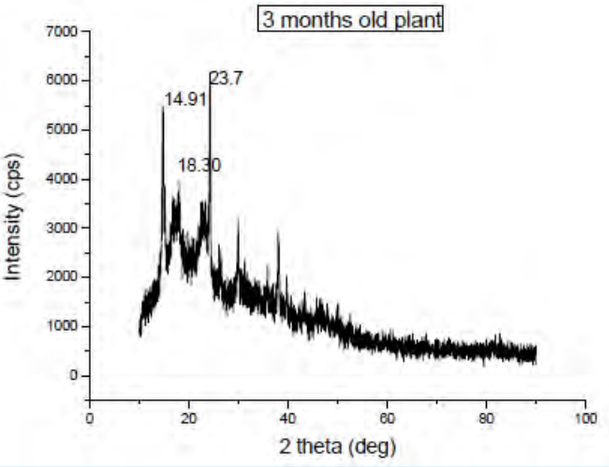

FIGURE 4. X-ray diffractogram of stem starch extracted from 3 months old pineapple plant

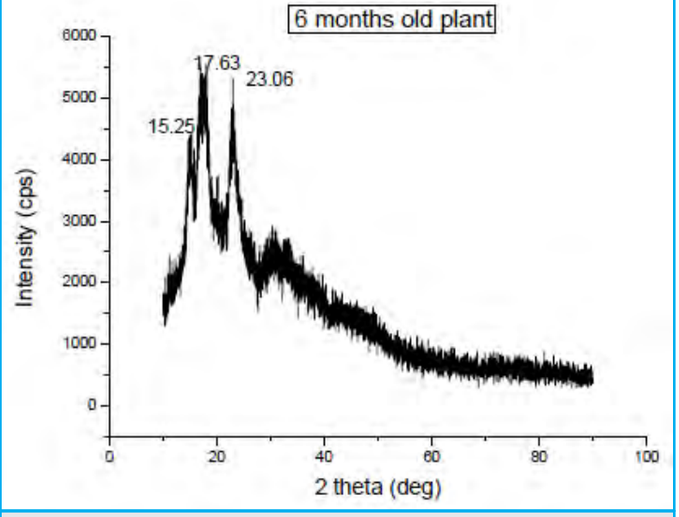

FIGURE 5. X-ray diffractogram of stem starch extracted from 6 months old pineapple plant

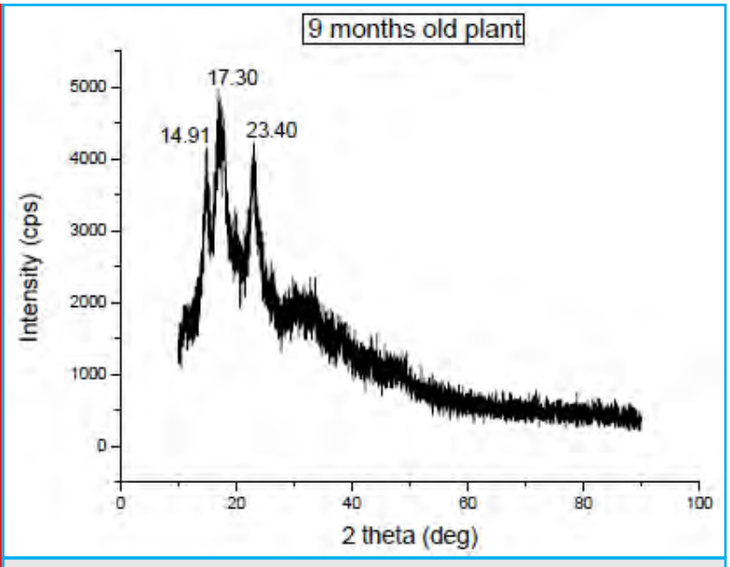

FIGURE 6. X-ray diffractogram of stem starch extracted from 9 months old pineapple plant

RESULTS AND DISCUSSION

The maximum starch yield obtained from the stem at nine months age (before flowering stage). After that yield decreased and then there is almost constant starch content observed. Because of shoot extension growth, utilization of photosynthate increases which causes lower carbohydrate availability for storage and starch content will decrease (Von Fircks and Sennerby-Forsse, 1998).

Three months old plant showed peaks at 2θ angles 14.91, 18.3 and 23.7 (Fig: 4), six months old plant had peaks at 2θ angles 15.25, 17.63 and 23.06 (Fig: 5), nine months old plant showed at 14.91, 17.30 and 23.40 (Fig: 6). Starch from 12 months old plant showed major peaks at 2θ angles 15.16, 17.25 and 23.2 (Fig: 7). Fifteen months have peaks at 14.91, 17.30 and 23.06 (Fig: 8), 18 months have 15.4, 17.26 and 23.21 (Fig: 9). There are A, B and C type starches according to the packing

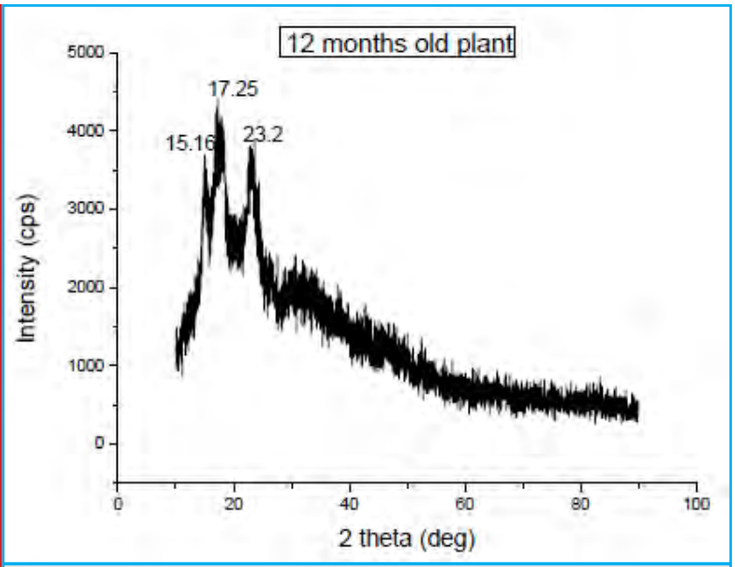

FIGURE 7. X-ray diffractogram of stem starch extracted from 12 months old pineapple plant

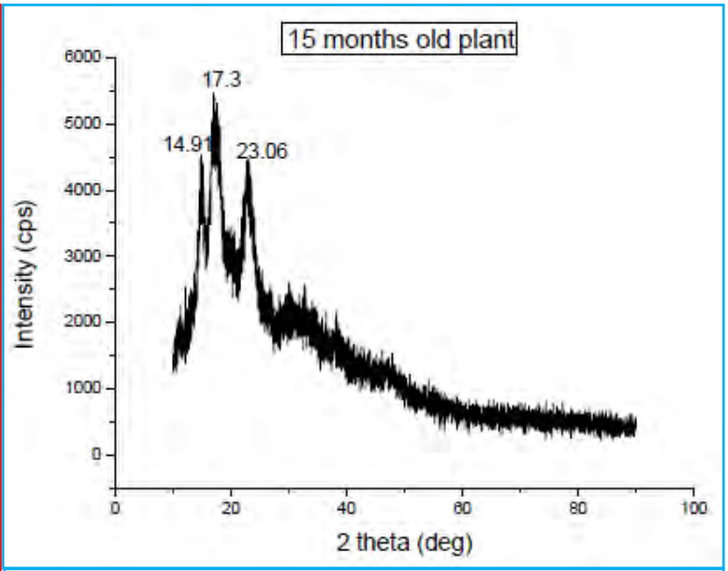

FIGURE 8. X-ray diffractogram of stem starch extracted from 15 months old pineapple plant

of amylopectin double helices. 'A' type starch granule shows peaks around 15°, 17°, 18°, 20° and 23° 2θ angles, 'B' type granules shows around 5°, 15°, 17°, 20°, 22° and 24° 2θ angles and C type starch has the mixture of A and B (Zhou, Wang, Zhao, Fang, & Sun, 2010), (Nwokocha, Nwokocha, & Williams, 2012).

'A' type starches are mostly found in cereal starches. B type in the tuber and root starches and C type, which is the mixture of A and B, found in particular root, legume, and seed starches. The packing of the helix in A type starch is more compact and less hydrated than B type (Wang et al., 2011) (Guimarães, Wypych, Saul, Ramos, & Satyanarayana, 2010) (Delcour et al., 2010). From the result obtained, it can be concluded that this stem starch possesses 'A' type crystals, and it is same in all the growth stages.

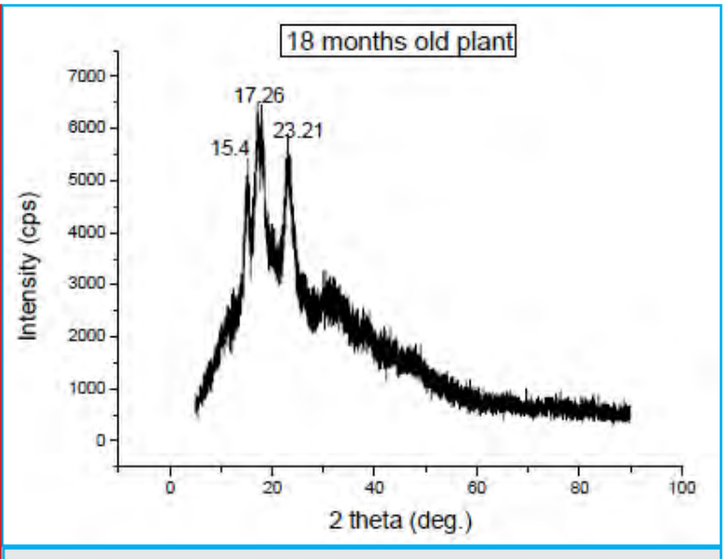

FIGURE 9. X-ray diffractogram of stem starch extracted from 18 months old pineapple plant

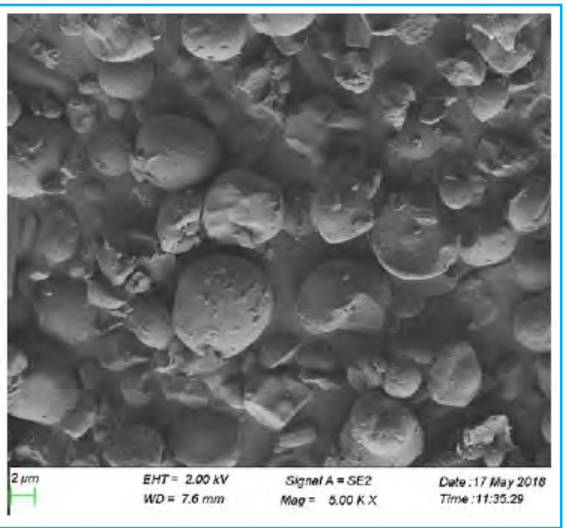

FIGURE 10. SEM image of stem starch extracted from 3 months old pineapple plant

Fig. 10-15 shows the SEM photographs of the stem starch at their different growth stages. The analysis revealed that there is no uniform distribution in granule size and shape. An increasing trend was observed in granule size with age. The shape is mainly polyhedral; surfaces are smooth, and round-shaped granules are also present. This observation is the same in all the observed stages of plant growth. These types of granules can be seen in the tuber and root starches (Hoover, 2001) (Lindeboom et al., 2004).

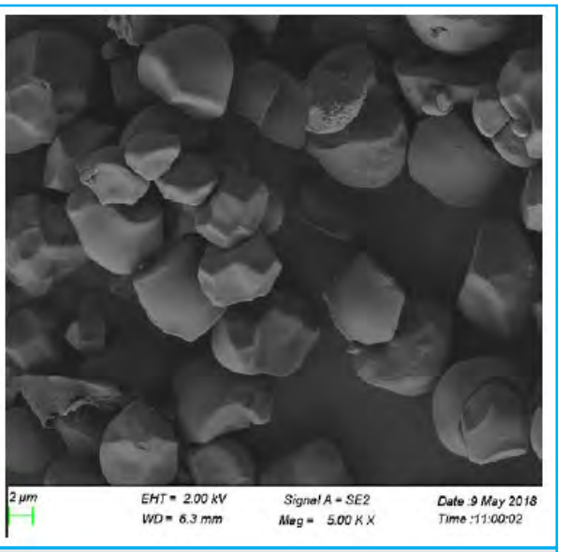

FIGURE 11. SEM image of stem starch extracted from 6 months old pineapple plant

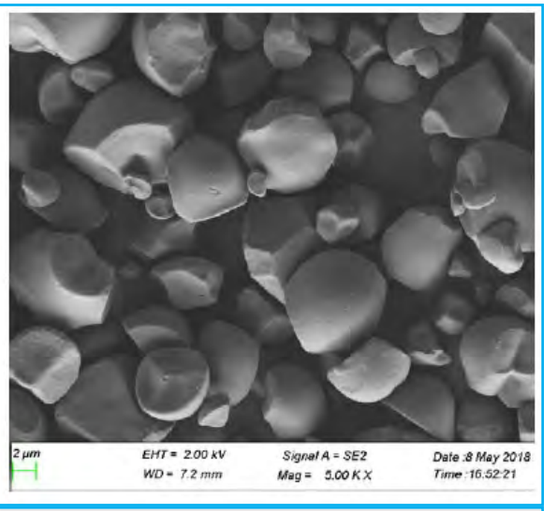

FIGURE 12. SEM image of stem starch extracted from 9 months old pineapple plant

CONCLUSION

The pineapple plant stem has a varying concentration of starches with growth and maximum starch yield obtained at nine months age. After that the yield decreased and then there is no considerable change in starch content. XRD analyses revealed this stem starch have A-type starch granules in all the growth stages. SEM study indicates that the starch granules are irregular polygonal in shape with a smooth surface. There is a slight increase in granule size with age. The results of

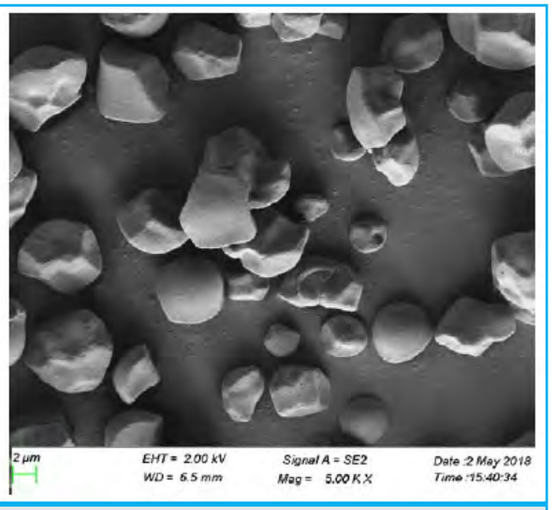

FIGURE 13. SEM image of stem starch extracted from 12 months old pineapple plant

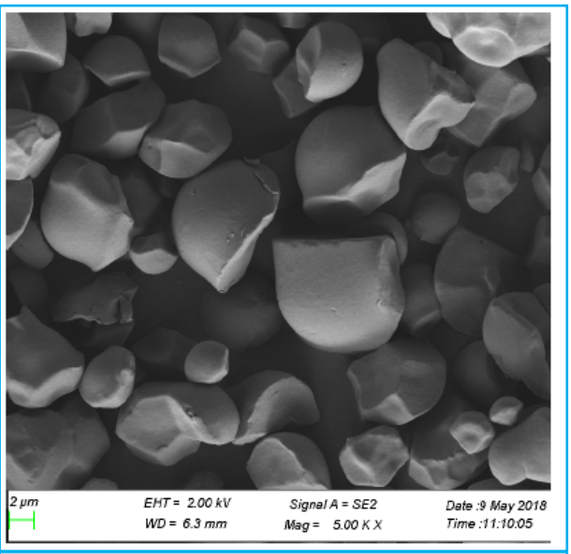

FIGURE 14. SEM image of stem starch extracted from 15 months old pineapple plant

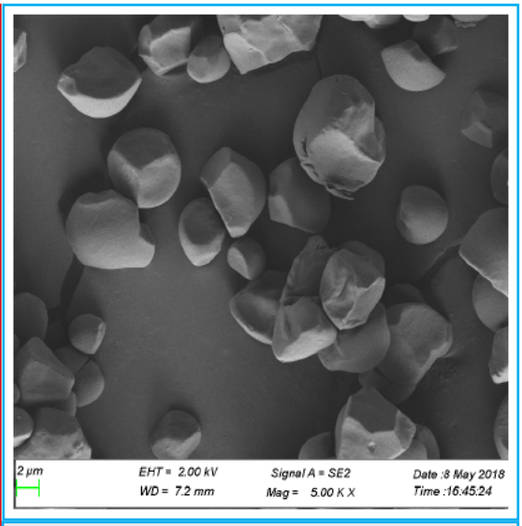

FIGURE 15. SEM image of stem starch extracted from 18 months old pineapple plant

Table 2. Size of starch granules at different growth stages

Age of the plant	Size of the starch granules (µm)
3months (Before flowering)	1-7
6months (Before flowering)	1.2-7
9months (Before flowering)	1.5-8
12months (After flowering)	2.8-7
15months (After flowering)	2.2-8.5
18months (After fruiting)	3-7

this research provide useful information about the characters of pineapple plant stem starch at their different growth stages. These data can enhance their application in various starch industries as a substitute for commercially available starches.

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Air Pollution Tolerance Index (APTI) of Some Plants Growing on the Roads of Abha, Saudi Arabia

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ABSTRACT

Ten plant species from the roads with heavy traffic in Abha Saudi Arabia were collected and studied for their phytomonitoring potential by calculating their Air Pollution Tolerance Index (APTI). Physiological as well as the biochemical parameters like Relative Water Content (RWC), Ascorbate Content, leaf extract pH as well as the Total Chlorophyll Content (Chl) were used to calculate the APTI values. *Bougainvillea glabra* and *Ricinus communis* plants showed highest tolerance to vehicular pollution, *Shinus molle*, *Catharanthus roseus*, *Hibiscus rosa-sinensis*, *Myoporum pictum*, *Juniperus procera*, *Phoenix caespitora* showed moderate tolerance while as *Tagetestanui folia* and *Vitis vinefera* were least tolerant species, thus making *Bouganivillea glabra* and *Ricinus communis* plants the ideal candidates to be used for green belt development in Abha region of Saudi Arabia.

KEY WORDS: AIR POLLUTION TOLERANCE INDEX (APTI), AIR POLLUTION, ASCORBIC ACID, PLANTATION PROGRAM

INTRODUCTION

Pollution, particularly the atmospheric pollution has emerged as one of the leading problems that has resulted from the rising human population and the industrialization (Odilara *et al*. 2006). Air pollutants including the particulate matter (PM), the vehicular exhausts as well as the industrial emissions result in deteriorating health effects in humans, create disturbances in plant ecosystem in addition to the global impact by changing the atmosphere (Raabe et al., 1999). According to the World Health Organization estimates, that air pollution results in around a million premature infant deaths around the world (Litchfield et al., 2018).

The fast urbanization and industrialization in the kingdom of Saudi Arabia has led to environmental concerns due to the heavy traffic and industrial activities. Experts at King Abdul Aziz University (KAU) have esti-

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mated that the consumption of benzene and diesel in the kingdom is 811,000 barrels every day for the 12 million cars that ply on the Saudi Arabian roads. Vehicular pollution is the major contributor of air pollution in Saudi Arabia, because of majority of population (which experts put at 95%) use cars and the population in Saudi Arabia was 32.94 million in 2017. Air Pollution Tolerance Index (APTI) is an inherent quality of plants to encounter air pollution stress. Owing to the contributions of parameters like ascorbic acid, the chlorophyll content, relative water contents as well as the leaf-extract pH in the pollution tolerance in plants, these parameters were computed together in a formulation to obtain an empirical value signifying the air pollution tolerance index (APTI) of species, (Singh and Rao, 1983 Aghaiee et al., 2019, Bellini and Tullio 2019 Manjunath and Reddy 2019).

To measure the air pollution tolerance indices of different plant species has become necessary owing to the increasing air pollution in urban localities. The Categorization of pants species as sensitive and tolerant has huge importance as the susceptible plant species can be used to serve as indicators while as the tolerant ones can work as sinks to monitor the air pollution in urban areas (Singh et al., 1991, Aghaiee et al., 2019). World-Wide significant work has been done to identify the plants suitable for phytomonitoring the vehicular pollution but nothing significant has been done to see the phytomonitoring capacity of Saudi Arabian flora. So this research aims at screening the Air Pollution Tolerance of Saudi Arabian flora to know about the susceptible and tolerant Plant species.

MATERIAL AND METHODS

Study Area: Abha city is situated in the south of Asir elevated at 2270 meters above sea level. It is located on the western edge of Mount al-Hijaz near the mount Alsouda, which is considered to be the highest mountain peak in Saudi Arabia.

The climate of Abha city is cold and semi-arid and the temperatures are influenced by the city's high altitude.

Bougainvillea glabra, *Ricinus communis*, *Shinus molle*, *Catha ranthusroseus, Hibiscus rosa-sinensis, Myoporum pictum, Juniperus procera*, *Phoenix caespitora, Tagetestanui folia* and *Vitis vinefera* plant species from Abha city were collected near traffic signals of Abha city for Polluted site while as the same plants were selected from garden away from the road which served as Control. The mature leaves were sampled from all the plant species. Collected leaves were packed in polythene bags and immediately stored in ice box for the analysis of various biochemical characteristics like total chlorophyll, ascorbic acid, pH of leaf extract, and relative water content. All the experiments were done in triplicate. The sampled leaves were analyzed in laboratory for their ascorbic acid content, Total chlorophyll, and Relative Water Content (RWC) as well as Leaf extract pH.

Ascorbic Acid Content: Leaf ascorbic acid content was calculated by the method of Bajaj and Kaur (1981) 4 ml oxalic acid-EDTA (0.05M oxalic acid, 0.2 M EDTA) was added to one gram leaf. To this one ml of o-phosphoric acid and 1ml sulphuric acid was added. 2ml of ammonium molybdate as well as 3ml of water were added to the mixture. The reaction was left alone for 15 minutes and later the OD was taken at 760 nm.The concentration of ascorbic acid in samples was then calculated from a standard ascorbic acid curve. For this 0.1 ml to 0.6 ml aliquots of standard ascorbic acid solution was taken in a series of test tubes and chemicals were added as before. After incubation period, absorbance was measured at 760 nm and standard graph was prepared.

pH: To determine the leaf extract pH, a 2 g leaves were ground in 20 ml de-ionized water and the reading was taken in a pH meter after filtration of the liquid.

Relative Water Content (RWC): The relative water content (RWC) was determined following the method of Singh et al. (1991). Fresh leaves (FW) were weighed to determine the fresh weight. The turgid weight (TW) was calculated by immersing the leaves in water over night, while as the dry weight (DW) after drying in the oven at 70^0 C was calculated as dry weight.

$$RWC= (FW-DW/TW-DW) \times 100.$$

Total Chlorophyll Content: The Total chlorophyll content in the leaves was analysed by the method of Hiscox and Israelstam (1979). The method recommends the evaluation of plant pigments. Washed leaves were chopped and 0.1g was put in vials. A volume of 5ml from dimethyl sulfoxide (DMSO) was put in these vials. The plant samples with chemicals were incubated at 65˚ C to extract the pigments. Thereafter, the vials were taken out and the OD was recorded at 663, 645 nm on Uv-Vis spectrophotometer. Values of optical densities (ODs) were used to compute the total chlorophyll contents by using the following formulae given in Arnon (1949).

Air Pollution Tolerance Index: The APTI values were calculated by the formula of Singh and Rao (1983). APTI= *A (T+ P) + R/10*A signifies the leaf ascorbic acid content (mg g^{-1} FW), T is the leaf total Chlorophyll (mg g- FW), P is the pH of leaves, and R is the percentage relative water content of leaf tissue.

RESULTS AND DISCUSSION

Ascorbic Acid Content: The Ascorbic Acid Content of the plant species is given in Fig 1. *Ricinus communis*

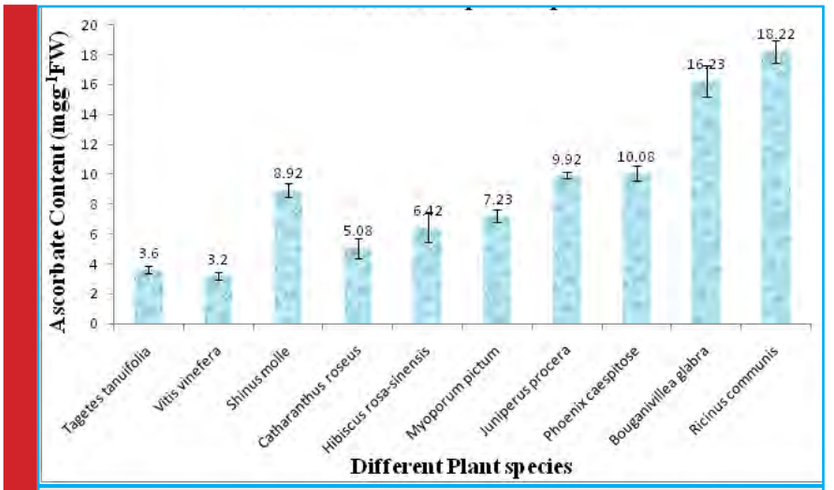

FIGURE 1. Ascorbate contents (mgg^{-1} FW) in the selected different plant species

showed the highest ascorbic content while as the *Vitis vinefera* showed the lowest Ascorbic acid content. Ascorbic acid an antioxidant is presentin all growing plant parts to prevent the plant from various types of stress. Ascorbic acid is said to be able to decrease the amount of ozone penetrating the cell wall to reach the plasma membrane (Roshchina and Roshchina, 2013, Bellini and Tollulio 2019). Ascorbic acid also has a role in carbon fixation in the process of photosynthesis where it works as a reducing agent (Pasqualini et al 2001, Wheeler et al., 2015). Plants have been researched to have a decline in ascorbic acid under pollution (Keller and Schwager, 1977). Increased ascorbic acid content in plants despite after being under pollution stress, therefore, indicates the high tolerance in the plant against the pollution (Singh et al. 1991, Manjunath and Reddy 2019).

pH of leaves: The leaf extract of the plant species is given in Fig 2. *Ricinus communis* has the highest pH of 7.11 while as *Myoporum pictum* has the lowest pH of 5.32. Elevated pH in plant leaves has a rolein conversion from hexose sugar to Ascorbic Acid (Escobedo, 2008) while as decreased pH is thought to be associated sensitivity to air pollution. Bharti et al. (2018) and Rathore et al. (2018) are of the opinion that the exposure of leaves to acidic pollutant like Sox and NOx result in decrease in the leaf pH and this decrease is moreprominent in the sensitive plants in comparison to the tolerant ones.

Total Chlorophyll Content: Total Chlorophyll Contents ranged from 0.84 mgg^{-1} FW in *Tagetes tanuifolia* to 6.32 mgg^{-1} FW in *Hibiscus rosa-sinensis*.Total chlorophyll content of leaves has been used commonlyfor evaluation of the effect ofair pollutants on photosynthesis

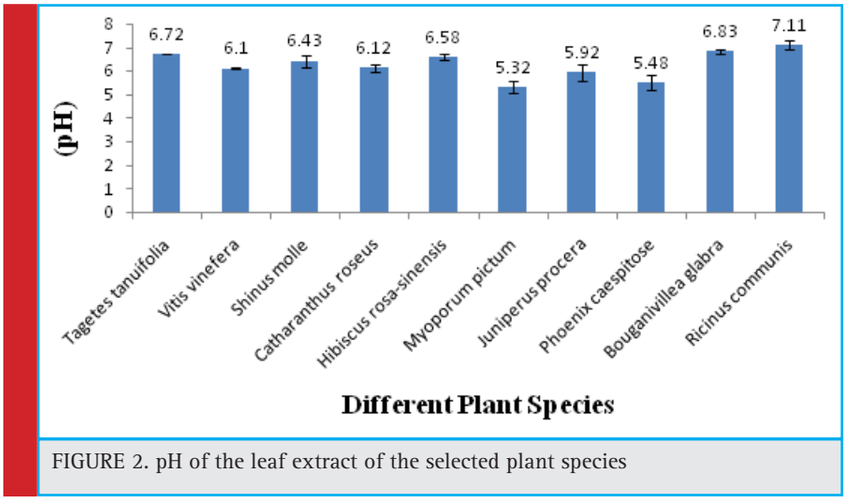

FIGURE 2. pH of the leaf extract of the selected plant species

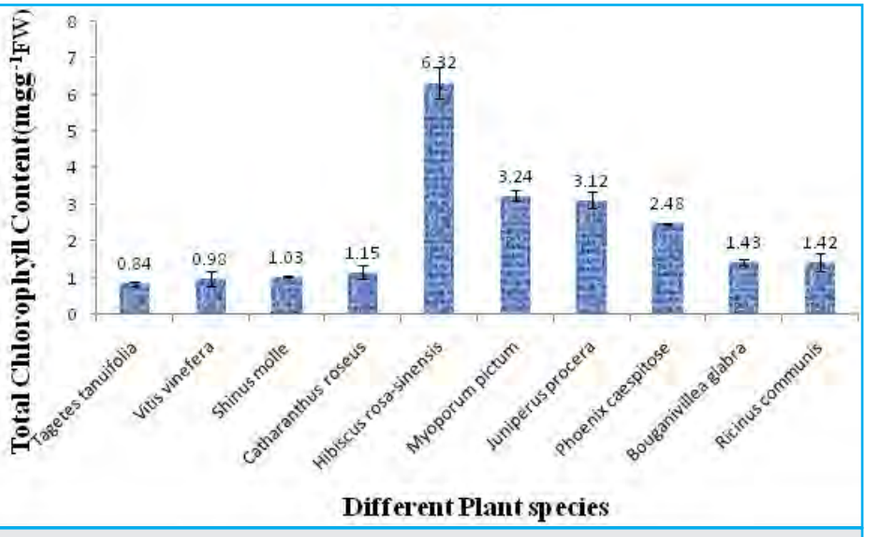

FIGURE 3. Total Chlorophyll Content (mgg^{-1} FW) of the selected plant species

rate in plant leaves (Sharma et al., 2019). Total Chlorophyll is thought to be an index of growth, photosynthetic activity and biomass production. Kameswaran et al. (2019) are of the opinion that the plant tolerance to the SO_2 from vehicular exhaust might be responsible for decreased chlorophyll synthesis or degradation in the already present chlorophyll content, thereby, suggesting that the plants with a high chlorophyll content even after subjected to vehicular pollution are usually tolerant to the pollutants.

Relative Water Content (RWC): The Relative Water Contents of the plant species are shown in Fig 4. *Ricinus communis* showed the greatest RWC (92.15%) while as the *Hibiscus rosa-sinensis* (41.15%) showed the least RWC. High Relative water content (RWC) in the leaves helps plants in the maintenance of the physiological balance under stress conditions. So a high value of RWC means a high resistance to stress in plants (Aghaiee et al., 2019).

Air Pollution Tolerance Index (APTI): The APTI values of the selected plant species is shown in Fig 4. *Bougainvillea glabra* and *Ricinus communis* plants showed highest APTI values, *Shinus molle*, *Catharanthus roseus, Hibiscus rosa-sinensis*, *Myoporum pictum, Juniperu procera*, *Phoenix caespitora* showed moderate values while as the *Tagetes tanuifolia* and *Vitis vinefera* plants showed the least APTI value. The APTI values offer a credible method to screen the flora of any location for

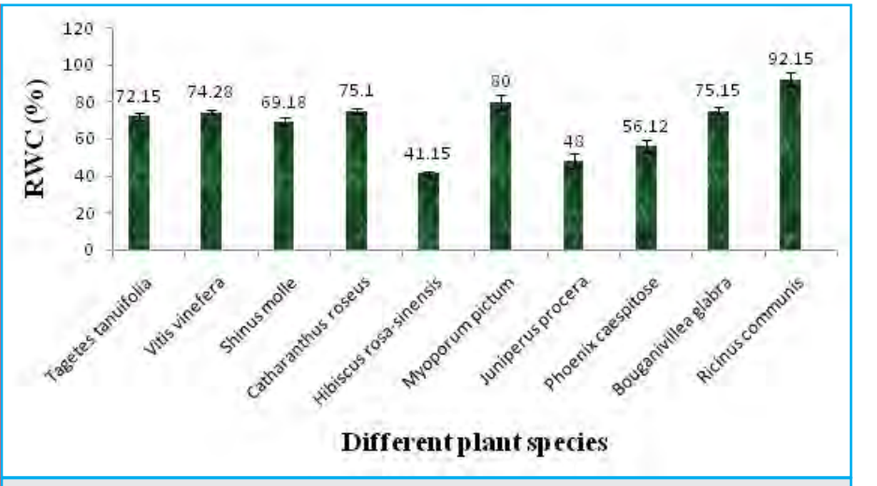

FIGURE 4. RWC (%) of the selected plant species

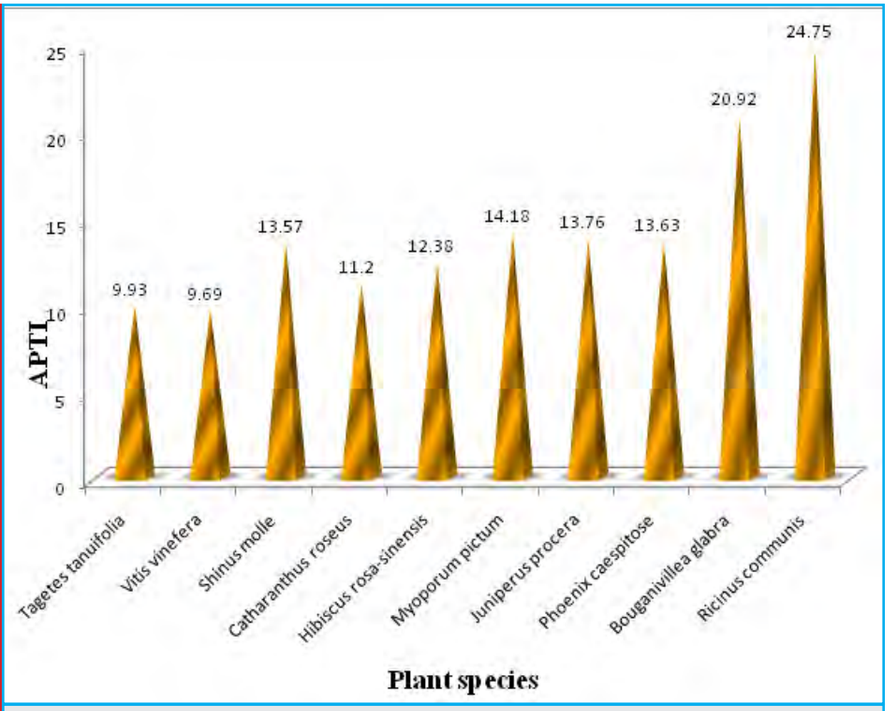

FIGURE 5. Air Pollution Tolerance Index values for the selected plant species

their tolerance and susceptibility to vehicular pollution. Plants with APTI value ≤11 are considered to be sensitive, while those with APTI value ranged from 12 to 16 classified as intermediate, and APTI value of ≥17 are known to be tolerant (Bharti et al., 2018, 1991).

SUMMARY AND CONCLUSIONS

The APTI values were in the order *Ricinuscommunis (*24.75) > *Bougainvillea glabra (*20.92) > *Myoporumpictum (*14.18*)* > *Juniperus procera (13.76)*>*Phoenix caespitora*(13.63) > *Shinusmolle* (13.57) > *Hibiscus rosa-sinensis* (12.38) > *Catharanthus roseus* (11.2) > *Tagetes tanuifolia* (9.93) > *Vitis vinefera* (9.69). This study is very important to choose tolerant speciessuitable to monitor the vehicular pollution in Saudi Arabia.

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Four-vector Efficiency of Infrastructure in the System of Providing Regional Socially Significant Needs Taking into Account the Concept of Marketing of Changes

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ABSTRACT

Current economic and social development of world regions, especially the territories of former USSR countries needs using modern technologies such as marketing of changes, innovative approaches to regional infrastructure development. Successful combination of different instruments together with adequate evaluation of their effectiveness impetus for the introduction of positive changes in the regions'position. The authors of the study developed the method for evaluating the potential of regional infrastructure with the formation of an integral indicator with four directions. The scientific novelty of the research is to substantiate the proposals for formulating an approach to assessing the effectiveness of regional infrastructure. The methodological coherence of the evaluation of indicators, unlike their separate analysis, greatly expands the possibility of objectively calculating the synergistic effect of the functioning of different infrastructure activities as components of the system. The totality of evaluations within the framework of the author's methodology makes it possible for qualitative comprehensive evaluation, adherence to the principles of hierarchy, complexity and universality of the evaluated criteria. The practical significance of the obtained results is the ability to use the results of this study in the practical activities of the entity managing territories that are aimed at providing socially significant services to the population. The proposals of the authors will be useful to the regional authorities in developing measures to enhance the development of local infrastructure as well as in the management of private social institutions.

KEY WORDS: INFRASTRUCTURE, INFRASTRUCTURE EFFICIENCY, INNOVATION, MARKETING OF CHANGES, REGIONAL DEVELOPMENT

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INTRODUCTION

Regional innovation systems formation, creation of targeted programs and projects concerning local social and economic development, marketing of changes implementation in local infrastructure objects management are tasks that require innovative approaches to their realization. It should be borne in mind that every innovative solution in the system of regional governance have both economic and social consequences, which necessitates perfect scientific and practical forecasting of the innovative marketing tools effectiveness. In modern conditions regional territorial policy should be based on the cooperation of state, private and local business infrastructure on the basis of their common goals. At present, the issue of innovation management and marketing of innovations, in particular in the regions activity, has been thoroughly researched in domestic economic science (Fedulova, 2015, Kainova, 2014, Lyulov, 2019, Oliinyk 2017, Orlatyi *et al*, 2013, Pepchuk, 2015, Poliakova, 2016, Prokopenko, 2017, Syhyda, 2018, Vasylieva, 2018, Goltvenko *et al*, 2019), as well as in the work of foreign scientists (Uyarra, 2010 Schwerdtner, 2015, Huang, 2013, Kolehmainen, 2016, Eder, 2017, Fridman, 2017). However, the subject of regional innovation management, in particular in the area of infrastructure provision of territories, remains poorly developed.

There is a clear tendency to intensifying the competition between regions, cities and territories for financial and information flows, highly qualified specialists, investors. Competitive bases for receiving funds from the state budget for the implementation of local projects are being implemented. More and more territorial units are developing projects and submitting them to competitions. Such competitive atmosphere forces territories to compare themselves with others, determine what they are best at, and demonstrate these benefits in the region's innovative passport. The use of marketing technologies for regional development is a new phenomenon for Ukraine, but the need for them is increasing. The authors highlight the main factors of social progress in modern conditions, Figure 1.

The figure purposely combines factors of social development socialization and information revolution, which on the one hand are influenced by scientific and technological progress as a separate fundamental factor of changes in society and the emerging factor of marketing of changes, the use of which causes changes in the parametric factors of socialization (social changes which are driven by the influence of marketing tools) and on the other hand by information revolution (revitalizing the progress of the information component due to the impetus of active marketing tools implementation to manage the process of social development). Marketing

FIGURE 1. Factors of social progress

tools are a solid foundation for streamlining strategic decision-making to build a positive image of countries and regions, enhancing their competitiveness. The infrastructure of the region is one of the dominant parameters that affects the reproduction potential of the territory, improving the quality of life, branding and more. In order to formulate appropriate priorities for the infrastructure development, it is advisable to develop criteria that will determine its current effectiveness and criteria of changes (strategic benchmarks that determine the further development of each infrastructure institution, regardless of its subordination). By aggregate estimates of each infrastructure item, it is possible to form a general map of the infrastructure provision of a particular region. An integrated assessment of the infrastructure development provides an opportunity to rank individual regions in terms of their infrastructure security, taking into account the impact of each type of institution on the social and economic situation of each region.

On the basis of determining the current state of infrastructure development level with the accession of foresight, it is possible to predict possible structural and dynamic changes in infrastructure development in the future. The consolidated assessment methodology makes it appropriate to compare the development of the infrastructure of a particular territory with the same assessments of it in the country as a whole.The level of regional development is determined by a number of aggregated, generalized and isolated indicators. Human Development Index has a fundamental role when calculating the countries' competitiveness. According to this indicator, Ukraine is in rather low positions – its value is lower than the average for the countries with high index of human development and below the average for the countries of Europe and Central Asia. In the period from 1990 to 2014, the value of the Human Development Index in Ukraine increased slightly from 0.705 to 0.747, but only by 6%, which is below the average level of its growth in the world (Chela, 2017).

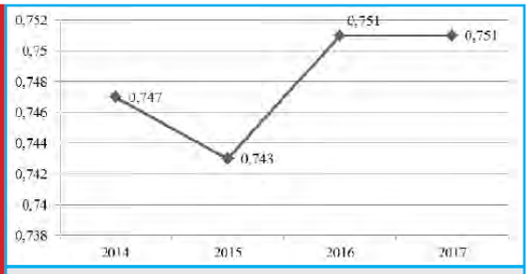

FIGURE 2. Dynamics of the Human Development Index for Ukraine

The dynamics of this indicator in recent years is presented in Figure 2. In general, we can note the unstable dynamics of this indicator.

The Human Development Index is directly related to the well-being of the population, which is also determined by the level of infrastructure development. Employee security with all the necessary social components through the use of social assets, is determined by the "decent work" in the figure below.

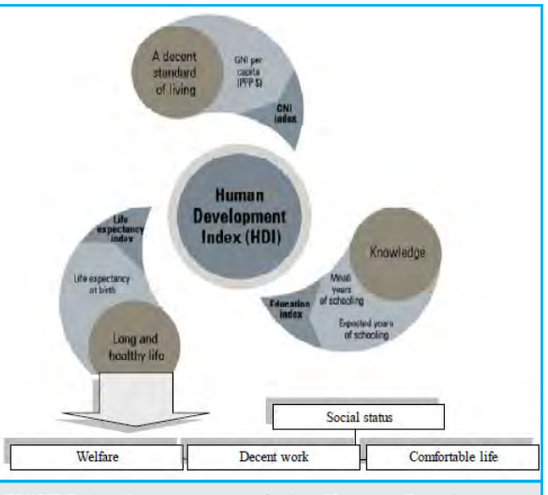

FIGURE 3. Components of the Human Development Index with parametric definition of the "Long and healthy life factor"

There are a number of useful projects implemented in Ukraine related to the infrastructure provision of regions. The first open source portal for local communities, GIS DATA, has been initiated and implemented to help decision-makers in various projects plan their follow-up more effectively. The tools of this portal allow to manage the network of infrastructure in each territory. Considering that 40% of school buildings in Ukraine are unfit for pupils' education, the urgent effective infrastructure solutions are badly needed. In the area of consumer services, there is a decline in the number of objects and the share of services provided to the public, although almost two thirds of all services are owned by private companies. In the trade and restaurant industry, there is a steady decline in retailers, especially in rural areas. There is a decline in restaurant network while increasing the fast service facilities. This is despite the fact that these are the objects of vital importance in the sphere of hospitality, which is identified as promising in Ukraine. The territorial accessibility of social infrastructure is, in fact, a criterion for optimizing the location of these objects and the effectiveness of their territorial organization. The average radius of social infrastructure accessibility in Ukraine is 5.7 km, with its highest mark 8.0 km in Kherson region and the smallest value 1.4 km in Kyiv. The range of accessibility of social infrastructure in Ukraine is permanently increasing (Shpyliova, 2006). Moreover Ukraine is characterized by a significant disproportion between housing and social infrastructure in cities. The dynamics of the obtained permits and the number of construction objects indicate that the pace of housing construction in Ukraine will not fall in the nearest future (Zaderei, 2019).

The industry is attractive to investors, as evidenced by the announcements of major projects. A study of Ukrainian households (Novikov, 2018) on the availability of individual goods and services shows that a lot of urban and rural households are deprived of their social needs, such as personal development, quality rest, medical care. A number of other problems with the accessibility of social services can be observed among the rural population.

The building norms of Ukraine state that the infrastructure of the city needs to be developed with the increase of population both in large and peripheral cities. However, the inconsistency of some provisions allows developers to circumvent such requirements. According to Article 40 of the Law of Ukraine "On Regulation of Urban Planning Activity", construction companies are obliged to pay a share contribution to the construction of infrastructure before the commissioning of the facility to the limit of up to 4% of the estimated cost of the project. Developers can make this contribution with money or in the form of utilities, kindergarten, school or other infrastructure objects. Therefore, often construction companies include educational institutions in the plans for construction of large residential complexes. Multiple companies can combine and build schools and kindergartens with spot-building in one array. Developers explain that they are ready to create local social infrastructure at the expense of a share contribution, but the city does not always accept on its balance built educational institutions, so new residential complexes often open private kindergartens and schools. This helps companies improve their reputation and make their assets more attractive to investors. In addition, there have been

TABLE 1. Survey results of Ukrainian households assessing their availability of infrastructure services (fragment)

Signs of unavailability of social services	Percentage of households deprived of goods and services, %	
	urban	rural
Lack of funds to pay for the services of a doctor in a medical institution (in the absence of such services on a free basis), analyzes, examinations, procedures	27.4	31.3
Insufficient funds to pay for inpatient treatment services (in the absence of such services on a free basis)	26.6	32.0
Insufficient funds for vocational education	7.2	8.7
The impossibility of a family vacation not at home for at least one week a year	52	52.0
Absence of retail stores near housing	2.9	13.7
Absence of establishments providing domestic services (hairdressing salons, dry-cleaners, repair of clothes, etc.)	5.6	51.5
Absence of pre-school facilities near housing	1.3	4.6

attempts to cancel even such a share contribution to the development of local infrastructure, which is explained by the lack of such practices abroad, the deterioration of the Doing Business ranking in terms of increasing the cost of administrative procedures, the lack of unit participation for the repair of infrastructure objects. From 2017 the sanitary norm "Equipment, maintenance of preschool educational institutions and organization of children's life" ceased to operate in Ukraine.

This document set out the requirements for the permissible walking distance for kindergartens. Instead, the Sanitary Regulations for Preschool Institutions entered into force, in which these requirements are absent. This means that it is no longer regulated how many kindergartens should be built in a particular settlement. Many decisions to be made do not go in favor of local infrastructure. At the beginning of the twentieth century many social needs including medical, cultural, professional, etc., have traditionally provided by regional town-forming enterprises for their employees and their families through their own infrastructure network (sanatoriums, medical centers at the enterprise, recreation centers, children's camps, vocational training, etc.). Currently, there is only a small proportion of infrastructure assets in very poor condition remains. They have potential utility and effectiveness for the enterprise if to manage them adequately (Sager, 2014). The authors of this study have attempted to evaluate the potential of regional infrastructure with the formation of an integral indicator, which can be included in the system of general indicators for assessing the level of social and economic development of regional systems.

MATERIAL AND METHODS

This research was carried out based on an integrative review methodology where relevant articles based on the research scope with key words such as infrastructure, regional infrastructure, infrastructure assessment, and infrastructure development were used (Kyrychenko, 2016, Sadchikova, 2017, Panasiuk, 2012, Malchykova, 2016). Such integrative review methodology allows to outline relevant papers both past and present for review in other to give better understanding to the topic of local infrastructure assessment from the standpoint of its development.

RESULTS AND DISCUSSION

Infrastructure efficiency is proposed to be evaluated in four components: economic, image, social and developmental capacity. The last component takes into account principles of marketing of changes. These components form the multiplicative efficiency of regional infrastructure, Figure 4.

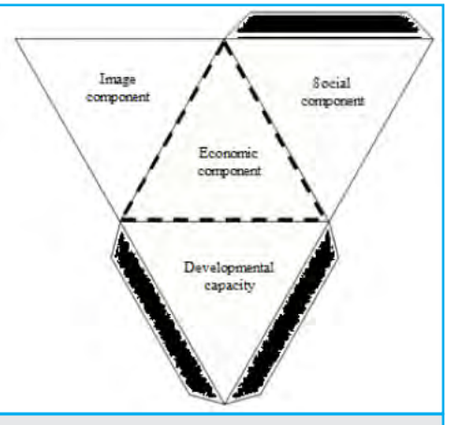

FIGURE 4. A four-way pyramid of components that determine the effectiveness of an infrastructure object

The generalized model for evaluating infrastructure effectiveness is as follows:

$$E_{infr.} = \{C_e; C_i; C_s; C_{d.c.}\} \quad (1)$$

where $E_{infr.}$ – four-dimensional indicator of infrastructure effectiveness; C_e – economic component; C_i – image component; C_s – social component; $C_{d.c.}$ – component of developmental capacity.

The economic component includes profitability indicators, which give information on whether a particular infrastructure item is profitable. The image component is manifested in the active support by the local population, the frequency of citizens visit, priority of their development in the eyes of the local population. The social component is to provide society with a set of services especially those services that are the most needed due to the lack of them in a particular region. The state of the material and technical base is a significant factor of competitiveness for the institutions of cultural and public services. This is the main factor that attracts consumers. Therefore, the analysis of indicators such as deterioration coefficient and replacement coefficient must be taken into account in the economic component of performance analysis.

The next step is to build a model for calculating the integral index of the economic component. To calculate the integral metric based on the above indicators, we propose to use the universal metric – Harrington's desirability function, which is characterized by such properties as adequacy and statistical sensitivity, which allows

Table 2. Indicators for determining the economic component of regional infrastructure efficiency

No.	Indicator	Formula	Optimal value (k_{opt})	Desired orientation of the indicator
1.	Profitability of the object (P)	$P = \frac{NP}{NPA} \cdot 100\%,$ where NP– the net profit of the institution; NPA – net proceeds from all activities	1	max
2.	Profitability of the specialized services provided by the infrastructure institution (C_p)	$C_p = \frac{P_p}{C_{serv}} \cdot 100\%,$ where P_p – profit from the sale of specialized services of the institution; Cserv– costs of services	1	max
3.	Coefficient of implementation of the service plan ($C_{i.p.}$)	$C_{i.p} = \frac{AV}{PV},$ where AV – the actual volume of services provided in the reporting period; PV – the planned volume of services provided in the reporting period	1	max
4.	Expense coefficient of the institution (C_{exp})	$C_{exp} = \frac{C_f}{C_p},$ where C_f– all expenses incurred by an institution in the reporting period; C_p – planned costs of the institution in the analyzed period (including costs for repair, modernization, costs due to the increase in the number of clients, etc.)	1	min
5.	Institution utilization coefficient (C_d)	$C_u = \frac{V_a}{AAC},$ where V_a – the volume of services provided by the institution in the analyzed period; AAC – average annual capacity to providing services	1	max
6.	Recovery coefficient (C_R)	$C_R = \frac{VF}{CB},$ where VF – the value of fixed assets at the end of the analyzed period; ACF– average annual cost of fixed assets	1	max
7.	Depriciation coefficient (C_D)	$C_D = \frac{AD}{ACF},$ where AD – accumulated depreciation; ACF – average annual cost of fixed assets	1	min

it to be used as an optimization criterion. The basis for constructing this generalized function is the idea of converting the natural values of individual indicators into dimensionless form, with the following subtraction of partial functions on the Harrington scale and the integral index of economic component E:

$$E = \sqrt[n]{\prod_{i=1}^{n} d_i}, \quad (2)$$

$$d_i = exp\ (-exp(-y_i)), \quad (3)$$

where n – the number of indicators used to evaluate the economic component of an institution's efficiency; d_i – partial function that is determined according to the Harrington scale; y_i – economic component in dimensionless form.

In order to use the Harrington scale, it is necessary to transfer the studied indices to a dimensionless form and to calculate the values of partial functions by formula (3).

We use such formulas to give dimensionless form to the indicators:

$$y_i \uparrow (\max) = \frac{k_i}{k_{opt}}, \quad (4)$$

$$y_i \downarrow (\min) = \frac{(1-k_i)}{k_{opt}}, \quad (5)$$

where k_i – the estimated value of the indicator; k_{opt} – the critical value of the indicator; *max/min* – criterion of maximization (minimization) of the indicator. To characterize the level of economic component of a particular institution of infrastructure we will use the scale given in Table 3.

Table 3. Standard marks on the Harrington scale

Function value	Marks on the scale
1.00-0.81	Very good (excellent)
0.80-0.64	Good
0.63-0.38	Satisfactory
0.37-0.21	Bad
0.20-0.00	Very bad (critical)

The image component involves the study of the consumers' attitude to infrastructure services, which is expressed in the frequency of requests for services, as well as the desire of clients to recommend the institution to other people. In this component it is expedient to take into account such criteria as the convenience of receiving services, the adequacy of the premises of institution, the facilities, the level of service, the openness and accessibility of information about institution, the variability of services, etc. The image of infrastructure objects is often determined by the small details. It should be convenient for the local population to receive the service of institution. An important role has the lack of queue, simplicity of registration and so on. Regarding the adequacy of the premises, this is a subjective criterion according to the opinion of the clients of institution, although for all infrastructure objects there are very specific normalized indicators of the area sufficiency. The variability of services (the ability to choose an alternative offer from an institution) is essential. Determining the quality of service indicator makes it possible to adjust the conditions of their provision and the attitude of consumers towards them. Indicator of consumer confidence in the institution is determined by the formula:

$$I_T = I_S + N_D + N_A, \quad (6)$$

where I_T – the indicator of trust; I_S – index of satisfaction with services, which is determined by the formula (6); N_D – the number of customers who have expressed a desire to use the services again; N_A – the number of service consumers who are willing to advise them to other people.

$$I_S = \frac{N_{total} - N_{n.s.}}{N_{total}}, \quad (7)$$

where N_{total} – the total number of analyzed consumers; $N_{n.s.}$ – the number of customers who are dissatisfied with the services.

The indicators in formulas (6-7) can be conveniently determined by interviewing consumers directly after the end of the service period and by the number of complaints and positive feedback received from customers. Other quantitative indicators that determine the image component of infrastructure facilities include the following:

$$S_p = \frac{RC}{TN} \cdot 100\%, \quad (8)$$

where S_p – the proportion of regular customers among the users of institution; RC – the number of repeat consumers (users of infrastructure services who have used service of a particular institution more than once); TN – total number of consumers of institution's services.

This indicator looks similar to the one calculated in formula (5), but in fact the indicators differ because formula (6) shows only the desire of consumers to use the services again, and the indicator of formula (8) shows the number of consumers who have already used the institution's services again

$$I_g = \frac{RC_c}{RC_p}, \quad (9)$$

where I_g – index of the number of regular clients growth compared to the previous analyzed period; RC_c –

Table 4. Indicators for determining the social component of regional infrastructure effectiveness

Indicator	The essence / formula to determine
Provision of typical infrastructure institution in the region (S)	The number of institutions providing similar services in the region where the analyzed object is located, units
The part of the region' population that needs this institution (S_p)	$S_p = \frac{AP}{TA} \cdot 100\%$, where AP – the number of people who use institution, people; TA – total population of the region, people
The importance of infrastructure object (IO)	How this institution is positioned in the region: as one of the attributes of the region, or no different from other institutions
Territorial provision of institutions providing similar services to the population (TS)	$TS = \sqrt{\frac{AT}{N_e}}$, where AT – area of the territory, sq. km; N_e – the number of infrastructure institutions of this type, units

the number of regular customers in the current period; RC_p – the number of regular customers in the previous period.

The social component of infrastructure efficiency is determined by the indicators which are shown in Table 4.

Regarding the development ability component, it can be assessed on a set of criteria expertly on a 5-point scale (0 points – institution does not meet the defined criterion; 1 point –institution is very poorly meets this criterion; 2 points – institution does not meet the specified criterion; 3 points – institution fits well with the selected criterion; 4 points – institution very well fits the defined criterion; 5 points – infrastructure object fully meets this criterion). The indicators are estimated by the formula:

$$R = \sum_{i=1}^{n} W_i \cdot B_i, \quad (10)$$

where R – the readiness of institution management to implement development measures; W_i – the weight of the i-th criterion for assessing readiness for changes; B_i – evaluation of the i-th criterion in points.

The level of readiness for changes can be determined on a scale of 0 to 5 in a step determined by the formula:

$$SS = (B_{max} - B_{min}) / n, \quad (11)$$

where SS – step scale; B_{max} – maximum scale score; B_{min} – minimum scale score; n – number of intervals.

The list of criteria for evaluating the component of readiness for development is shown in Figure 5.

To determine the multiplicative indicator of the effectiveness of each infrastructure institution, it is advisable to use a complex formula:

$$E_a = \frac{(E \cdot w_e + S \cdot w_S + I \cdot w_I + A \cdot w_A)}{w_e + w_s + w_I + w_A}, \quad (12)$$

where E_a – multiplicative efficiency of infrastructure institution; E – value of the component of economic efficiency; S – the value of the social component; I – he value of the image component; A – he value of a component of developmental ability; w_e – the weight of the component of economic efficiency; w_S – the weight of the social component; w_I – the weight of the image

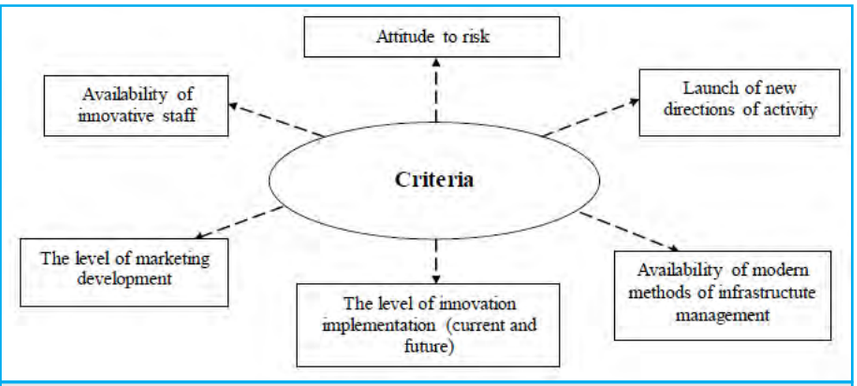

FIGURE 5. Criteria for evaluating the readiness of institution to develop

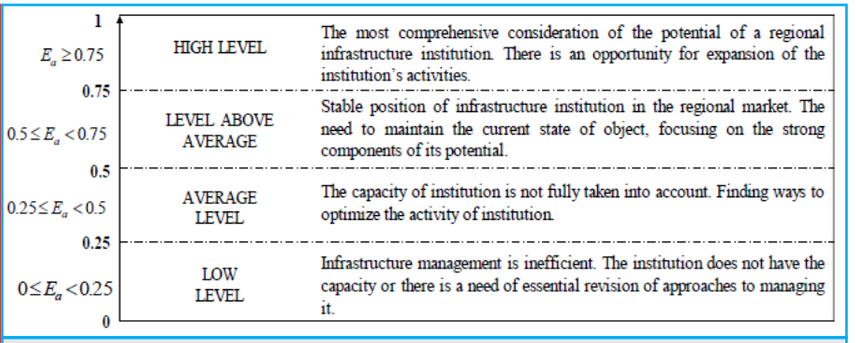

FIGURE 6. Efficiency levels of infrastructure management

component; w_A – the weight of a component of developmental ability.It is possible to distinguish the value of the multiplicative indicator, within which we can state the effectiveness or inefficiency of a particular infrastructure institution.

The authors' approach to assessing an individual institution of infrastructure over four different components can be the basis for determining a comprehensive indicator of the effectiveness of regional infrastructure in certain territories. The effectiveness criteria may be revised and supplemented or modified according to specific motives of infrastructure development in the regions.

CONCLUSION

The scientific novelty of the research is to substantiate the proposals for formulating an approach to assessing the effectiveness of regional infrastructure. The article presents an approach to assessing the effectiveness of infrastructure institutions. The methodological coherence of the evaluation of indicators, unlike their separate analysis, greatly expands the possibility of objectively calculating the synergistic effect of the functioning of different infrastructure activities as components of the system. The totality of evaluations within the framework of the author's methodology makes it possible for qualitative comprehensive evaluation, adherence to the principles of hierarchy, complexity and universality of the evaluated criteria. The practical significance of the obtained results is the ability to use the results of this study in the practical activities of the entity managing territories that are aimed at providing socially significant services to the population. The proposals of the authors will be useful to the regional authorities in developing measures to enhance the development of local infrastructure. Further research requires development of proposals for profiling of the estimated indicators, taking into account the specific activity of infrastructure objects, as well as focusing on the study of the conditions of direct competitors' activity of local state infrastructure institutions – private institutions providing socially significant services to the population.

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Research Communications

Students Perception of Teaching Methodologies Practiced in an Academic Institution in Majmaah, Saudi Arabia: A Unified Perspective

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ABSTRACT

Medical education aims to provide valuable knowledge through effective learning and teaching strategies to students so that they can become competent caregivers. This study seeks to determine students' perception regarding those strategies and preferences for different available teaching methodologies. This is an observational, cross-sectional, and institution-based study conducted using a pre-structured, pre-validated, close-ended questionnaire. Three hundred fifty students participated in the study. Most of the students (78.3%) preferred interactive lecture through multimedia which was perceived to be the most effective method of delivery. More than 45% respondents considered the use of PowerPoint presentations as an efficient tool and 58% considered the multiple-choice question format in examination as the best evaluation method as compared to essay questions. Student's perceptions should be evaluated further through longitudinal and follow up studies which could help in corroborating their effectiveness. This would help in bridging the gap between the knowledge taught and gained by the students, thus improving academic excellence and reducing average outcomes.

KEY WORDS: LECTURE, MEDICAL EDUCATION, MULTIMEDIA, MEDICAL STUDENTS, SEMINARS

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INTRODUCTION

The arena of medicine is a very competitive field, and medical students encounter various teaching strategies over the course of their studies.. Medical institutions worldwide face the challenge of catering to a growing student population while maintaining the quality of education. Teacher-centered and student-centered approaches are broad categories of approaches to education. Lecture is a conventional teacher-focused strategy, though in a student-focused approach, students play a functional role in learning and concentrate on deep thinking (Lida and Mona, 2013). The introduction of technology in education has changed the way that ideas are delivered and conveyed. Electronic media like PowerPoint is becoming more and more popular in medical institutions (Muttappallymyalil et al, 2016). Research about efficient teaching techniques is a pressing issue, and the implications of this research can shape the nature of education (Jafri and Keramati, 2012, Withers et al 2016, AyeMon et al 2014, Raj and Kanagasabapathy, 2019).

Student evaluations of teaching methodologies contribute significantly to improving teaching standards. Students are the end beneficiaries of education, and their opinions regarding teaching methods are indicators of their satisfaction with a particular learning experience (Withers et al, 2016; Theall and Franklin, 2001). Papanna et al (2013) conducted a study involving 286 students and found that problem-based learning (PBL) was most favored (71.4%), while didactic lectures were the least preferred (32.8%). Blackboard was observed to be another favored teaching aid, preferred by 46.9% of students (Papanna et al, 2013). Salwani et al (2014) conducted a study involving 50 students. In his study, 72% of students chose lecture as the most-preferred teaching and learning method, tutorials were preferred by 10% of students, while 6% of students preferred PBL and practical respectively (Salwani et al, 2014). Atif et al (2011) conducted a study involving 200 students and found that 40% of the students favored PowerPoint presentations as a reliable mode of teaching as it was interesting and interactive as compared to PBL (28.8%), audio-visual aids (18.6%), and Whiteboard (12%). Shreemanta et al (2013) conducted a study among 337 students and found that 77.02% selected standard lectures as the most efficient mode of teaching, followed by group discussion (68.02%), tutorials (58.94%), and seminars (49.05%) (Shreemanta et al, 2013).

Therefore, this study aims to evaluate the perceptions and preferences of students regarding various learning methodologies used in medical colleges. Evaluating students' opinions can help address student learning issues, identify students' preferred learning methods, and help teachers improve the efficiency of their teaching. The findings of this study will help overcome deficiencies in medical education and improve the credibility of medical graduates as future caregivers.

MATERIAL AND METHODS

This is an observational, cross-sectional, and institution-based study employing a pre-validated, close-ended questionnaire. A total of 350 students participated in the study. The participants were all MBBS students at various stages of their studies. Approval for the study was granted by the ethical and research committee, allowing the participants to be approached for the study. Informed consent was obtained from every student and confidentiality was assured. A carefully designed questionnaire comprised of four sections was distributed among the students. In the first section, they were instructed to indicate their unbiased opinion regarding various teaching methods on a Likert scale ranging from 1–5 (1 = strongly disagree to 5 = strongly agree). The respondents were asked to select the option that they believed was most appropriate. In the second section, they were encouraged to propose the reason they preferred a particular teaching method. The third section consisted of questions regarding students' preferences for various teaching aids, including PowerPoint, Blackboard, various other audio-visual aids, and problem-based discussions. Finally, the fourth part was framed to elicit responses regarding student preferences for various evaluation methods used in examinations on a Likert scale ranging from 1–5 (1 = strongly disagree to 5 = strongly agree). There were clear instructions to participants not to disclose their identity nor to write any individualized remarks about staff members. Responses were collected from students within a time-bound period. On the basis of analysis and observation, results were extrapolated and were compared with other relevant literature.

RESULTS AND DISCUSSION

Undergraduate medical education is undergoing major changes, and efforts are being made to make it more interesting (Desy et al, 2017). In our study, the majority of the medical students strongly agreed or agreed that teacher-centered approaches were better than student-centered approaches. Teacher-centered activities were defined as conventional lectures, whereas student-centered activities were teaching methodologies that promoted active student participation, such as small-group discussions, tutorials, and seminars prepared and delivered by students (Kim and Hwang, 2017). The student-centered approach has challenged the traditional role

Table 1. Preference for Teaching Methods

Teaching method	Strongly Agree (5)	Agree (4)	Neutral (3)	Disagree (2)	Strongly disagree (1)	Mean Likert-scale score
Lecture	83 (23.7%)	191 (54.6%)	50 (14.3%)	16 (4.5%)	10 (2.9%)	3.9
Seminars	46 (13.2%)	111 (31.7%)	99 (28.3%)	54 (15.4%)	40 (11.4%)	3.1
Small-group discussion	78 (22.3%)	132 (37.7%)	60 (17.2%)	54 (15.4%)	26 (7.4%)	3.5
Tutorial	50 (14.3%)	114 (32.6%)	88 (25.1%)	68 (19.4%)	30 (8.6%)	3.2

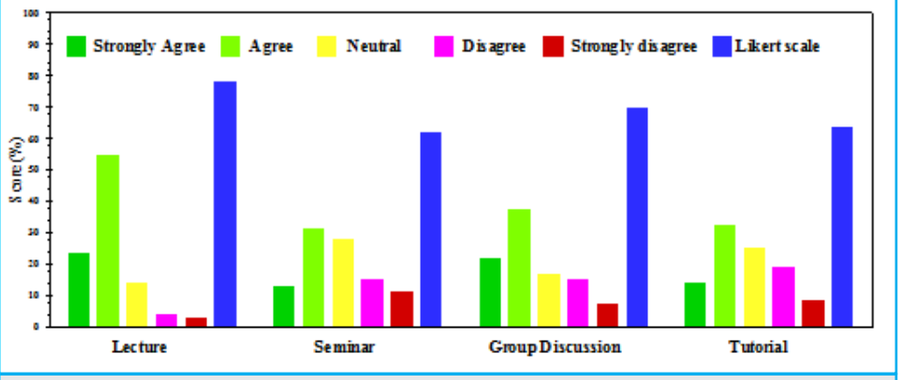

FIGURE 1. Students' preferred teachingmethods

of teachers as experts in their field who thus determine how and what a student needs to learn (Vizeshfar and Torabizadeh, 2018).

Regarding the results of the first section of the questionnaire, which addressed students' preferred teaching methods, 78.3% of students favored lecture, followed by small-group discussion (60%) and tutorials (46.9%). Seminar was the least popular teaching method (44.9%).

The participants in our study favored lectures because they felt that they are more informative, their delivery by content experts with good communication skills and grasp of the subject matter made them interesting and beneficial, and students received more attention in these. This result is similar to those of the studies conducted by Zinski et al (2017); El-Belbasy et al (2018); and Stirling (2017). In contrast, the studies conducted by Schwartzstein and Roberts (2017); Ramnanan and Pound (2017); Abdul et al (2015); and Vimal et al (2015) showed that e – learning, flip method teaching and also small group discussion (as shown in fig. 2) were the preferred teaching mode. This is because they feel that it could enhance students' reasoning, gives them the opportunity to provide their opinion, a good stage for student-teacher cooperation and develops confidence.

Figure 3 shows that small-group discussions were the selected as the best for interactive participation (36%). Tutorials scored highly for being informative (25.7%), and seminars scored highly for confidence-building (36.6%).

Figure 4 shows a graphical representation of the teaching tools included in our study. Many students, 48% and 46.6% of the participants, responded that PowerPoint was the best tool for learning and teaching, as it provides a better learning experience. A study conducted by Shigli et al (2016) concluded that PowerPoint presentations deliver a multisensory experience, foster better understanding of charts, diagrams, tables, and various other concepts and helps improve memory. McBride and

Table 2. Comparative overview of findings on student teaching-method preferences

Teaching Method	This study	Shreemanta et al.	Salwani et al.	Vimal et al.	Abdul et al.	Papanna et al.
Lecture	78.3%	77%	72%	67%	59.1%	32.8%
Seminar	44.9%	49%	-	48%	54.3%	-
Small group discussion	60%	68%	6%	89%	80%	71.4%
Tutorial	46.9%	58.9%	10%	57%	-	-

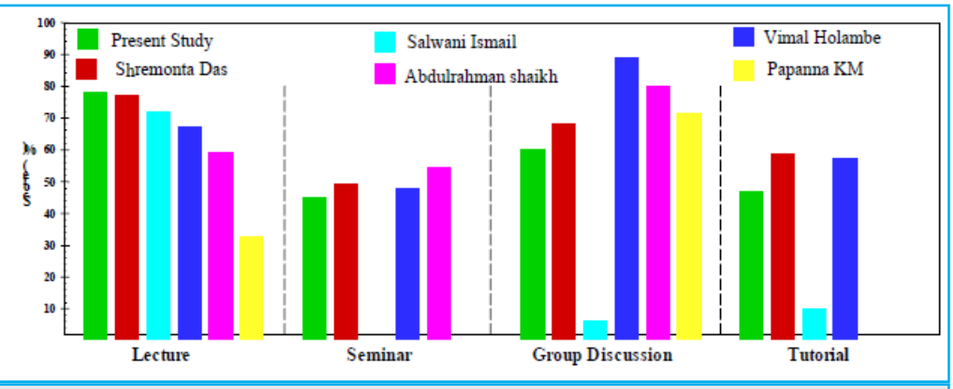

FIGURE 2. Comparative overview of selected studies on teaching methodologies with present study

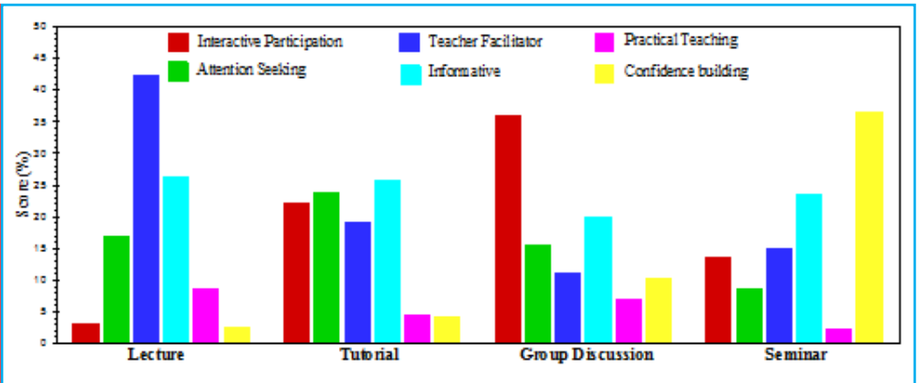

FIGURE 3. Reasons for teaching-method preferences

Drake (2017) in their research, found an increase of 24% to 29% in classroom attendance as compared to a similar study done Aye Mon et al (2014).

PBL was the second most-favored teaching tool, with 26.3% respondents stating it to be their preferred learning method. A study conducted by Merritt et al (2017) showed that PBL increased students' enthusiasm as it upgraded their reasoning skills, academic achievement, knowledge retention, conceptual development and attitude.

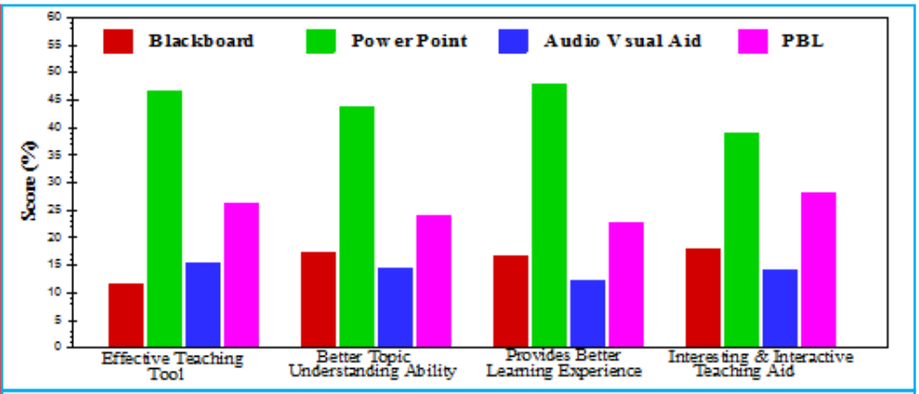

FIGURE 4. Perceived benefits of teaching aids

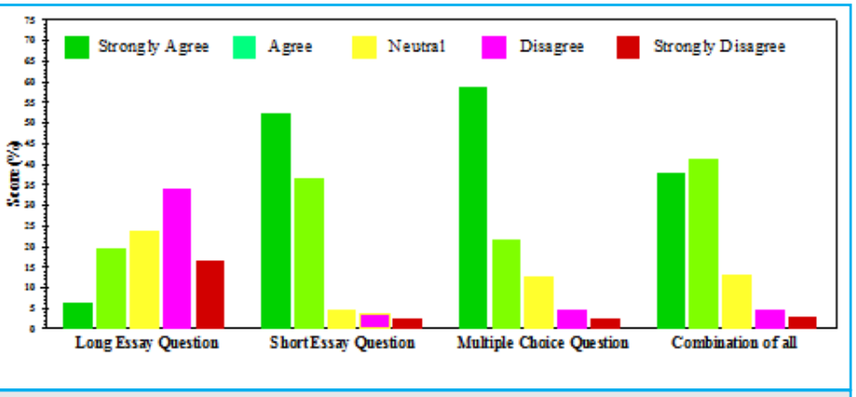

FIGURE 5. Preferred evaluation methods

Figure 5 describes students' preferred evaluation methods. The majority chose multiple-choice questions (MCQs) (58.6%), followed by short essay questions (52.3%). Long essay questions were rated the worst evaluation method, as only 6.2% of students strongly agreed with this method of evaluation. The finding is similar to a finding in a study (Ibrahim et al, 2015) conducted at King Abdul Aziz University in Jeddah, wherein the majority preferred MCQs as their assessment procedure of choice. Contrary to our findings, Lalvarmawi et al (2015) found that majority of the students preferred revision cum self-study, tutorials, and terminal exams These students, for whom English is their first language, do not experience the language problem faced by Saudi students. Our participants' mother tongue is Arabic, and they find it difficult to express themselves. Therefore, assessment approaches in Saudi Arabia need to be designed according to regional preferences.

CONCLUSION AND RECOMMENDATION

This study revealed that lecture-style teaching accompanied by the use of additional aids such as multimedia software presentations was the method most preferred by the participants. Participants emphasized that its benefits include increasing the clarity of teaching materials and offering a more engaging presentation style. They also welcomed the opportunity to have more interactive sections or student involvement during lectures. Therefore, we recommend that several similar studies be conducted among the wider community of medical students to discover innovative and effective educational interventions that will benefit medical students through identifying their concerns and preferences and formulating an action plan suited to their needs.

Ethical Approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Nutritional Communication

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BBRC Bioscience Biotechnology Research Communications

Effect of natural and synthetic antioxidant on shelf life of different Sudanese *Pennisetum glaucum* L. flour

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ABSTRACT

Development of rancidity in pearl millet flour even after a shorter period of storage time is the major limitation for its acceptability by the consumers. So, our aim of this study was to improve the shelf life of pearl millet flour obtained from two cultivars, (Ashana and Hreahry) using natural (ascorbic acid) and synthetic (butylated hydroxytoluene) antioxidant. Flour samples were evaluated for free fatty acids (FFA), peroxide value and fat acidity for a time period of 0, 10, 30, 60 and 90 days. We found that, untreated samples had significant increase in FFA compared to samples treated with ascorbic acid and butylated hydroxytoluene. Moreover, peroxide values in butylated hydroxytoluene treated samples were found to be low compared to untreated flour as well as ascorbic acid treated flours for both the cultivars. From our results it was observed that, butylated hydroxytoluene and ascorbic acid treated samples were able to maintain shelf life for 30 days, respectively. However ascorbic acid being a natural antioxidant could be a potential source of preservation and it could provide an effective and natural way for improving the shelf life of pearl millet flour.

KEY WORDS: PEARL MILLET; BHT; ASCORBIC ACID; ANTIOXIDANT; FREE FATTY ACID

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INTRODUCTION

Pearl millet (*Pennisetum glaucum* L.) is one of the oldest cereals known to human being and consumed in various parts of the world as a staple food since hundreds years back (Deepak *et al*., 2012; Goyal and Chug 2017). It has been one of the major food sources for millions of people, especially those who live in hot, dry regions of the world other than wheat and maize. In contrast, millet is the major food sources of protein for billions of people in Africa and Asia. Millet has been reported to have many nutritional as well as therapeutic properties (Amadou *et al.,* 2013; Sarita and Singh, 2016). Nutritionally pearl millet is on a par or even superior to other cereals such as rice, maize and wheat with respect to energy value, proteins, fat and minerals. Various macronutrients like amino acids, vitamins, minerals, dietary fibers and antioxidants presents in a more balanced ratio in a pearl millet than in other cereals. It makes an important contribution to human diet due to high levels of calcium, iron, zinc, lipids and high quality proteins. The level of stored energy in pearl millet is approximately equal to that of maize. The most prominent feature of pearl millet is relatively higher lipid content, which gives more energetic feed than maize, wheat, or shorgum (Deepak *et al*., 2012; Devi *et al*., 2014). Different phytochemicals such as phytic acid, tannins, and phenolic compounds contribute to antioxidant activity, which makes it very important for health, ageing and metabolic diseases (Alghamdi et al., 2108; Ahmed et al 2011, Gull *et al*, 2015, Kulthe et al 2017 Goyal and Chugh 2017 Al Ghamdi et al 2018).

Antioxidants are substances that protect cells from damage caused by unstable molecules known as free radicals. Antioxidants acts by retarding autoxidation of triglycerides. The amounts of protection provided by antioxidant depend on its concentration, its reactivity towards the particular reactive oxygen species being considered, and the status of the antioxidants with which it interacts (Odusola *et al*., 2013; Pushparaj & Urooj, 2014). Few antioxidants such as butylated hydroxyl toluene (BHT) and ascorbic acid acts by scavenging for oxygen or chelating pro-oxidant metal ions. But the use of synthetic chemicals such as BHT has also been met with skepticism from consumers; because of this many of us today demand food products without synthetic additives (Eskin and Przybylski, 2001). When pearl millet is grinded into flour, the resulting flour tends to become rancid due to oxygen exposure as well as high moisture content. This is attributed to the deterioration of its triglycerides through lipolysis and subsequent oxidation of de-esterified unsaturated fatty acids. These chemical changes manifest themselves as off-odors and off-taste of the flour (Palande *et al*., 1996; Yadav *et al*., 2012; Ashraf *et al*., 2016). Thus, it becomes unsuitable for the consumers to store these flours for longer period of time. There are various reports, which indicate storing of millet flour for even few days causes rapid rancidity and produces off-flavor and bitter taste. In order to minimize losses occurring during storage, the chemical conventional treatment could emerge as an alternative method of storage (Mohamed *et al*., 2010; Mohamed *et al*., 2011, Al Ghamdi et al 2018).

Moreover, various research studies were carried out for proximate composition and mineral accessibility, but information on effect of using natural and synthetic antioxidant activity on the keeping quality in pearl millet is limited. Our objective was to extend the shelf life of pearl millet flour using natural and synthetic antioxidants.

MATERIAL AND METHODS

Material: Two Sudanese pearl millet cultivars (Ashana, Hreahry), were obtained from the local market of Elobied, North Kordofan State, Sudan. Polyethylene bags, butylated hydroxytoluene (BHT) and ascorbic acid were obtained from a local chemical supplier in Khartoum, Sudan.

Treatments of pearl millet flour: The grains of both the cultivars were cleaned and milled using traditional stone mill. Pearl millet flours were divided into three groups of about 5 kg for each one of the three treatments. One group was left untreated and considered as control, the second group was treated with BHT (0.02%) and the third group was treated with ascorbic acid (0.5%). The required quantity of antioxidants was first mixed by hand in a small portion of flour sample. The mixture was then added to the bulk flour and mixed well to ensure uniform distribution. The treated and untreated flour samples were stored for 90 days at prevailing room temperature (37 $\pm$ 4°C) in polyethylene bags. The samples were periodically tested for 0, 10, 30, 60 and 90 days (Kapoor and Kapoor *et al*., 1990).

Proximate analysis: The determination of moisture, crude fiber and ash were carried out according to the AACC (2008) standard methods, while crude fat and crude protein were determined according to the AOAC (2005) standard methods (Ibrahim *et al.,* 2018).

Determination of Free fatty acid, fat acidity and Peroxide value*:* Free fatty acid (as oleic acid) was determined according to the (Majid *et al*., 2015). Fat acidity was measured according to the standard method of the AACC (2008). The peroxide value was determined according to the standard method (Bashir *et al.,* 2015).

Statistical analysis: The analysis of variance (ANOVA) was performed to examine significant effect in all

Table 1. Proximate composition of pearl millet (Ashana and Hreahry) cultivars

Cultivar	Moisture (%)	Ash (%)	Crude Proteins (%)	Crude Fibre (%)	Lipid (%)	Available carbohydrate (%)
Ashana	4.69 ± 0.44^{b}	2.28 ± 0.46^{b}	12.84 ± 0.58^{b}	1.52 ± 0.70^{a}	5.12 ± 0.32^{a}	73.55 ± 2.14^{a}
Hraehry	6.28 ± 0.07^{a}	2.80 ± 0.74^{a}	14.99 ± 0.22^{a}	1.48 ± 0.03^{a}	5.43 ± 0.40^{a}	69.02 ± 0.40^{a}

Means (s) values (± SD) having different superscripts in the same column are not significantly different (P ≤ 0.05); n.s: not significant

parameters measured. Duncan Multiple Range Test was used to separate the means (Montgomery, 2001).

RESULTS AND DISCUSSION

Proximate analysis of raw material: The moisture contents of Ashana and Hreahry cultivars were found to be 4.69% and 6.28 %, respectively as shown in table 1. Statistical analysis of the results showed a significant difference (p ≤ 0.05) in moisture contents between Ashana and Hreahry cultivars. Previous studies reported that, moisture content of pearl millet varies from 5.4 % and 6.48 % (Eltayeb, 2006), which was in consistent with the previous studies. Moreover, ash contents for the Ashana and Hreahry cultivars were found to be 2.28 % and 2.80 % respectively. Statistical analysis of the ash % showed significant difference (p ≤ 0.05) between the two cultivars. These results were higher than the reported values 0.73% by Gull *et al.*, 2015. Crude protein content of Ashana and Hreahry cultivars were found to be 12.84 % and 14.99 % respectively. However, Amadou *et al.* (2013) reported 14.8% protein content, which was in comparable with Hreahry cultivars. Ashana cultivar flours were found to be having less protein content than Hreahry cultivars. Crude fiber of Ashana and Hreahry cultivars were found to be 1.52 % and 1.48 % respectively. Percentage of crude fiber was found to be lower than the range of 2.4% and 8.6% as reported by Eltinay *et al.* (2005). Nambiar *et al.* (2011) reported that, the lipid content of pearl millet varied from 2.4% to 5.0 %. While our investigation found that, 5.12 % and 5.43 % lipid content for Ashana and Hreahry cultivars respectively. Carbohydrate content in Ashana and Hreahry cultivars were found to be 73.55% and 69.02% respectively. These values of the available carbohydrates were higher than 67.67% and 68.55% reported by Eltayeb, (2006).

Stability studies*:* Shelf life study for pearl millet flour of two cultivars were carried out in which three parameters were selected and checked viz, Free fatty acid (FFA), peroxide value and total acidity. FFA of both the cultivars was presented in table 2. Which shows that, Ashana cultivar had significantly increased (P ≤ 0.05) in the untreated flour sample at the storage of 0, 10, 30, 60 and 90 days and values recorded were 0.465, 0.640, 1.170, 1.700 and 3.030 %, respectively (Table 2). On the other hand, ascorbic acid treatment indicated lower value of FFA and significantly different than untreated flour at same days of analysis, were found to be 0.42, 0.54 0.79, 0.93 and 1.70 %, respectively. Treatment with BHT recorded lowest value compared with untreated flour and ascorbic acid treatment, whereas values obtained were 0.43, 0.52, 0.77, 0.90 and 1.09 %, respectively. While, Hreahry cultivar flour results were presented in Table 2. Which showed that FFA were significantly increased (P ≤ 0.05) in the untreated flour sample at the 0, 10, 30, 60 and 90 days, were found to be 0.35, 0.71, 1.38, 1.53

Table 2. FFA (% oleic acid) of Hreahry cultivar flour as affected by the addition of antioxidants

Storage period (days) / Treatment	0	10	30	60	90
	Hreahry cultivar flour				
Control	0.35 ± 0.02^{p}	0.71 ± 0.04^{lm}	1.38 ± 0.08^{f}	1.53 ± 0.13^{de}	3.17 ± 0.06^{a}
BHT	0.42 ± 0.06^{op}	0.45 ± 0.04^{op}	0.79 ± 0.08^{jkl}	0.88 ± 0.08^{hijk}	1.53 ± 0.08^{de}
Ascorbic acid	0.46 ± 0.00^{op}	0.52 ± 0.01^{no}	0.74 ± .08^{klm}	0.96 ± 0.01^{hi}	1.64 ± 0.14^{c}
	Ashana cultivar flour				
Control	0.47 ± 0.02^{op}	0.64 ± 0.03^{lm}	1.17 ± 0.30^{ef}	1.70 ± 0.06^{de}	3.03 ± 0.13^{a}
BHT	0.43 ± 0.06^{op}	0.52 ± 0.01^{op}	0.77 ± 0.06^{jk}	0.90 ± 0.01^{hijk}	1.09 ± 0.16^{de}
Ascorbic acid	0.42 ± 0.06^{op}	0.54 ± 0.07^{no}	0.79 ± 0.07^{jkl}	0.93 ± 0.04^{hi}	1.70 ± 0.14^{c}

Mean values ± SD. sharing same superscript(s) are not significantly different (P ≤ 0.05)

Table 3. Peroxide value (mEq/kg) of Ashana cultivar flour as affected by the addition of antioxidants

Storage period (days) / Treatment	0	10	30	60	90
	Hreahry cultivar flour				
Control	3.28 ± 0.15^{m}	5.24 ± 0.11^{jk}	7.95 ± 0.09^{g}	10.76 ± 0.39^{d}	17.07 ± 0.30^{a}
BHT	3.63 ± 0.26^{m}	3.77 ± 0.21^{m}	5.72 ± 0.13^{ij}	8.55 ± 0.01^{f}	10.59 ± 0.14^{d}
Ascorbic acid	3.49 ± 0.38^{m}	4.52 ± 0.05^{l}	6.16 ± 0.21^{i}	8.78 ± 0.07^{f}	12.88 ± 0.15^{c}
	Ashana cultivar flour				
Control	3.22 ± 0.09^{p}	4.95 ± 0.11^{n}	8.30 ± 0.16^{hi}	10.74 ± 0.35^{d}	16.69 ± 0.23^{a}
BHT	3.14 ± 0.05^{p}	3.18 ± 0.14^{o}	5.49 ± 0.45^{m}	7.99 ± 0.40^{ij}	10.72 ± 0.28^{d}
Ascorbic acid	3.15 ± 0.04^{p}	4.07 ± 0.06^{o}	6.15 ± 0.23^{n}	8.22 ± 0.28^{hi}	12.06 ± 0.00^{c}

Mean values ± SD. sharing same superscript(s) are not significantly different (P ≤ 0.05)

and 3.17 %, respectively. On the other hand, ascorbic acid treatment indicated lower value and significantly different from the untreated flour at the same days of analysis, were found to be 0.46, 0.52, 0.74, 0.96 and 1.64 %, BHT treatment recorded lowest value compared with untreated flour and ascorbic acid treatment, were found to be 0.42, 0.45, 0.79, 0.88 and 1.53 %, respectively. A high FFA value is mainly due to hydrolytic changes associated with the action of lipolytic enzymes. Further, an increase in lipase activity during storage may have led to a significantly higher FFA value in control than the treated flour (Yadav *et al.*, 2012).

Peroxide Value: Ashana cultivar flour were found to be increased significantly (P ≤ 0.05) in the untreated flour sample at the storage of 0, 10, 30, 60 and 90 days and values recorded were 3.22, 4.95, 8.30, 10.74 and 16.69 mEq/kg respectively as presented in table 3. On the other hand, ascorbic acid treatment indicated lower value of PV than untreated flour at same days of analysis, and it was observed 3.14, 3.18, 5.49, 7.99 and 10.72 mEq/kg, respectively. Treatment with BHT recorded lowest value compared with untreated flour and ascorbic acid treatment, whereas values obtained were 3.15, 4.07, 6.15, 8.22 and 12.06 mEq/kg, respectively. While, Hreahry cultivar flour results were mentioned in table 3. Which showed that, PV were significantly increased (P ≤ 0.05) in the untreated flour sample at the 0, 10, 30, 60 and 90 days, were found to be 3.27, 5.23, 7.94, 10.76 and 17.07 mEq/kg, respectively. Additionally, BHT treatment indicated significantly different from the untreated flour at the same days of analysis, and found to be 3.49, 4.51, 6.16, 8.77 and 12.88 mEq/kg, respectively. Furthermore, ascorbic acid treatment recorded lowest value compared with untreated flour and ascorbic acid treatment, were found to be3.62, 3.76, 5.71, 8.55 and 10.59 mEq/kg respectively. PV increased significantly (P ≤ 0.05) in control flour from 3.22 to 17.08 mEq/kg fat after 90 days of storage. The increasing trend of PV during storage was in agreement with the observation of Chaudhary and Kapoor, (1984).

Fat Acidity: In table 4 fat acidity changes during storage of the pearl millet flour at ambient temperature (37

Table 4. Fat acidity (mg KOH/ 100g) of Ashana cultivar flour as affected by the addition of antioxidants

Storage period (days) / Treatment	0	10	30	60	90
	Hreahry cultivar flour				
Control	26.92± 0.29^{n}	63.64±6.40^{ghi}	88.93±6.55^{f}	125.20±18.46^{c}	183.40±13.04^{a}
BHT	21.99± 0.26^{n}	40.72± 0.78^{m}	51.22±0.13^{jkl}	67.71 ± 0.84^{gh}	111.10± 1.40^{d}
Ascorbic acid	26.72± 0.57^{n}	48.72±0.81^{klm}	53.05±0.10^{jkl}	68.63 ± 0.71^{g}	127.80 ± 3.44^{c}
	Ashana cultivar flour				
Control	22.42± 0.40^{n}	37.06±0.49^{ghi}	95.41±4.65^{f}	115.60±21.91^{c}	186.50 ± 8.92^{a}
BHT	24.01±2.98^{m}	29.57 ± 0.63^{m}	51.53±0.32^{jkl}	65.51 ± 1.12^{gh}	118.30±5.52^{ac}
Ascorbic acid	24.45± 3.03^{m}	34.79±0.88^{klm}	58.17±4.32^{lm}	72.54 ± 2.70^{hi}	125.10±4.26^{ad}

Mean values ± SD. sharing same superscript(s) are not significantly different (P ≤ 0.05)

± 4˚C). A gradual, yet significant (P ≤ 0.05) increase was observed in the fat acidity of the three treatments. The mean values of fat acidity at the 10 days of storage for untreated, BHT, ascorbic acid treatments were found to be 37.06, 29.57 and 34.79 mg KOH/ 100g, respectively and no significant differences (P ≤ 0.05) were found. At 60 days of storage, the values of fat acidity were found to be 115.60, 65.51 and 72.54 mg KOH/ 100g for untreated, BHT, ascorbic acid treatments, respectively and significant difference (P ≤ 0.05) were found between them. Untreated flour reported the highest values at all time during storage period, followed by ascorbic acid treatment and the BHT treatment recorded the lowest value. Similarly, Table 4 shows the changes in fat acidity during storage of the pearl millet flour at ambient temperature (37 ± 4 ˚C).

Results at the begging of the storage were 26.92, 21.99 and 26.72 mg KOH/ 100g for untreated, BHT and ascorbic acid treatments, respectively and indicated no significant difference (P ≤ 0.05). A gradual, yet significant (P ≤ 0.05) increase was observed in the fat acidity of the three treatments. The mean values of fat acidity at the 30 days storage of the untreated, BHT, ascorbic acid treatments were 84.18, 44.82 and 48.46 mg KOH/ 100g, respectively and significant differences (P ≤ 0.05) were found between them, at 60 days of storage, the values of fat acidity were 125.20, 67.71 and 68.63 mg KOH/ 100g for untreated, BHT, ascorbic acid treatments, respectively and significant difference (P ≤ 0.05) were found between them. Untreated flour reported the highest values at all time during storage period, followed by ascorbic acid treatment and the BHT treatment recorded the lowest value. Jalgaonkar *et al.* (2016) reported that fat acidity of flour from the untreated grain increased from 30.3 to 123.7 mg KOH/100g. Additionally, Tiwari *et al.* (2014) found that fat acidity was above 30 mg KOH/100g in the untreated pearl millet flour.

CONCLUSION

Pearl millet flour has been well known staple food source which is not only providing major nutrients like protein, carbohydrate and fat but also have important vitamins and minerals. However, due to development of rancidity in pearl millet flours it has been not well accepted from the consumers. Based upon our results, we found that BHT and ascorbic acid treatments were able to reduce both hydrolytic and oxidative reaction. Furthermore BHT was found to be better than ascorbic acid treatment, in retardation of the lipid degradation in pearl millet flour. The BHT and ascorbic acid treatment was able to maintain the keeping quality of flour up to 30 and 30 days respectively. Our study would be useful for the scientist; miller, retail seller, as well as consumer, as utilization of this antioxidant will help to keep the quality of millet flour for longer duration. In addition to that, it would also encourage utilization of pearl millet grains, which is still untapped despite its various nutritious and therapeutic benefits.

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Bio-efficacy of acetonic leaf extract of *Murraya koenigii* with reference to its antibacterial spectrum against food-borne bacteria

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ABSTRACT

The food-borne antibacterial activity of *Murraya koenigii*, acetone leaves extract (ALE) was investigated against standard strains of *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus* causing various food-borne diseases in human. The ALE showed a significant inhibition of the *L.monocytogenes*, *S. auresus* but the low inhibition of *E.coli.* The bioactive metabolites analysis of ALE by TLC, HPLC, UV-Vis spectroscopy, FTIR and NMR exhibited the presence of diverse types of bioactive phyto-constituentssuch as flavonoids, saponins, phenolic bioactive compounds etc. which might be responsible for bacterial inhibition in-vitro. FTIR studies of ALE, revealed the presence of functional groups peaks such as phenol, alkanes, alkenes, aromatic, aliphatic and amine plant bioactive compounds. NMR showed the presence of aliphatic groups and –OH groups compounds which were structurally and functionally similar due to the chemical arrangement of functional groups. Thus, *M. koenigii* provided a natural remedy for the control of food-borne pathogens.

KEY WORDS: ANTIMICROBIAL ACTIVITY; FTIR; HPLC;NMR; PHYTO-CHEMICAL ANALYSIS

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INTRODUCTION

Food-borne bacterial diseases in worldwideare proliferate day-by-day and becomes a serious concerns for both consumers and the food fabrication. Majority of food-borne diseases are caused by food spoilage bacteria and other microorganism (Sousa, 2008). Hence, the problem of food contamination arising through bacterial infectionis a matter of major concern for the public health in both developed and developing nations (Shi and Zhu, 2009). Food spoilage is a sundry process involving food-borne microorganisms causing loss of 25% world's food supply and a vast degree of infection illness. Fresh products such as fruits, vegetables, dairy products etc. aremost likely to be contaminated by food-borne pathogens such as *Campylobacter*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, etc. (Dhama et al., 2015). The majorly pathogenic bacteria (66%) have played main part in food spoilage or food borne illness (Khare and Rawat, 2018).

A good number of such toxicogenic bacteria have been discovered and few of them such as *L. monocytogenes* among, causing their survival at even extremely low temperatures where the other bacteria do not grow. The economic burden and food-borne diseases by contamination of food is continuing in the contemporary technical advancement (Ray and Bhunia, 2013). It has been estimated that in developing countries like India, 30% of people suffer from outbreaks of food-borne pathogens (Scallan et al. 2011). Traditional remedy, especially the medicinal plants and their extracts, still play a major role in the developing countries to combat bacterial infections (Pirzada et al., 2009). According to the WHO, the majority of worldwide population depends on traditional remedial system as a resource of natural drugs for human healthcare (Agyare et al. 2018).

The folk medicine makes valuable remedial for several diseases due to very low side effects (Zhang et al. 2016). The active phytochemical constituents of medicinal plants havebio-efficacy such as antioxidant, insecticidal and antibacterial activities. The folk medicine and its different parts extract exhibit significant anti-bacterial activity without any adverse serious side effects to host. The leaves of *M. koenigii* contain good sources of antioxidant activity, revamp the food protection and diminish food-borne infections, and can be used as preservatives (Genena et al. 2008). *M.koenigii* is a medicinal plant belongs to the family Rutaceae, is native to India and Sri Lanka. Its leaves are used in recipes in India and neighboring countries as spices due to aromatic nature. The leaves of *M.koenigii* work in relief from thefood-borne pathogenic infection, vomiting and dysentery. The present work is therefore, aimed to study the bio-efficacy of ALE against both Gram-positive and Gram-negative food-borne bacteria because of their significance its combat human food-borne pathogens.

MATERIALS AND METHODS

Plant material: The healthy leaves of *M.koenigii* were collected from their natural habitat growing at different locations of district Haridwar, Uttarakhand (29.945°N North 78.163° East) during September to October 2015 and 2016. The plant was identified following of authentic literature based on its characteristic features and a herbarium was kept in the Department of Botany and Microbiology, GurukulaKangriVishwavidyalaya, Haridwar, India.

Bacterial strains: The standard cultures of *Escherichia coli* MTCC 25922, *Staphylococcus aureus* MTCC 25923 and *Listeria monocytogenes* MTCC 657 were procured from the Microbial Type Culture Collection, (MTCC), Chandigarh, India. The test bacteria were sub-cultured onto nutrient agar medium in order to determine their viability. Stock cultures were maintained on nutrient agar slants at 4°C and inoculated in nutrient broth at 37°C prior to further use.

Extraction of plant material: The *M. koenigii* leaves were washed with distilled water and the dried leaves were pulverized to get powder in form. 200 g leaf powder was used for bioactive chemical extraction using acetone as solvent. Acetonic leaf extract (ALE) wasconcentrated by vacuum evaporator under the control temperature and pressure to obtain a gummy/semi-solid mass, which was preserved in a refrigerator at 4°C for further uses (Oniszczuk and Podgórski, 2015).

Antibacterial activity: The selected food-borne bacterial strains were prepared by transferring microbial inocula from stock cultures to test tubes containing Mueller-Hinton Broth (MHB) and incubated at 37˚C for 24 h. Antibacterial activity was tested by agar well- diffusion method (Correa et al., 2017). 100 µL of diluted inoculum of 10^5cfu mL^{-1} of 24 h old cultures of *E.coli, L. monocytogenes* and *S. aureus* were separately mixed in Mueller Hinton Agar (MHA) medium, with thorough shaking. Medium was poured in sterilized Petri plates and were allowed to solidify. A sterile cork borer of (6 mm diam.) was punch wells in medium. The stock extract of ALE (100 %) was diluted using acetone solvent to get 25, 50 and 75% concentrations for measuring antibacterial activity. DMSO (dimethyl sulphoxide) was used as control. The plates were incubated at 37°C for 24 h for the antibacterial activity (Balouiri et al., 2016). The data was interpreted on the basis of the size of the diameter of zone of inhibition (mm).

Phytochemical screening: The acetonic leaf extract (ALE) was subjected for qualitative analysis of phytoconstituents viz., alkaloids, amino acids, flavonoids,

saponins and tannins following Trease and Evans (1983). Dragendorff's test for alkaloid was carried out in which diluted hydrochloric acid (0.1 mL) and Dragendorff's reagent (0.1 mL) were added separately in 2 mL of ALE in test tubes. After proper mixing, formation of orange brown colored precipitate indicated the presence of alkaloids. For saponins test ALE (1 mL) was separately diluted with distilled water up to 20 mL and vigorously shaken in a graduated cylinder for 15 min. Development of stable foam indicated the presence of saponins; this test is also known as foam formation test. ALE (5 mL) was separately treated with 1 mL of 10% aqueous lead acetate solution. Formation of yellow colored precipitate indicates the presence of flavonoids. For tannins analysis, ALE (5 mL) were separately allowed to react with 1 mL of 5% ferric chloride solution. Appearance of greenish black color indicated the presence of tannins. ALE (2 mL) and 0.25% ninhydrin reagent were added in fresh test tube and boiled for few minutes. Formation of blue color indicated the presence of total amino acids.

Thin layer chromatography (*TLC*)*:* For quantitative analysis of different bioactive marker, ALE was applied on prepared TLC role and developed in a TLC chamber saturated with different suitable mobile phase such as ethyl acetate: methanol (3:1), hexane : chloroform : methanol (5:1:1) and chloroform : methanol (5:1). Based on clear bands and proper R_f values, the chloroform: methanol (5:1) was found the most suitable solvent system for the separation of bioactive metabolites. (Ramalloet *al.,* 2006).

Fourier transform infrared spectroscopy (*FTIR*)*:* FTIR was conducted following Kumar *et al.* (2014). The Infrared spectra of acetoneextractwas analysis for the determination of functional groups responsible for biological activities. ALE was mixed with KBr (spectroscopic grade) and pressed to form 1-mm pellet. Perfectly dried powder of the ALE was placed on the sample chamber of Nicolet Avatar 330 FTIR spectrometer (Thermo Electron Co., Madison, WI, USA) for the record of spectra and in the range of 600–3600 cm^{-1}. The absorption frequencies appeared in functional group region as well as fingerprint region of the spectra was observed to record FT-IR spectral (Liu *et al.,* 2006).

UV-Vis spectrophotometry: For the UV-VIS spectrophotometer (Perkin Elmer, USA Model: Lambda 950) analysis, the sample was prepared by diluting to 1:10 within the same solvent used in extraction of material (Do et al., 2014). The extract was examined under visible and UV light in the wavelength range 200-800 nm. The UV-visible spectra were performed to identify the compounds containing σ-bonds, π-bonds, lone pair of electrons and aromatic rings. The UV-VIS spectrum was observed both in visible and UV-VIS light lambda 200-800 nm as given by Maji et al (2016).

HPLC analysis: HPLC analysis was performed by Perkin Elmer Series 200 system in isocratic conditions using a C-18 (250mm x 4.6 mm, 5µm) at 25°C. Running conditions included injection volume, 20µl; mobile phase, methanol: acetic acid (0.4%) (800: 200 v/v); flow rate (1 ml/min). *M. koenigii*ALE (2.5 mg) was dissolved with 5 ml acetone. Bioactive compounds present in test sample were identified by chromatographic peaks with the retention time (RT) at 220 nm and 254 nm by UV detector. HPLC analysis was performed according to the method of Altun et al.(2002).

1H and 13C NMR analysis: Sample was dissolved in respective dutrirated solvents (CDCl3), 600 µl was poured in NMR tube and observed on the applied magnetic field (Tachibana et al., 2001), to obtain the Nuclear Magnetic Resonance (DRX-300Mega Hz Bruker, Switzerland).

RESULTS AND DISCUSSION

Phytochemical screening: Preliminary phytochemical analysis of ALE of *M. koenigii* revealed the presence of saponins, alkaloids, amino acids and flavonoids. However, terpenes was not detected in ALE of *M. koenigii* (Table 1).

Antibacterial activity: ALE caused broad zone of inhibition. Significantly, wider zone of inhibition of actively growing bacteriaon surface of Petri plates indicated the antibacterial potentials of ALE. ALE had the maximum activity against *L. monocytogenes* (12 mm), while the minimum inhibition was recorded against *E. coli* (7 mm). The zone of inhibition at 100% concentrations of ALE was most prominent followed by 75, 50, and 25 %. Which was corresponding to concentration the order of effectiveness of ALE was *L.monocytogenes*>*S. aureus*>*E. coli* on the basis of sensitivity (Table 1).

Thin layer chromatography: Different active metabolites such as the alkaloids, flavonoids, glycosides, terpenoids and saponins with many high resolution bands appeared with different $R_f$values (Figure 1).

Table 1. Antibacterial activity of ALE of *M.Koenigii* against food-borne bacteria.

ALE	Zone of inhibition		
%	*L.monocytogenes*	*S. aureus*	*S. aureus*
100	11 ± 2.5	9 ± 1.02	9 ± 1.02
75	10 ± 1.01	8 ± 0.87	8 ± 0.87
50	8 ± 0.75	5 ± 0.72	5 ± 0.72
25	4 ± 0.45	3 ± 0.25	3 ± 0.25
Control	NI	NI	NI
Erythromycin	25	23	23

Values are mean of three independent observations ± SD; NI= No inhibition

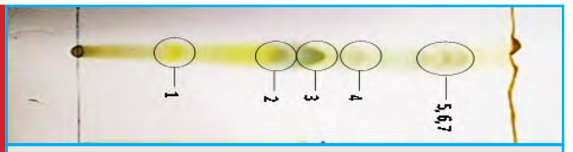

FIGURE 1. TLC of ALE of *M. koenigii* with band pattern of secondary metabolites.

Fourier transform infrared spectroscopy (FTIR): The functional groups of bioactive chemicals of ALE both known and unknown bands appeared at 3959.59 – 553.53cm^{-1}. The intense broad absorbance at 3515.99 cm^{-1} was the characteristic of the hydroxyl functional group in alcohols and phenolic compounds. The absorbance was relatively intense and broad at 3515 cm^{-1} characterized for hydroxyl functional groups. Two bands at 3444.63, 3259.47 and a weaker band at 1600.81 cm^{-1} exhibited amine group. Further, intense and weaker absorptions were observed in the C-H aromatic bands at 3130.25, 2960.53 cm^{-1}, respectively. On the other hand, low intensity of the absorption was observed in the two aldehyde bands at 2812.02 and 2704.01 cm^{-1} similar to another two alkyne bands at2329.85 and 2189.06 cm^{-1}. Single intense absorption peak appeared that determines the hydroxyl band at 3515.99 cm^{-1}, while alkene band at 1361.65 cm^{-1} has low absorption spectra. The appearance of ester band at 995.20 cm^{-1} along with others having 4 small alkyl halide bands at 862.12, 754.12, 678.90 and 553.53 cm^{-1} were significantly diverse but showed majority of alkaloids with distinct secondary metabolites of unknown functional groups referred to the absorption spectra.On the other hand, spectral data of most of the extract confirmed the presence of bioactive groups such as –O-H, -C-H, =C-H, -C≡C, N-H, –C-O, –COOH, C-Cl, C-Br and alkene. Important IR absorption frequencies displayed the presence of C, H, Br and alkane string bioactive compounds (Figure 2).

UV spectroscopy: The ALE was examined by UV spectroscopy for proximate analysis. The UV-VIS profile in the range of 200 - 800 nm wavelength exhibited the sharpness of the peaks and proper baseline. The flavonoids spectra typically consisted of two absorption maxima in the ranges 200-290 nm and 300-530 nm. The precise position and relative intensities of these maxima gave valuable information in the nature of flavonoids. Occurrence of peaks at 207-557 nm reveals the presents of flavonoids in the *M. koenigii* (Figure 3).

HPLC analysis: HPLC profiling of ALE showed the presence of bioactive metabolites in different peaks observed at 220 nm and 254 nm in UV. The eight compounds were separated at different retention times at 220 nm and at 254 nm. These spots showed different bioactive constituents (Table 2).

Nuclear magnetic resonance (NMR) spectrophotometry: 1H NMR and 13C NMR spectrum of isolated compound reveals a one strong solvent (CDCl3) peak at 7.264 ppm. NMR spectrum of ALE extract resulted 11 peaks due to the presence of –OH group, C-H and C=O group. In 1H NMR spectrum a less intensive peak appeared at 1.6, 1.7 ppm due to R-CH_2-R group and 4.0 ppm for =CH group. The four broad spectrum peaks constitute 2.1, 2.2 ppm for C=O and 2.3, 2.6 ppm spectrum due to the presence of C-N group and one 1.25 ppm for –OH in ALE of *M. koenigii* (Table 3).

Foods-borne infectious diseases are serious concern of worldwide. About 250 different food-borne diseases

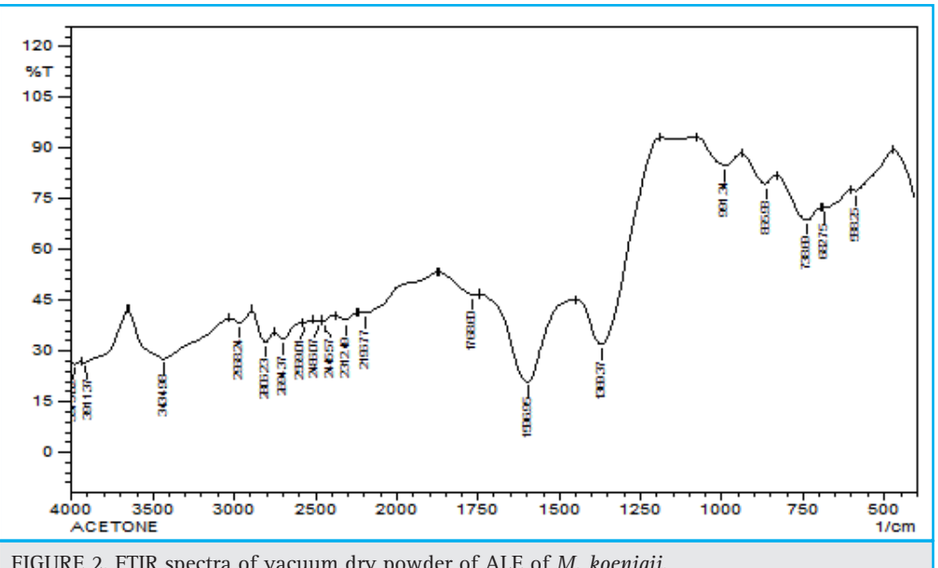

FIGURE 2. FTIR spectra of vacuum dry powder of ALE of *M. koenigii.*

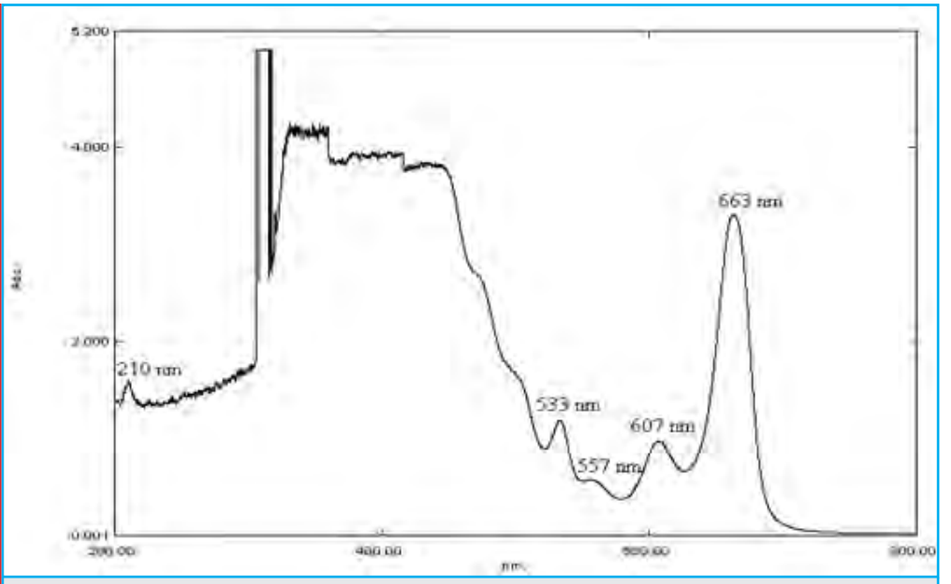

FIGURE 3. UV-VIS spectra of ALE from *M. koenigii*.

Table 2. Peak list and Rf value of the chromatogram of the ALE of *M. koenigii*.

RT	Area	Height	Purity Angle	UV range (nm)
4.364	3102730	195562	6.639	220
9.628	266428	41190	3.636	220
18.936	24814	178512	2.54	220
20.110	364394	64676	0.375	220
21.309	762224	135192	0.222	220
22.782	1052445	192003	0.232	220
4.365	47873872	2730580	6.430	254
7.550	126028	25924	3.784	254

have been reported, and bacteria are the major and common causative agents of two thirds of food borne infectious disease outbreaks (Le et al., 2003). Medicinal plants are widely used against dysentery and food-borne infection throughout the world but only selected plants have been validated by scientific community. *M. koenigii* leaves are used mainly for flavor and medicinal purpose worldwide shown its potential to serve as food ingredients in India and other Asian countries (Perera and Li., 2012; Shanthala and Jamuna., 2005). We have observed that the leaves of *M. koenigii* have highly anti-bacterial activity against *L. monocytogenes*, *S. aureus* and *E. coli*.

Table 3. ^{1}H-NMR and ^{13}C-NMR spectral data of bioactive constitutents of ALE.

Type of proton	Chemical shift (δ) ppm	Description of proton
ROH	1.25	Alcohol
C-H	1.4, 1.5	Alkane
R-CH2-R	1.6, 1.7	Alkyl
C=O	2.1, 2.2	Carbonyl
C-N	2.3, 2.6	C attached to N and Halogens (Cl, Br, and I)
C=O	3.9	C attached to O
=CH	4.0	Alkene

Recently, Sablania et al. (2019) found that the *M. koenigii* leaves have antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli* and other microorganism. The most significantly inhibitory effect has been observed against *L. monocytogenes*. ALE resulted in the maximum zone of inhibition against *L. monocytogenes* followed by*S. aureus* and quite less against*E. coli*. Due to the presence of OH (hydroxyl group)at 1.25ppm in ALE, it may be have similar mode of action against bacteria like erythromycin as stated by (Kohanski et al., 2007). Some of the bioactive chemicals may have similarmode of action such as DNA damaging, protein synthesis inhibition and bacteriostatic activity against food-borne bacteria. The anti-bacterial effects against Gram-positive bacteria and Gram-negative may be attributed due to the presence of several bioactive chemicals in ALE (Table 2). The natural bioactive chemicals which contain -OH groups, that werecausing of protein damage and membrane lipid inhibition of bacteria (Brogden., 2005).

Similarly, Gupta et al. (2018) reported strong antibacterial properties by *M. koenigii* leaves extract mainly due to the saponin and active protein. But the action of additional anti-food-borne bioactive compounds of *M.koenigii*

cannot be ruled out. The microbicidal activity of ALE was higher against the Gram-positive bacteria incomparison to the Gram-negative bacteria. Majority of Gram-positive bacteria have been drastic food-borne pathogens in comparison to that of Gram-negative bacteria. The data of UV-VIS spectral absorption showed the presence of flavonoids at absorbance 207-280 nm also reported by Kavitha and Uduman (2017) whereas phenolic acid derivatives at 317-340 nm (Zavoi et al., 2011). In the present study, ALE spectral absorption sharp baseline and peaks were clearly shown in spectral graph. The Preliminary plant bioactive metabolites screening and TLC Rf values ALE of *M. koenigii* revealed to the presence of diverse type of bioactive metabolites such as alkaloids, amino acid, saponins, flavonoids. On comparison of the leaves shows that the ALE has similar flavonoids and glycosides compounds reported. A similar finding has also been reported as also by Kumar et al., (2013).

Since, the functional groups of bioactive metabolites describe the nature and behavioural characteristicstherefore, it isvaluable for the preliminary separation and determination of bioactive metabolites constituents, Meepagala *et al.* (2013) have reported that HPLC chromatograms of ethyl acetate extract of leaves of *M. koenegii*relative with the peaks of extract retention time 8.2, 9.1 and 10.3 as isomahanine and mahanine according to their chemical name. Shah et al. (2018) found the prominent peak of tannic acid at a retention time of 3.21 min. During our study, the H1 and C-13-NMR spectroscopy analysis of ALE of *M. Koenigii* showed the presence of the aliphatic group, such as alkane, alkyl and alkene of bioactive chemicals which may be responsible for the anti-bacterial nature of ALE. The NMR spectrum of proton and carbonalso predicts the anti-food-bornebioactive metabolites structure on the basis of FTIR and UV-VIS data interpretation as evidenced by previous workers (Pretsch et al., 2013; Sarker et al., 2006)

The results ofALE are useful for the existing information for identification and validation of *M. koenigii*leaves for the future prospective against food-borne bacteria. The bioactivity of ALE and the therapeutic use of *M. koenigii*for various ailments and look promising for using the bioactive phyto-chemicals in pure form as an effective natural drug agent in future. Therefore, the leaves of *M. koenigii*can be used for developing commercial management in food industry; chemical preventative such as benzoic acid and sodium benzoate are also work as antimicrobial which are normally used to expand shelf-life of food products (Nair, 2001).

CONCLUSION

It may be concluded that the ALE of *M. koenigii* antibacterial efficacy against some food-borne bacterial pathogens due to the presence of several phytochemical compounds such as alkaloid, phenol, flavonoids and tannin were observed. The UV-VIS profile showed the absorbance of flavonoids in the ALE. FTIR analysis confirmed the presence of functional groups of bioactive metabolites such as phenol,alkanes, alcohol and amines, while HPLC outline of *M. koenigii* showed the presence active metabolites in ALE and the NMR spectrum predicted the structure of bioactive compounds based on data of FTIR and UV-VIS. The results of this study offer a platform for using *M. koenigii* leaves as herbal alternative for various food-borne disease and infections caused by bacterial pathogens after animal trials. Further work is in progress.

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Antibacterial activity of leaf extracts of *Spondias mangifera* Wild: A future alternative of antibiotics

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ABSTRACT

Antibacterial efficacy of both dry and green leaf extracts of *Spondias mangifera* was observed by using their methanol, ethanol, and aqueous extracts. Six human pathogenic bacterial strains were selected as test organisms and antibacterial activities were assessed by using disc diffusion method. Maximum inhibition of *Enterococcus faecalis* was observed by ethanolic dry leaf extract (25.00 ± 0.58). Similarly, methanolic dry leaf extract was very effective against *Shigella boydii* (25.17±0.44). Higher antibacterial activity was observed by green leaf extracts for other test organisms. Aqueous extract of green leaf showed maximum inhibition (11.50± 0.76) against *Staphylococcus aureus*. Ethanolic extract of green leaf showed maximum activity (10.17±0.44) against *Escherichia coli*. Similarly, methanolic extract of green leaf against *Klebsiella pneumoniae* and *Proteus vulgaris* showed maximum antibacterial activities, i.e. (15.50±0.29) and (12.50±0.29) respectively.

KEY WORDS: ANTIBACTERIAL, BACTERIA, EXTRACT, INHIBITION, SOLVENT

INTRODUCTION

Since ancient times, we depend on plants or plant products for medicines. Plants serve as source of many chemicals and many of them have been identified as pharmaceutically important. Presence of secondary metabolites such as alkaloids, tannins, flavonoids, terpenoids, etc. contribute significant role in developing antimicrobial properties. After the discovery of antibiotics, our dependence on antibiotics had minimized the use of such plants. Many microbes have developed resistance against several antibiotics and the treatment of patients

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infected with such microbes has become a big concern for medical practitioners. Improper use of antibiotics is also one of major cause for rising number of patients with resistance of antibiotics and for their treatment an alternative of antibiotics is urgently required. The use of plant products as antimicrobial is an efficient way to combat above problem. There is another advantage that plant products show almost negligible side effects, (Li and Webster, 2018).

Spondias mangifera Willd. is also called as *Spondias pinnata* (Linn. F.) and belongs to Anacardiaceae. The plant is cultivated for its edible fruits and it is known by different names in different localities and languages such as hog plum, wild mango, amra, etc. Its leaf, bark and fruits are used for the treatments of various ailments (Tripathi and Kumari, 2010). Exocarp of the fruit has shown the presence of various activities such as antioxidant, antimicrobial and thermolytic (Manik et al, 2013). Similarly, its resin also showed antimicrobial activity (Gupta et al, 2010). The present investigation was done to evaluate antibacterial activities of fresh and dried leaf extracts in different solvents.

MATERIAL AND METHODS

Material Collection and Preparation of Extracts: For green leaves: Green young leaves of *Spondias mangifera* were collected from the campus of Banaras Hindu University (BHU), Varanasi, India. The surface of leaves was cleaned by running tap water and then blotted by blotting papers. 1g of leaves was crushed in mortar and pestle in 5 ml of different solvents like ethanol, methanol and double distilled water separately. Solutions were centrifuged at 3000 rpm for 10 minutes to remove all the cell debris. Supernatant was separated out and final volume was maintained up to10 ml. The extract solutions were stored at 4 °C.

For dry leaves: Leaves were shade dried for 6-7 days, oven dried at 45-50 °C for 2-3 hrs and then grinded in mechanical grinder to make coarse powder. For the preparation of extract, 20 g of powdered leaf were mixed in 200 ml of solvent by using a Soxhlet apparatus. Ethanol, methanol and Double distilled water (DDW) were used as solvents for extraction. Extracts were then filtered and dried at 45 °C on rotary evaporator. Extracts were stored at -20 °C for further use.

Preparation of samples: Stock samples of leaf extracts were prepared in dimethyl sulphoxide (DMSO) and the concentration of stocks was 100 mg ml^{-1}. About 5 µl extracts were dispensed on sterile disc for susceptibility test.

Test Microorganisms: Six human pathogenic bacterial strains were selected for screening of antibacterial activity. Two Gram positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and four Gram positive bacteria (*Klebsiella pneumoniae, Escherichia coli, Shigella boydii and Proteus vulgaris*) were taken for the investigation. Microbial cultures were obtained from Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India.

Medium preparation: For the preparation of medium, 38 g Muller Hinton agar (MHA) and 10 g bacteriological agar were dissolved in 1 litre double distilled water. Saline was prepared by dissolving 8.5 g NaCl in 1 litre double distilled water. The medium was autoclaved for 15 min at 1.1 kg/cm^2 and 121°C. Approximate 20 ml autoclaved molten medium was poured in autoclaved Petri dishes in laminar flow.

Preparation of Inoculums: Bacterial inoculums were prepared by growing cells on MHA (Himedia, Mumbai) for 24 h at 37°C. The turbidity of the bacterial suspension was adjusted to about 0.5 McFarland turbidity standard (~1 x 10^7 CFU/ml).

Antibacterial Sensitivity: The disc diffusion method (Zaidan et al, 2005 and Singh et al, 2016) was used to screen antibacterial activity. The test cultures were swabbed on the top of the solidified media and dried for 5 min. About 5 µl of extract was loaded to each disc. The loaded discs were placed on the surface of the medium. Dimethyl sulphoxide (DMSO) was used as negative control. Specific standard drugs Streptomycin was used against all Gram positive and Gram negative bacteria. The plates were incubated for 24 h at 37 °C for bacteria in BOD incubator (REMI). Zone of inhibition (diameter) was recorded in millimeters.

RESULTS AND DISCUSSION

Frequent use of antibiotics and development of resistant varieties of microbes have become a major concern for medical practitioners (Kourkouta et al, 2017). Many patients show resistance for several antibiotics and their treatments pose a major challenge for physicians. Antimicrobial activities have been reported by many plants, but their use as alternative to antibiotics are not in practice. There is utmost need to identify the plants with such activities, so that it could replace the use of antibiotics effectively. Use of antibiotics shows many side effects, but plant products have less or negligible side effects. Present work used Mueller Hinton Agar medium and disc diffusion method (Fig 1). Mueller Hinton Agar is considered best medium for reactive antibiotic susceptibility test. In the present study, both dried and fresh leaf extracts have shown antimicrobial activity (Table 1 & 2). Previous reports also showed antimicrobial activities of

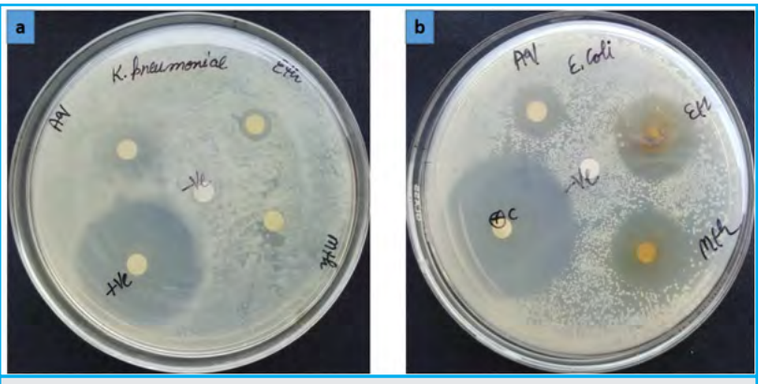

FIGURE 1. (a) & (b) Inhibition zones shown by plant extracts in different solvents Aq = Aqueous extract, Eth = Ethanolic extract, Mth = Methanolic extract, (+Ve) = Used antibiotic streptomycin as positive control (-Ve) = Dimethyl sulphoxide (DMSO) used as negative control

Table 1. Antimicrobial activity of dry leaf extract of *Spondias mangifera*

Test organisms	Inhibition zone diameter (mm)				
	Ethanolic extract	Methanolic extract	Aqueous extract	Control	Standard drugs (5µl/disc)
S. aureus	8.83 ± 0.17	6.83 ± 0.17	5.67 ± 0.67	0.00 ± 0.00	35.50 ± 0.29
E. faecalis	25.00± 0.58	8.83 ± 0.44	23.88± 0.60	0.00 ± 0.00	42.67 ± 1.20
E. coli	7.83 ±0.17	8.67± 0.33	6.67 ± 0.33	0.00 ± 0.00	39.17 ± 0.44
K. pneumoniae	8.67 ± 0.33	11.50± 0.29	5.83 ± 0.17	0.00 ± 0.00	38.67 ± 0.33
P.vulgaris	10.67± 0.33	10.5±0.29	4.5 ± 0.29	0.00 ± 0.00	23.00±0.58
S. boydii	23.50±0.29	25.17±0.44	11.50±0.29	0.00 ± 0.00	36.17±0.60

fruit and leaves of *S. mangifera* (Tripathi and Kumari, 2010 and Jain et al, 2014). Here antimicrobial properties of both fresh and dried leaf extracts in different solvents have been tested for different test organisms.

The antimicrobial activity depends upon the type of extract, concentration of the extract, type of solvents and type of test organisms. Maximum inhibition for *E. faecalis* (25.00±0.58) was seen in ethanolic extract of dried leaves, whereas aqueous extract also showed high inhibition activity (23.88± 0.60) (Table 1 & 2). Inhibition of *K. pneumoniae* was maximum (11.50±0.29) by methanolic extract of dried leaves. Poor inhibition was observed for *S. aureus* and *E. coli* by different extracts of dry leaves (Table 1). However, hexane extract of leaves was reported highly effective against *S. aureus* (Jain et al, 2014). Maximum inhibition of *P. vulgaris* (10.67± 0.33) was seen by the methanolic extract of dried leaves. High antibacterial activities were seen against *S. boydii* by methanolic (25.17±0.44) and ethanolic (23.50±0.29) extracts of dried leaves. Maximum antimicrobial

Table 2. Antimicrobial activity of green leaf extract of *Spondias mangifera*

Test organisms	Inhibition zone diameter (mm)				
	Ethanolic extract	Methanolic extract	Aqueous extract	Control	Standard drugs (5µl/disc)
S. aureus	8.50 ± 0.29	4.67 ± 0.33	11.50± 0.76	0.00 ± 0.00	35.50 ± 0.29
E. faecalis	9.33± 0.33	11.50± 0.29	24.17± 0.44	0.00 ± 0.00	42.67 ± 1.20
E. coli	10.17±0.44	8.17± 0.73	9.17 ± 0.60	0.00 ± 0.00	39.17 ± 0.44
K. pneumoniae	7.83 ± 0.17	15.50± 0.29	7.00± 0.58	0.00 ± 0.00	38.67 ± 0.33
P.vulgaris	6.00± 0.58	12.50±0.29	5.17±0.60	0.00 ± 0.00	23.00±0.58
S. boydii	6.50±0.76	13.8±0.44	11.16±0.73	0.00 ± 0.00	36.17±0.60

(±) = showing for standard error

response was seen in hexane extract of leaf but present report shows methanolic extract of dry leaf more effective. The solvents dissolve phytochemicals of similar polarity and dissolved chemicals play significant role in showing antimicrobial activities (Altemimi et al, 2017 and Ngo et al, 2017)

Therefore, a wide range of solvents should be tried to get the best response. In general, extracts prepared from green leaves showed less antibacterial potential compared to the extracts of dried leaves. But, aqueous extract of green leaf showed significantly high antibacterial activity against *E faecalis* (24.17±0.44). Similarly, higher antibacterial activities against *E coli* were seen by both ethanolic (10.17± 0.44) and aqueous (9.17±0.60) extracts of green leaves. Methanolic extract of green leaf showed maximum inhibition (15.50±0.29) for *K. pneumoniae*. Thus, present work shows significant antibacterial activity of both green and dried leaf extracts of *Spondias mangifera*.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Biodegradation of Textile Effluent Containing Azo Dye using Individual and Mixed Adapted Bacterial Strains

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ABSTRACT

Wastewater from the textile industries contains a variety of pollutants, particularly dyes. Azo dyes are the largest group of synthetic aromatic dye used in the textile industry for dyeing purpose and are highly water soluble in nature. The removal of azo dyes from industry effluents is desirable not only for aesthetic reasons but also because azo dyes and their breakdown products are toxic to aquatic life and mutagenic to humans. The treatment of these dyes have been carried out by many methods such as physical, chemical and biological as individual and in combination to reduce the effect of pollution. The biodegradation of azo dyes is difficult due to the complex structure, synthetic nature and some are highly resistant to microbial attack. To overcome this problem, this study aims in developing a consortium to degrade complex azo dyes present in the effluent. The current work aims in screening and identification of potent azo dye degrading bacteria, studying azo dye degradation efficiency of the isolates, preparing the microbial consortia and to determine the efficiency in decolourization and degradation in both chosen dye (Amido black 10 B) and effluent. The degradation efficiency of the effluent using mixed dyes were significantly higher than the individual strain which was evident from reduction in colour and COD which was 76 % and 79 % respectively. The degradation of the effluent using consortium was studied using FT-IR which revealed change in the peak characteristics.

KEY WORDS: AMIDO BLACK, AZO DYES, CONSORTIUM, DECOLOURIZATION, FT-IR

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INTRODUCTION

Textile industry is considered as one of the chief export earnings and employment providing sector of the nation. Though it helps developing the economy of the country it also burdens it by polluting the environment through the processes involved in its production especially the dyes and chemicals used. This industry also consumes a generous amount of water during its manufacturing processes especially in the dyeing and finishing operations of the plants. The textile wastewater is considered the most polluter among all the industrial sectors based on the volume generated and the composition of the effluent (Mansour et al 2012). Since, there is an increase demand for textile products the production also increases relatively and this has led to the use of synthetic dyes which in turn has led to be a source of severe pollution (Ogugbue and Sawidis 2011 Yin et al., 2019).

A wide range of dyes are being used in the textile industry among which synthetic dyes play an important role due to their cost effectiveness and dyeability. Synthetic dyes are polyaromatic molecules that give a permanent coloring to materials like textile fabrics. A variety of synthetic dyestuffs released by the textile industry pose a threat to environmental safety. The degree of coloring process continues even after the dyeing process, leading to effluents containing azo dye (Lu & Liu 2010). The chemical structure of the dyes makes it resistant to most types of physical, chemical and biological treatments (Mansour et al. 2011b). The removal of azo dyes from industry effluents is desirable not only for aesthetic reasons but also because azo dyes and their breakdown products are toxic to aquatic life and mutagenic to humans. Therefore, treatment of textile effluent is necessary before discharging in the environment. The treatment of textile effluents is also essential to protect the ecosystem and allow the subsequent recycling of the treated effluent for irrigation or reuse within the procedures of the textile plant (Yaseen and Scholz, 2019).

The treatment of these dyes have been carried out by many methods such as physical, chemical and biological as individual and in combination to reduce the effect of pollution. Physical or chemical methods for textile wastewater pretreatment are of high cost, extremely energy consuming, and environmentally low efficient and generate toxic sludge (Sakar *et al.,* 2017). These dyes are generally recalcitrant to biodegradation due to their xenobiotic nature. However microorganisms, being highly versatile, have developed enzyme systems for the decolorization and mineralization of azo dyes under certain environmental conditions. The complete mineralizations of organic pollutants by biological methods are cost effective and eco-friendly (Nachiyar et al., 2016)

The biodegradation of azo dyes is difficult due to the complex structure, synthetic nature and some are highly resistant to microbial attack. To overcome this problem, several studies using microbial consortia were attempted to achieve not only dye decolourization, but also degradation of the aromatic amines due to the efficiency of the microorganisms (Chan et al., 2011). Thus, this work aims in screening, identification of potent azo dye degrading bacteria, studying azo dye degradation efficiency of the isolates, preparing the microbial consortia and to determine the efficiency in decolourization and degradation in both chosen dye and effluent.

MATERIAL AND METHODS

Sample Collection: The textile effluent was collected from Chinnakkari Common Effluent Treatment Plant (CETP), Tiruppur and was used for the study.

Physiochemical analysis: The physiochemical parameters such as color, pH, BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), TSS (Total Suspended Solids), TDS (Total Dissolved Solids) were determined immediately after being brought to the laboratory as per the standard APHA methods (Rice, 2012)

Isolation of autochthonous bacterial strains from effluent sample: The spread plate assay method was used for the enumeration of aerobic bacteria from the collected sample. This was done by serial dilution of the sample (10^{-1} to 10^{-8}), and placing 0.1ml of the diluted samples (10^{-6}, 10^{-7}) in an minimal media (64 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl and 5.0 g NH_4Cl, 1M $MgSO_4$ and 1M $CaCl_2$) agar plate containing dyes. The dyes used for the study were Alizarine red, Amido black 10 B, Reactive red and Brilliant violet (SIGMA, India). The diluted samples were spread on the surface of agar plate using L- rod and was incubated at 37˚C as for 48 hours. The cultures which showed a zone of clearance around their colonies were isolated and used for further screening (HeFang et al., 2004).

Screening of microorganisms capable of decolorizing and degrading the azo dye: The minimal broth containing four different dyes (100 mg/L) were prepared; the isolated organisms were inoculated and incubated at 37°C for 7 days. The degradation patterns were observed for each organism in different dyes using visible spectrophotometer in the wavelength ranges between 400 - 650 nm (Omar, 2008). The strains showing decolourization efficiency in chosen all four dyes, where studied for their potency in Amido Black 10 B dye and their percentage decolourization was recorded using the formulae below at 610 nm:

$$\%\ decolourization = \frac{Initialabsorbance - Finalabsorbance}{Initialabsorbance} \times 100$$

Identification of Selected Microorganisms: The screened bacterial strains were identified by using standard biochemical (Cappucino and Sherman, 2014) and microscopic techniques according to Bergey's Manual of Systemic Bacteriology.

Strain Identification – Molecular characterization: The three most active isolates were identified by 16S rRNA sequencing after extracting the genomic DNA. The 16S region of ribosomal rRNA gene was and amplified using the universal primers 27F and1492R primer. The PCR amplification was done by initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30seconds, 60°C for 30 sec, 72°C for 60 sec and final extension at 72°C for 10 min. PCR purification was done by SolGent PCR Clean up kit (Millipore).The PCR product was sequenced using the 27F/1492R primers. Using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), sequencing were performed. The sequences were assembled and edited using Bioedit software and deposited in the NCBI database for accession numbers. The sequences obtained were compared to find sequence similarity using GenBank program MUSCLE 3.7. PhyML 3.0 aLRT was used for phylogeny analysis. The program Tree Dyn 198.3 was used for tree rendering. The sequences obtained in this study were deposited in the GenBank database.

Test for synergism: In order to develop a bacterial consortium the selected strains must be tested for their compatibility. The test was performed as follows: three nutrient agar plates were taken and each plate was bored with two wells. The first plate was smeared with one of the three selected culture (TMB 2) and added with 10µl of the supernatant from TMB 6 and TMB 7 in the two wells respectively. The plates were then kept for incubation at 37˚C for 24 hours. Absence of any zone of inhibition around the wells showed that the cultures are compatible. The test was repeated by changing the swabbed organisms with the other two selected bacterial isolates and changing the supernatants from the organisms added in the bored wells accordingly (Ammar *et al.*, 1998).

Development of Consortia for treatment textile effluent: Based on the mutuality of the isolates, the Cayley's tables were made. According to the Table-1, the culture consortium were developed and used for further study.

Treatment of effluent by individual strains and developed consortia: About 5ml of mixed culture was inoculated into 95 ml of textile effluent sample and incubated at 37°C in shaking condition for 5 days. The degradation patterns of the effluent were measured in spectrophotometer in the range of 423nm.

Table 1. Cayley's table for consortia preparation

Organisms	TMB 2	TMB 6	TMB 7
TMB 2	TMB 22	TMB 26	TMB 27
TMB 6	TMB 62	TMB 66	TMB 67
TMB 7	TMB 72	TMB 76	TMB 77

$$\%\ of\ decolorization = \frac{Initialabsorbance - Finalabsorbance}{Initailabsorbance} \times 100$$

Fourier Transformed Infrared Spectroscopy analysis: IR-spectra were obtained using a SHIMADZU FTIR 8400S. The untreated textile effluent (raw) and treated effluent with the potent consortia were assessed by FT-IR for the chemical modification. Effluent samples was recorded from 4000 – 400 as scanning range between wave number (cm^{-1}) and % Transmittance. All samples were run in triplicate and the data presented are the average of the three measurements.

RESULTS AND DISCUSSION

The textile effluent sample was collected in sterilized container from CETP, Chinnakarai, Tiruppur and stored at 4°C for further studies.The autochthonous bacterial strains were isolated in minimal media plate containing the azo dye using spread plate technique. The result showed around 7 different colonies had the ability to degrade the azo dye which was observed through the zone around the colonies. The obtained colonies were designated as TMB1, TMB 2, TMB 6, TMB7, TMB 8, TMB 10 and TMB 11. It was clear from the results that the natural adaption of microorganism enabled them to survive in the presence such recalcitrant compounds (azo dyes). The ability of the organisms to decolourize was due to the utilization of the toxic dye which resulted in the breakdown of chromophore of the dyes. The isolated strains were then subjected to quantitative screening by inoculating the isolated strains in minimal media broth containing azo dyes. The results revealed that among the 7 strains screened, three strains were found to have ability to degrade all the four azo dyes used in the study. These strains were chosen because they showed percentage reduction more than 45% in 48 hours of incubation in all the four dyes used for the study (Fig. 1). Therefore, the strains TMB2, TMB 6 and TMB 7 were chosen for further study and were identified. The isolated bacterial strains from effluent sample collected from the CETP were identified through morphologically different bacterial isolates cultures were preserved on nutrient agar medium at 4˚C. The three organisms which showed significant decolourization of the dye degradation were

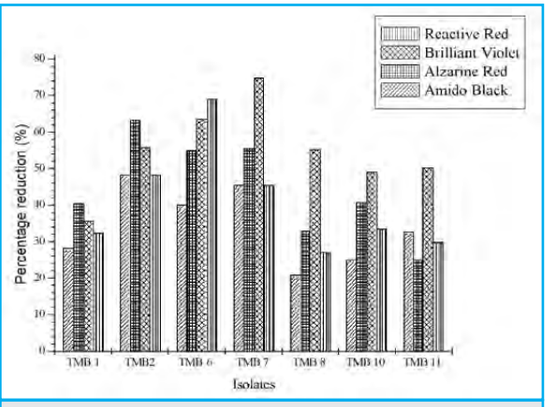

FIGURE 1. Quantitative decolourization efficiency of isolates using various dyes under shaking condition for 72 hours

identified through morphological and biochemical analysis. The isolated bacterial cultures – TMB2, TMB 6 and TMB 7 were tentatively identified to be *Pseudomonas* sp., *Klebsiella* sp., *Shigella* sp., through biochemical test.

This was reconfirmed with 16s rRNA sequencing after extracting DNA.The sequences obtained were compared using GenBank program-Basic Local Alignment Search Tool (BLAST). The phylogenetic trees based on 16S rRNA gene sequences were constructed by the neighbour-joining method. The sequences obtained in this study were deposited in the GenBank database. GenBank accession numbers for the nucleotide sequences are KY788338,KY788341 and KY789442. The isolates obtained were found to be *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Shigella dysenteriae*. From the results it is clear that the obtained strain are pathogenic to human, but still have the potency to degrade the chromophore of the toxic dyes. Several studies have reported that these pathogenic strains have efficiency in degrading the azo compounds. The decolourization Direct Blue 6 by *Pseudomonas desmolyticum* in 72 h under microaerophilic conditions was reported by Kalme et al. (2007). Similarly the decolorization and biodegradation of four different azo dyes, Reactive Yellow 107 (RY107), Reactive Black 5 (RB5), Reactive Red 198 (RR198), and Direct Blue 71 (DB71), in a sequential microaerophilic– aerobic treatment in 72, 120, 96, and 168 h, respectively, by a facultative *Klebsiella* sp was accounted by Franciscon et al. (2009).

The recent findings of Dixit and Garg (2018) clearly state the pathogenic strain posses' potency to degrade the azo dye and completely mineralize them. In their study which is a two-stage sequential process that utilizes the isolated *Klebsiella pneumoniae* and successfully degrade the azo dyes and their metabolites completely in much less time as compared with the reported strains

Table 2. Physico – Chemical Characterization of Untreated Textile Effluent

Parameters	Value	TNPCB standards
pH	8.9	5.5-9.0
Temperature	35°C	40°C at the point of discharge
Conductivity (µS/cm)	13330	2250
Turbidity (NTU)	45.3	10
Color (Hazen units)	1200	400
COD (mg/L)	1160	250
BOD3 (mg/L)	70	30
Hardness	960	300
Total suspended solids (TSS) (mg/L)	3700	100
Total dissolved solids (TDS) (mg/L)	5970	2100
Chloride (mg/L)	2080	600
Phosphate (mg/L)	1.4	5
Sulphate (mg/L)	1745	1000

(Dixit and Garg, 2018). Decolourization of Acid Red 131 by using *Shigella* sp was reported by Sivaranjani et al (2013) in which they stated that 99% degradation was observed by the isolated strain (Sivaranjani et al., 2013). Therefore, further study of treating the effluent using the strains were carried out as to determine the efficiency of degradation and mineralization of azo dye containing effluents. The untreated textile effluent was characterized and its physico – chemical parameters was determined and tabulated below (Table 2).

The collected sample had an unpleasant fishy odor and was turbid with greenish color. Unpleasant smell and taste in water, perhaps due to declining vegetation, inorganic constituents / organic substances, wastewater discharge into water bodies (Mohabansi et al., 2011). As reported by Patel et al., (2015) the primary sample has fishy, secondary aerated and processed has rotten egg as well as stagnant sample has fortified odor (Patel et al., 2015). The strong coloration of the effluent was sometimes influenced by the pH and temperature of the dyeing process which strengths the chromophore group. The presence of highly colored components affects the dissolved oxygen of the water. pH of a effluent is very important in determination of water quality since, it affects other chemical reactions such as solubility and metal toxicity (Fakayode, 2005). The dissolved oxygen levels are found to be very low and hence a lot of oxygen has been used up, which shows the increased concentration of organic matter. The collected effluent sample had a BOD_3 70 mg/l which implies the content of organic matter is too high and oxygen gets depleted rapidly.COD is a critical parameter in assessing the water

quality as it indicates the presence of non-biodegradable and organic matter present in the effluent. In the present study the investigations made indicate high COD value of around 1160 mg/l which is higher than the permissible level. As the physico-chemical parameters of the collected effluent are higher than the allowable limits, proper treatment is necessary before discharging. The elevated amount of industrial effluent pollution creates environmental issues that impact plant, animal and human life (Kolhe et al., 2011).

Analysis of compatibility was performed to verify whether the chosen strains used in biodegradation were suitable for effective bioremediation when used as a consortium. The results showed that there was no inhibition zone around the wells for any of the plates after incubation. The main reason for this compatible nature of the selected bacterial strains may be that they have co-existed for a longer period of time in a common environment. This clearly illustrates that the compatible nature of these strains would be effective in degrading the recalcitrant azo dyes and the complex organics present in the effluent. About 4 different consortia containing equal amount of the strains were prepared and designated as TMB 26, TMB 27, TMB 67 and TMB 267 and used for the study. On observing the reduction in the various physico – chemical parameters of the treated effluent sample using bacterial consortia, it could be concluded that the bioremediation efficiency of the consortium TMB 27 was found to be reduced to a significant extent (Table 3) compared to the other strains or developed consortia. This could be due to the fact that the consortium of adapted microorganism were able to exert more enzymes that have a wide spectral range which in turn could have degraded the complex organic compounds and dye content present in the effluent. Similarly investigation done by Puvaneshwari *et al.*, (2006) revealed that mixed bacterial strains showed an efficient degradation of the organic compounds.

Table 3. Percentage Reduction of Physicochemical Parameters of Effluent Treated using Individual Strains and Developed Bacterial Consortia

Treatments	Percentage of reduction in physico-chemical parameters (%) after 72 hours of treatment			
	COD	TDS	Colour	Turbidity
Untreated	-	-	-	-
TMB 2	71.1	48.07	65.7	39.2
TMB 6	64.9	29.6	50.5	49.2
TMB 7	62.8	27.1	52.6	38.1
TMB 26	62.8	35.5	57.8	53.6
TMB 27	79.3	59.7	73.6	66.8
TMB 67	73.1	53	68.4	67.9
TMB 267	75.2	56.4	71.05	56.9

FTIR gives information regarding the structural changes that occurs during biodegradation process with the help of functional groups present (Ladwani et al., 2016). IR Spectra of the untreated effluent showed many bands of 3394.72 cm^{-1}, 3174.83 cm^{-1}, 2607.76 cm^{-1},2110.12 cm^{-1}, and 1269.16 cm^{-1} which are representatives of functional group OH, N-H, O-H with strong and very broad intensity, and C-N. The peak position of 3498.12 cm^{-1}, 2858.51 cm^{-1}, 2607.76 cm^{-1}, 2144.84 cm^{-1},1597.059 cm^{-1}, 1435.04 cm^{-1}, 1095.57 cm^{-1} and 898.82 cm^{-1}which are the characteristics of O-H, C-H, O-H of strong and very broad intensity, , C=C, ether C-O and C-Cl alkyl halide group respectively.

IR spectrum of the textile effluent treated with consortia shows difference in bandings when compared with the untreated sample. During the biodegradation of effluent, the IR spectrum of the treated sample shows variations such as appearance and disappearance of peaks 2754.34, 3429.43, 2144.34, 1693.50, 1435.03, 1269.16,898.82 in treated sample. The absorption bands showed variation in 3930.27, 3853.77, 3722.61,3649.31, 3251.98, 3194.12, 3140.11, 1577.77,1107.14 cm-1, due to N-H stretching, O-H stretching (alcohol), N-H stretching (Amide), N-H (bending), C-O stretching (Alcohol)respectively (Figure 2). From the above results it is clear that changes in the FTIR spectrum are evidence for the degradation of the dyes and complex organic matter present in the effluent into simpler molecules which is due to

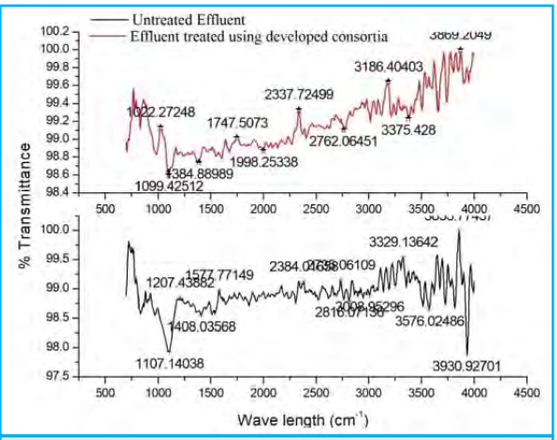

FIGURE 2. FTIR Spectra of untreated effluent and effluent treated using developed consortia

the metabolism of the mixed bacterial strains. Therefore, the study concludes that when mixed bacterial population when used in degradation of effluents containing recalcitrant compounds, the rate of degradation would be faster as more number of various enzymes produced by the strains would act on the molecules and aid in degradation. Similar studies on degradation of recalcitrant's by mixed microbial strains rather than individual have shown better degradation efficacy (Lade et al., 2015; Nachiyar et al., 2016; Chan et al., 2011) The strains isolated in the study are said to be pathogenic in nature which might pollute the environment further, in order to overcome this biotechnological tools can be used and the gene responsible for degradation can be cloned into non-pathogenic strains.

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Analysis on the level of well-being among Indian secondary school adolescents

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ABSTRACT

Well-being in adolescence is an increasing field of study. Well-being, as a component of quality of life, has been a field of important developments during the last two decades. Adolescent well-being is a comprehensive construct that includes the ability to acquire knowledge, skills, experience, values, and social relationships, as well as access to basic services, that will enable an individual to negotiate multiple life domains, participate in community and civic affairs, earn income, avoid harmful and risky behavior, and be able to thrive in a variety of circumstances, free from preventable illness, exploitation, abuse and discrimination. The purpose of the research is to find out the level of well-being of adolescents. Participants of the study were 640 secondary school adolescents from the state of Punjab. Survey was used to study the level of well-being of adolescents. The findings show that out of total 640 adolescents, 196 adolescents i.e. 32.67% of adolescents have high well-being, 392 adolescents i.e. 65.33% have average level of well-being and only 12 i.e. 2% have low level of well-being. A majority of adolescent boys and girls have average level of well-being. Majority of rural adolescents have average and majority of urban adolescents have high level of well-being. This study highlights the importance of considering well- being of adolescents. These results have strong implications for adolescent's positive mental health promotion, including school-based policies and practices.

KEY WORDS: ADOLESCENTS, WELL-BEING, SECONDARY, SCHOOL, PUNJAB

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INTRODUCTION

Adolescence stage is the progressive transition from childhood to adulthood, it is a phase when many important biological, economic, social and demographic events set the stage for adult life. It is a transitional stage of psychological and physiological development from puberty to adulthood. There are more than 1.2 billion adolescents worldwide indicating that roughly one in every six persons is an adolescent. About 21% of Indian population is adolescents (about 243 million). It is well recognized that India's ability to achieve the Millennium Development Goals and to achieve its population stabilization goals will depend on the investment made in its young people. They are the future of the nation, forming a major demographic and economic force. They have specific needs which vary with gender, life circumstances and socio economic conditions. In India large number of adolescents face challenges like poverty, lack of access to health care services, unsafe environments etc. Adolescence is a period of preparation for undertaking greater responsibilities like familial, social, cultural and economic issues in adulthood. It is a critical developmental period with long-term implications for the health and well-being of the individual and for society as a whole. Each year, an estimated 20 % of adolescents experience a mental health problem, most commonly major depression or other disturbances of mood. Mental health problems in adolescence, if unaddressed, can carry over and negatively affect individuals over long term. A major depression experienced for the first time in adolescence, for example, can persist or recur through adulthood, (Essen & Martensson, 2014, Easow and Ghorpade 2017).

In India, data on adolescents from national surveys including National Family Health Survey III (NFHS-3), District Level Household and Facility Survey III and Sample Registration System call for focused attention with respect to health and social development for this age group. Therefore, it has been realized that investing in adolescent mental health will yield social and economic dividends to India. Thus well-being in adolescence is an increasing field of study. Well-being, as a component of quality of life, has been a field of important developments during the last two decades. However, its study in relation to childhood and adolescence has been, comparatively speaking, much more limited despite the fact that during the 1990's an increase of interest towards the development of adequate instruments has taken place, these instruments being more sensitive to age and the evolution period of the individuals (Casas et al., 2000 Viejo et al. (2018).

The concept of well-being has a multidimensional constitution, it could be a representation of positive feelings, individuals experience as well as aspects of life characterized by optimal functioning and flourishing (Fredrickson & Losada, 2005). It has been asserted that it is practical to assume that the concept of health is comparable to the concept of well-being (Essen & Martensson, 2014). Research in well-being has been growing in recent decades (e.g., Diener et al., 1999; Kahneman, s1999; Keyes, et al. 2002; Stratham & Chase, 2010; Seligman, 2011) yet the question of how it should be defined remains unanswered. In the research Ryff and Keyes (1995) identified that the absence of theory-based formulations of well-being is puzzling. The question of how well-being should be defined (or spelt) still remains largely unresolved. Thomas (2009) also argued that well-being is intangible, difficult to define and even harder to measure.

The well-being issues are a growing concern in the school and for the community counselors, and educators. Research studies have revealed an increasing incidence of depression and other mental health issues among the youth (adolescent health and development (WHO). In fact, increasing incidences of suicide in adolescents have attracted more attention of the concerned authorities (Sharma et al., 2008). Now a days adolescents are deeply concerned as to how others view them and are apt to display self consciousness and are embarrassed on being criticized by others (Mahajan & Sharma, 2008). High prevalence of behavioral & emotional problems are also found in Indian adolescents (Pathak et al, 2011). Research conducted in the state of Rajasthan of India by Easow and Ghorpade (2017) revealed that the majority of 84(84%) adolescents had adequate psychological well-being and 11(11%) of them had moderate and only 5(5%) of them had inadequate psychological well-being. Viejo et al. (2018) in their study of Spanish adolescents showed good scores of psychological and subjective well-being among the adolescents, with a significant impact of sex and age in both measures of well-being.

Due to the increasing maladjusted behavior manifested by adolescents and against the proven empirical facts that a person is not necessarily inherently stressful, it is necessary to have a look at the factors that contribute to well-being of individual. Last decade's research has highlighted the relationship between well-being and various other factors such as locus of control, stress, coping, meaning of life, family climate and parental autonomy-support & parent relationship with adolescent (Kunhikrishan & Stephen, 1992; Lekes et al., 2010; Rathi & Rastogi, 2007; Schlabach, 2013; Sehgal & Sharma, 1998; Seaton & Yip, 2009; Vandeleur et al., 2009; Vera et al., 2011 and Walsh et al., 2010). Well-being also found to be correlated with self-esteem, physical self identity, age, social support and positive psychological strengths (Jovic- Vranes et al., 2011; Karatzias et al., 2006; Khan, 2013).

MATERIAL AND METHODS

Research Questions: 1What is the level of well-being among Indian adolescents? 2 What is the relationship of well-being of adolescents in with gender and locale?

Sample: Descriptive survey method was used to investigate the level of well-being among adolescents. The present study was conducted on 640 secondary school adolescents from the state of Punjab. The total sample for the study was selected by multistage randomization, meaning thereby, randomization was followed at the district, tehsil, block, school and student level. The sample of the present study was raised from four randomly selected districts of Punjab viz., Ludhiana, Moga, Gurdaspur and Ferozepur out of the total twenty-two districts. For the study, ten schools (five rural and five urban) were picked up at random per district.

Data Collection: Quantitative method was used to collect and analyze data obtained from respondents. Well-Being Scale (WBS) by Singh and Gupta (2001) was used to address the research objectives. All permissions were requested and anonymity. Confidential use of information was guaranteed, and it was only used for statistical treatment and for the purposes of the research.

Data Analysis: The adolescents were classified into following three categories on the basis of the scores they obtained on the variable of well-being: 1. Adolescents with low well-being 2. Adolescents with average well-being 3. Adolescents with high well-being. For the classification of adolescents in the categories of high, average and low well-being, the classificatory scores given in the well-being scale by Singh and Gupta were used. As per the scale, the group of adolescents whose scores on the well-being scale were between 176-250 was termed as the group with high well-being. The group of adolescents whose scores on the well-being scale were between 125-175 was termed as the group with average well-being. The group of adolescents whose scores on the well-being scale were between 50-124 was termed as the group with low well-being.

Table 1 and Fig 2 show that out of total 640 adolescents, 216 adolescents i.e. 33.75% of adolescents have high well-being, 407 adolescents i.e. 63.59% have average level of well-being and only 17 i.e. 2.66% have low level of well-being.The distribution clearly indicates that majority of adolescents have average level of well-being. A substantial percentage of adolescents possess high well-being as well. However, a very small percentage of adolescents experience low well-being.Percentages of adolescents depicting different levels i.e. high, average and low levels of well-being were also calculated for the following group of adolescents: (i) Group of Adolescent Boys, (ii) Group of Adolescent Girls, (iii) Group of Rural Adolescents, (iv) Group of Urban Adolescents

Table 1. Showing percentage distribution of adolescents at different levels of well-being (n=640)

Category	Number of Adolescents	Percentage
High	216	33.75
Average	407	63.59
Low	17	2.66
Total	640	100

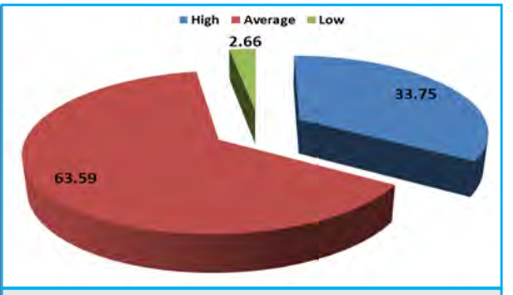

FIGURE 1. Showing percentage distribution of adolescents at different levels of well-being (n=640)

Table 2 and Fig. 2 shows that out of total 320 adolescent boys, 102 adolescents i.e. 31.88% of adolescent boys have high well-being, 215 adolescent boys i.e. 67.19% have average level of well-being and only 3 i.e. 0.93% have low level of well-being. The distribution

Table 2. Showing percentage distribution of adolescent boys at different levels of well-being (n=320)

Category	Number of Adolescent Boys	Percentage
High	102	31.88
Average	215	67.19
Low	3	0.93
Total	320	100

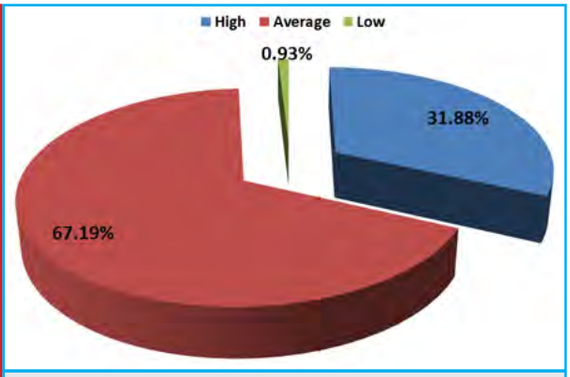

FIGURE 2. Showing percentage distribution of adolescent boys at different levels of well-being (n=320)

Table 3. Showing percentage distribution of adolescent girls at different levels of well-being (n=320)

Category	Number of Adolescent Girls	Percentage
High	114	35.63
Average	192	60
Low	14	4.37
Total	320	100

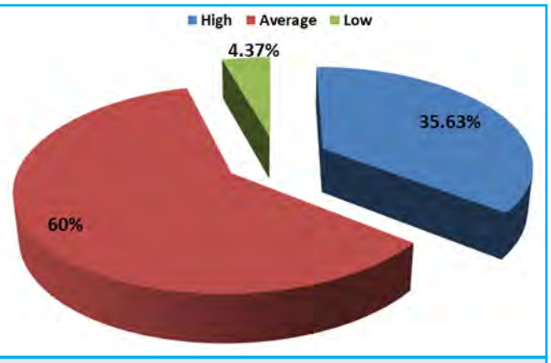

FIGURE 3. Showing percentage distribution of adolescent girls at different levels of well-being (n=320)

clearly indicates that majority of adolescent boys have average level of well-being. A substantial percentage of adolescent boys possess high well-being as well. However a very small percentage of adolescent boys experience low well-being.

Table 3 and Fig. 3 shows that out of total 320 adolescent girls, 114 adolescents i.e. 35.63% of adolescent girls have high well-being, 192 adolescent girls i.e. 60% have average level of well-being and only 14 i.e. 4.37% have low level of well-being.The distribution clearly indicates that majority of adolescent girls have average level of well-being. A substantial percentage of adolescent girls possess high well-being as well. However a very small percentage of adolescent girls experience low well-being.

Table 4 and Fig. 4 show that out of total 320 rural adolescents, 20 adolescents i.e. 6.25% of rural adolescents have high well-being, 284 rural adolescents i.e. 88.75% have average level of well-being and only 16 i.e. 5% have low level of well-being. The distribution clearly indicates that majority of rural adolescents have average

Table 4. Showing Percentage Distribution of Rural Adolescents at Different Levels of Well-being (N=320)

Category	Number of Rural Adolescents	Percentage
High	20	6.25
Average	284	88.75
Low	16	5
Total	320	100

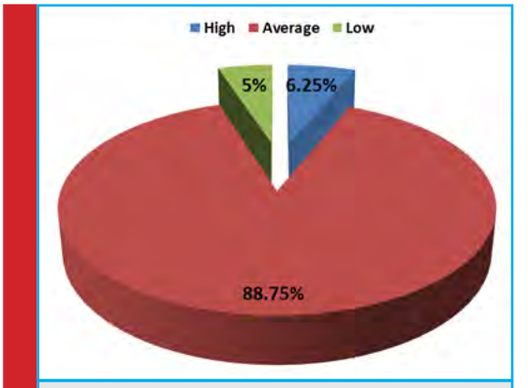

FIGURE 4. Showingpercentage distribution of rural adolescents at different levels of well-being (n=320)

level of well-being. A However only a small percentage of rural adolescents experience high as well as low well-being.

Table 5 and Fig. 5 show that out of total 320 urban adolescents, 196 adolescents i.e. 61.25% of urban adolescents have high well-being, 123 urban adolescents i.e. 38.44% have average level of well-being and only 1 i.e. 0.31% have low level of well-being. The distribution clearly indicates that majority of urban adolescents have high level of well-being. A substantial percentage of urban adolescents possess average well-being as well. However negligible percentage of urban adolescents experience low well-being.

Table 5. Showing percentage distribution of urban adolescents at different levels of well-being (n=320)

Category	Number of Urban Adolescents	Percentage
High	196	61.25
Average	123	38.44
Low	1	0.31
Total	320	100

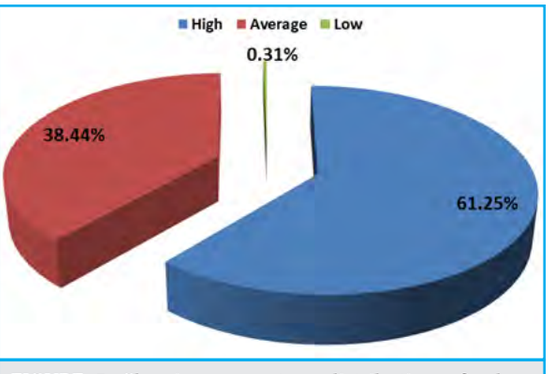

FIGURE 5. Showing percentage distribution of urban adolescents at different levels of well-being (n=320)

RESULTS AND DISCUSSION

The results of the study have implications for policy makers, parents and various social groups dealing with training of adolescents. Better understanding of the adolescent psychological problems and their early identification is need of the hour and the best way to a healthy society. Implications of this study can be carried forward into educational as well as counselling settings.The current study was conducted to assess the levels of psychological well-being among adolescents which revealed that out of total 640 adolescents, 216 adolescents i.e. 33.75% of adolescents have high well-being, 407 adolescents i.e. 63.59% have average level of well-being and only 17 i.e. 2.66% have low level of well-being A majority of adolescent boys and girls have average level of well-being. Majority of rural adolescents have average and majority of urban adolescents have high level of well-being. There can be several plausible reasons for the said results. Although, it is not possible to work out all the possible reasons, yet, it can be easily noted that life in urban areas is far more multifaceted than life in rural areas. There is better exposure for adolescents in urban areas than in rural areas. Urban environment has a more stimulating effect on learning and social interaction. Urban adolescents are more independent and are allowed to deal with their problems themselves. So they are better able to face the life situations and challenges. The adolescents from the rural perspectives are under intense pressure to act like adults. They are under social restrictions and they are not able to pursue their interest due to these restrictions and are expected to behave in an ideal manner as the social and cultural setups of villages expect them to be. Childhood spam is short for them. Thus they easily get affected by the physiological and psychological changes occurring during this period.

The study highlights the need to take some action in the educational scenario prevalent in rural areas as rural adolescents show lower level of well-being as compared to urban adolescents. The fact cannot be ruled out that majority of the population of India resides in rural areas and low well- being among rural adolescents will seriously affect our national development. For this purpose, suitable research works need to be initiated so that the real causes of the low well-being among rural adolescents can be identified and rural adolescents be helped accordingly.

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Modulation of biodiesel production by sodium bicarbonate and nitrogen deficiency in the microalga *Chlorella vulgaris*

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ABSTRACT

Algal biodiesel is a sustainable and a renewable biofuel. Therefore, it is important to optimize the lipid production in microalgae. To enhance bio-diesel production the unicellular alga *Chlorella vulgaris* was grown in ambient air and high CO_2 (5% CO_2, rest air) in the absence or presence of $NaHCO_3$ (12 mM) that provides additional carbon source for algal photosynthesis and biomass production. In ambient air the algal biomass increased 2.5 fold in the presence of $NaHCO_3$. High CO_2 purging of the algal growth medium in the presence of $NaHCO_3$ partially increased the biomass by 10%. In the same vein the chlorophyll content of algal culture increased (100% to 139%) due to the abundant availability of the carbon source. Addition of $NaHCO_3$ increased biodiesel amount by 12%. These demonstrate that addition of extra carbon source i.e., $NaHCO_3$ that is readily photosynthetically fixed by the coordinated action of carbonic anhydrase and rubisco increases the carbon skeleton to be used to optimize biodiesel production by microalgae. Further, the nitrogen deficiency decreased the algal biomass due to reduced N assimilation and amino acid synthesis. The carbon skeletons in N-deficient culture were diverted towards fatty acid synthesis that resulted in 50% increase in lipid content i.e., from 40% to 60% per algal dry cell weight. This approach could be further optimised for large scale biodiesel production.

KEY WORDS: BIODIESEL, *CHLORELLA VULGARIS*, $NAHCO_3$ SUPPLEMENTATION, NITROGEN DEFICIENCY

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INTRODUCTION

Microalgae are fast-growing photosynthetic organisms that fix CO_2 to generate millions of tons of algal biomass in nature. In addition to photosynthetic production of carbohydrates, several microalgae synthesize lipids by using carbon skeletons and reducing equivalents generated from carbohydrate metabolism. Lipids extracted from green algae can be utilized for biodiesel generation, and whatever is left of the biomass can be changed over into ethanol that may be used as biofuel. Biodiesel generation from microalgae is a desirable option. However, their lipid content is required to be high to be an economically viable option. The green alga *Chlorella vulgaris* is widely studied for biodiesel production (Chiu et al., 2008). It has higher productivities than land plants and generates a lot of fatty acids the precursors of biodiesel. To grow algae crop land is not required. It can be grown in ponds or lakes. However, a few challenges need to be overcome to use algae as a sustainable and economically viable option for commercial biodiesel production from *Chlorella*. Microalgae use bicarbonate as the exogenous carbon source for photosynthesis. $NaHCO_3$ is converted to CO_2 via carbonic anhydrase (Dixon et al., 1987; Nimer et al., 1997). Algae could use purged high CO_2 in the growth medium for photosynthesis via same mechanism (Baldisserotto et al., 2014). Nitrogen is a main element controlling species component, diversity, and primary productivity of species (Mandal et al. 2018). Limiting or starving the availability of essential nutrients such as nitrogen (N) can induce triacylglycerol's (TAGs) accumulation (Janssen et al. 2019).

In the present study, we have optimized biomass generation and lipid production of *Chlorella* by providing $NaHCO_3$ as an inorganic carbon source. In addition we have purged the growth medium with ambient air or high CO_2 (5% CO_2, rest air) in the absence or presence of $NaHCO_3$ as additional carbon source for photosynthetic carbon assimilation. These cells were further grown in the N-deficient conditions to increase their lipid content. We have shown than addition of $NaHCO_3$ doubled the algal growth and increase biomass production when purged with either air or 5% CO_2. Their lipid content increase by 12% by $NaHCO_3$ and it further increased in N-deficient growth condition by 50%.

MATERIAL AND METHODS

Photobioreactor and cultivation conditions: *Chlorella vulgaris* (an isolate of river Ganges, courtesy: Prof. Nirupama Mallick, Indian Institute of Technology, Kharagpur, India), was used in this research. Pure culture of *C. vulgaris* were grown in N11 medium (Soeder and Bolze, 1981) at pH 6.8 and maintained in a culture room at 25 ± 2˚C under a photoperiod of 14:10 h at a light intensity of 150 µmol photon m^{-2} s^{-1}. The photobioreactor was setup in a temperature-controlled environment fitted with nine LED fluorescent lamps (Philips, 36W) containing 6 photobioreactor units. Photobioreactors were 1.5 L borosilicate glass tubes with a working- volume of 1.3 L, fitted with sealed stoppers and autoclaved as complete units. Airflow into the photobioreactor was supplied via filtered hydrocarbon-free building air and 5% CO_2 through teflon tubing at a rate of 200 mL min^{-1} (i.e., 0.25 vvm, volume gas per volume media per min). The airflow was adjusted using a stainless steel micrometering valve (JTM LPA Air, Japsin Rotameter). Experimental replication was achieved by repeating each treatment 4 times. The initial biomass concentration for all cultures was 0.11 g L^{-1} and all runs studied for 14 days. When required 12 mM $NaHCO_3$ was added to the growth medium to increase the carbon source for biomass and biodiesel production. After addition of $NaHCO_3$ the pH was adjusted to 6.8. To cause 90% N deficiency (N10), 90% of KNO_3 was substituted by KCl.

Studies on nitrogen deprivation with and without $NaHCO_3$: To study the effects of nitrate, starvation, algal cultures pre-grown in N 11 medium were harvested by centrifugation, washed with N 11 medium without the specific nutrient for 2-3 times and transferred to the 90% N-deficient medium (N10). *C. vulgaris* was grown in ambient or 5% CO_2 without or with $NaHCO_3$ (12 mM) as additional carbon source to support photosynthesis. Cultures were grown under bicarbonate supplementation into early stationary growth phase (10–12 days) and samples were taken for analysis at desired time point to monitor biomass production, chlorophyll content and cellular lipid content.

Analytic determinations: Dry cell weight (dcw) was measured gravimetrically (Rai et al., 1991). To determine the quantity of total chlorophyll, extraction was done according to Sartory and Grobbelaar (1984) with small modifications. To 1 mL of pellet and beaded culture, 2 mL of methanol was added and heated for 10 min at 70 °C in a water bath in a sealed tube. After cooling, the samples were centrifuged for 10 min (4 °C; 6,000g). For chlorophyll a, chlorophyll b quantification, absorbance was recorded for the supernatants at 666 nm, 653 nm and estimation was done using the equations proposed by Wellburn (1994). Extraction of lipid from the dried biomass was done following the protocol of binary solvents using chloroform and methanol (Bligh and Dyer, 1959).

RESULTS AND DISCUSSION

In the present study, the $NaHCO_3$ was given as an inorganic carbon source for algal growth. Simultaneously, it

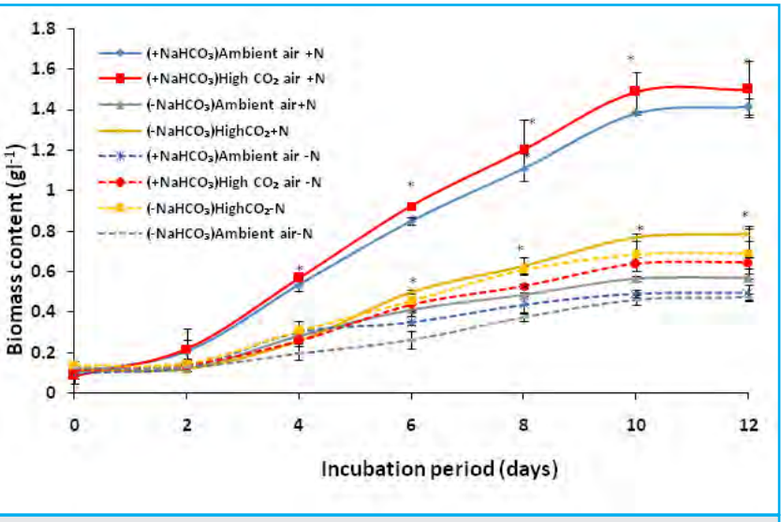

FIGURE 1. Modulation of *Chlorella vulgaris* biomass by $NaHCO_3$, high CO_2 and nitrogen deficiency. Algae were grown in N11 media with or without $NaHCO_3$ and high CO_2 in the N-deficient (N10) or N-sufficient conditions. Two ml of algal culture were taken from the bioreactor on alternate days and their dry biomass was determined as described in Methods. Each data point is the average of 3 replicates and the error bars represent sd. Asterisks indicate significant differences determined by t-test (*P<0.05)

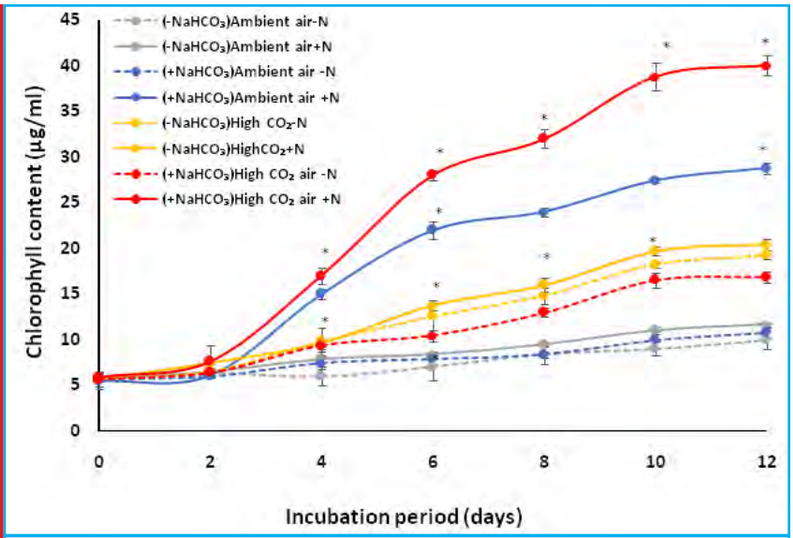

FIGURE 2. Chlorophyll content of *C. vulgaris* culture supplemented with and without bicarbonate in N-deficient and N-sufficient growth media. Algae were grown in N11 media with or without $NaHCO_3$ and high CO_2 in the N-deficient (N10) or N-sufficient conditions. One ml of algal culture was taken from the bioreactor on alternate days and their chlorophyll content was measured by spectrophotometry as described in Methods. Each data point is the average of 3 replicates and the error bars represent sd. Asterisks indicate significant differences determined by t-test (*P<0.05) In N-deficient (N10) growth condition the pigmentation of algal cells declined by 18%, 5.5%, 62% and 57% in -$NaHCO_3$ ambient air, -$NaHCO_3$ high CO_2, $NaHCO_3$ ambient air and +NaHCO3 5% CO_2 growth media respectively. Loss of Chl was previously reported in algal cells due to nutrient deficiency (Fredeen et al. 1990; Jacob and Lawlor 1993; Lippemeier et al. 2003).

was bubbled with air or 5% CO_2 to increase the carbon source. As shown in figure- 1, the algal growth, determined as dry biomass accumulation, saturated after 10 days of growth. The algal cells grown in the photo-bioreactor purged with ambient air accumulated 0.57 g of dry biomass l^{-1} on10th day of growth Upon purging the culture medium with 5% CO_2, the growth and biomass accumulation increased by 38%.

The biomass accumulation substantially increased in the presence of additional inorganic carbon source $NaHCO_3$. When the growth medium was supplemented with 12 mM $NaHCO_3$ and bubbled with air the biomass accumulation substantially increased i.e., by 2.5 fold to 1.41 g l^{-1}. Upon purging the $NaHCO_3$ supplemented growth medium with 5% CO_2 the biomass accumulation further increased to 1.5 g l^{-1}. This accumulation was 100% higher than biomass produced in high CO_2 grown minus HCO_3 samples. This demonstrates that by providing additional inorganic carbon source the algal growth could almost be doubled. Bubbling high CO_2 alone does not achieve the desired result. Our results further demonstrate that high CO_2 purging of algal culture could partially (8%) increase algal growth in the presence of 12 mM $NaHCO_3$. Increase of carbon source as $NaHCO_3$ could provide high CO_2 needed by Rubisco to efficiently fix carbon by the photosynthetic Calvin-Benson cycle. $NaHCO_3$ is not the substrate of Rubisco, rather it is CO_2. The $NaHCO_3$ would have converted to CO_2 by intracellular carbonic anhydrase (Raven, 1995) before its fixation by Rubisco.

Nitrogen deficiency is known to cause reduction of algal growth (Wen *et al.* 2003; Hejazi *et al.* 2004; Aro 2016). To probe the role of nitrogen deficiency on algal growth supplemented with additional sources of inorganic carbon, we monitored the biomass of *C vulgaris* in the absence or presence of $NaHCO_3$ + 5% CO_2 in 90% N-deficient media (Fig. 1). Due to N deficiency, i.e., in 90% N deficient state (N10), the biomass of algal culture declined by 17%, 11%, 62% and 57% in -$NaHCO_3$ ambient air, -$NaHCO_3$ high CO_2, +$NaHCO_3$ ambient air and +$NaHCO_3$ 5% CO_2 growth condition respectively. The highest percentage (61%) of reduction of biomass production in N -deficient condition was observed in high CO_2-grown and $NaHCO_3$-supplemented algal cells. These clearly suggest that N is a highly limiting factor for algal growth in the presence of high inorganic carbon sources. The nitrogen deficiency decreased the algal biomass due to reduced N assimilation and amino acid and protein synthesis. The reduction in biomass was predominantly due to reduced cell division in N-limited growth. This is in agreement with previous studies with *Chlamydomonas reinhardtii and Scenedesmus subspi-*

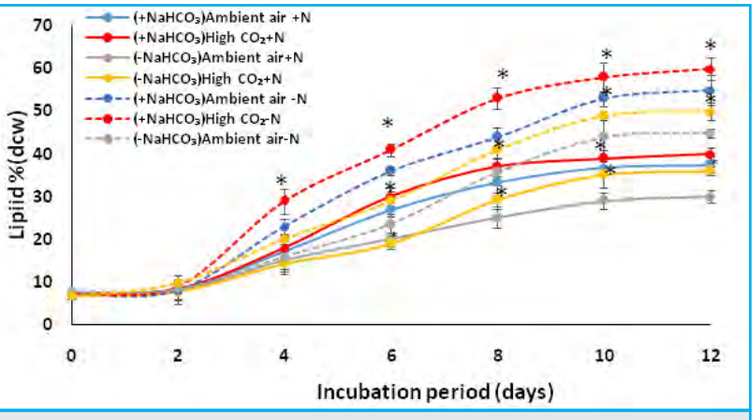

FIGURE 3. Impact of $NaHCO_3$ and high CO_2 and nitrogen deficiency on cellular lipid % (dry cell weight) in Chlorella vulgaris. Algae were grown in N11 media with or without $NaHCO_3$ and high CO_2 in the N-deficient (N10) or N-sufficient conditions. Fifty ml of algal culture were taken from the bioreactor on alternate days and their dry biomass was determined as described in Methods. Each data point is the average of 3 replicates and the error bars represent sd. Asterisks indicate significant differences determined by t-test (*P<0.05). The carbon skeletons in N-deficient culture were used towards fatty acid synthesis that resulted in 50% increase in lipid content i.e., from 40% to 60% per algal dry cell weight. The N deficiency possibly upregulated the genes responsible for fatty acid biosynthesis. In the absence of aminoacid synthesis this would have targeted carbon precursors generated in photosynthesis to be redirected towards fatty acid synthesis.

catus where nitrogen deficiency significantly inhibited their cell division (Dean et al., 2010).

As shown in figure 2, the chlorophyll content of algal cells after 10 days of grown in high CO_2 was higher than ambient. In the presence of $NaHCO_3$ the chlorophyll content of algal cells substantially increased (100% - 139%). This was probably owing to increased number of cells due to higher cell division in high CO_2 environment and higher Chl content per cell.

Addition of $NaHCO_3$ increased the lipid amount by 12%. These demonstrate that addition of extra carbon source i.e., $NaHCO_3$ that is fixed photosynthetically by the coordinated action of carbonic anhydrase and Rubisco to generate carbohydrates and carbon backbones could be used for lipid production by microalgae. Limitations of nitrate, under ambient and high (5%) CO_2 stimulated lipid accumulation (Fig. 3) in the microalga *C. vulgaris*. Lipid percent of dry cell weight increased up to 10^{th} day of culture both in ambient and high CO_2. N deficiency is known to increase lipid content i.e., triacylglycerol in algal cells (Illman et al., 2000) (Janssen et al. 2019). On 10th day of culture, the lipid percent of dry cell weight (dcw) of algal culture in N-deficient (N10) growth condition increased by 51.7 %, 40%, 47% and 48% in -$NaHCO_3$ ambient air, -$NaHCO_3$ high CO_2, $NaHCO_3$ ambient air and +$NaHCO_3$ 5% CO_2 growth media respectively.

CONCLUSION

Chlorella vulgaris grown photo-autotrophically is an excellent organism to generate biodiesel. The growth and biomass of the algal culture nearly doubled by adding $NaHCO_3$ (12 mM) in high CO_2 (5%) growth condition. The lipid production increased by 50% due to N deficiency. The growth of *Chlorella vulgaris* in high CO_2, $NaHCO_3$ in N-deficient conditions could be further optimized for large scale production of biodiesel in an industrial scale.

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Conflict of interest

Authors have declared no conflict of interest.

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Mycorrhizal Soil Development Using *Sorghum bicolor* for Rhizospheric Bioremediation of Heavy Metals

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ABSTRACT

Mycorrhiza is a mutualistic relationship between microorganism and plant roots. The best communal relationship is the vesicular-arbuscular, which creates fungus assemblies like arbuscules and vesicles in the cortex section of the plant roots. The present research work was carried out to assess the potential of *Sorghum bicolor* for development of mycorrhizal soil. The soil-based mycorrhizal inoculum was developed by *Sorghum* as a host plant in the greenhouse for two and a half months using pot culture technique. The physicochemical properties of soil like pH, electrical conductivity, soil moisture, water holding capacity, organic carbon, organic matter, available phosphorus, potassium, nitrate, nitrite, exchangeable ammonia were analyzed during the development of mycorrhizal soil. Bacterial and fungus populations were evaluated in the rhizospheric soil samples. Along these lines, developing mycorrhizae prompt a progression of changes in nutrients accessibility, microbial structure and enzymatic activities in the soil that may decide the result of a phytoremediation effort. In the present research work, the developed mycorrhizal Soil may contribute to building up a compelling mycorrhizosphere that can give nature to the improved degradation of toxins present in the soil. The findings revealed that the inoculum might comprise a very high percentage of organic carbon and other nutrients. The microbial populations were also increased with the progression of the study period.

KEY WORDS: HEAVY METALS, MYCORRHIZA, MYCORRHIZAL INOCULUM, RHIZOSPHERIC BIOREMEDIATION, *SORGHUM*

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INTRODUCTION

Plants in natural environments have fluctuating degrees of dependency on mycorrhizal associations, as inclined by the accessibility of nutrients in the soil in which they certainly occur (Hindumathi and Reddy 2011). Plants are reliant on the mycorrhizal colonization to show extreme progress at a specified level of soil productiveness is known as Mycorrhizal dependence (Gerdermann 1975). Development of phytoremediation procedures needs a comprehensive consideration and supervision of the composite communications in the mycorrhizosphere. The main role of mycorrhizosphere organisms may have been marginalized in intensive farming since bacterial populations in conventional farming systems have been improved due to tillage and great efforts of inorganic manures (Abbasi *et al*., 2015). To enrich nutrient limits and contaminant noxiousness benefit may be taken in increasing and handling rhizosphere (Dubey and Fulekar 2011). Vesicular-arbuscular mycorrhiza fungi are dynamic constituents of the environment for the conservation of soil assembly and restoration of tainted lands (Caravaca et al. 2005; Fulekar et al. 2017, Chen et al. 2018).

Vesicular-arbuscular mycorrhiza is well-known to enhance soil nutrients thus enlightening the development and healthiness of the plants. VAM has been designated for nutrient such as phosphorous which has limited movement in the soil. It is perceived that VAM populated plants accrue and engage more phosphorous compared to plants that are not colonized, grown in soil that have low phosphorous (Azcón et al. 2003). The Vesicular-arbuscular mycorrhiza colonized roots not only enhanced the uptake of phosphorous but these also help in the accumulation of macro and micronutrients like copper, calcium, magnesium, iron (Allen et al. 2003). Mycorrhizal interdependences not only profit to plant progress but also to plant safety, particularly against ecological strains. Insufficiency of important nutrients specifically phosphorus, mainly limits crop development and efficiency (Nagarathna et al. 2007). The cooperative relation offers numerous welfares, comprising higher uptake of poorly movable soil nutrients and reduced vulnerability of roots to soil-borne pathogens (Quilambo 2003). The plants, developed in rhizospheric soil are extra viable and more tolerable to ecological pressures than normal plants, possibly improving contaminant accessibility (Dubey and Fulekar 2011; Gaur and Adholeya 2004).

Numerous research studies showed that persistent organic pollutants like PAH, aromatic hydrocarbons and hexachloro cyclohexane may be converting into less poisonous form, in the mycorrhizosphere (Huang et al. 2006; Sainz et al. 2006; Volante et al. 2005). Subsequently, the expansion of the rhizosphere by immunization of mycorrhiza has shown leniency to poisonous mixtures and also transforms the quality and richness rhizo-microbial communities accountable for biodegradation of polluted soil. The profits of the symbiotic relationship between plants and AMF are accredited to the more quantity of soil explored by the fungal hyphae, which delivers maximum water and nutrients fascination by the plant, moreover the removal and immobilization of heavy metals by fungal tissues (Wang et al. 2017).

Although the potential of AM fungi for the protection of plant is generally acknowledged, it should be noted that in some cases, mycorrhizal crops have no benefits from AM, or may even exhibit reduced growth and fitness (Chen et al. 2018; Jacott et al. 2017). Sorghum bicolor has been cultured in sub-Saharan Africa and South Asia for over 5000 years and highly receptive to AM fungi, for the growth of plants, in low-fertility soil (Cobb et al. 2016). Some microbes have been used in relationship with plants to lighten the stress instigated by metal contamination(Leal et al. 2016). Arbuscular Mycorrhizal Fungi form effective cooperation with many plants and helps in progress and conferring better lenience to ecological strains, including heavy metal contamination (Ogar et al. 2015; Rajkumar et al. 2012; Wang et al. 2017 Chen et al. 2018).

For rhizospheric bioremediation studies, development of a cost-effective technique for the bulk invention of mycorrhizal inoculum is of extreme significance. Since the above-mentioned facts, current research work was carried out to develop and evaluate the potential of soil-based mycorrhizal inoculum for effective rhizosphere bioremediation of hazardous compound and to study the influence of mycorrhiza on soil physicochemical parameters during the development of mycorrhizosphere.

MATERIAL AND METHODS

Host Plant and Testing: The experiment was conducted in the greenhouse of the School of Environment and Sustainable Development, Central University of Gujarat, Gandhinagar, Gujarat. *Sorghum bicolor* commonly called as *Sorghum* a fibrous root grass which is widely used for culturing the mycorrhiza was chosen as a host plant in the current study for the development of mycorrhizal soil at laboratory scale. The sorghum seeds were procured from Department of Seed Technology, Indian Grassland and Fodder Research Institute, Jhansi, Uttar Pradesh. Prior to their use in the experiment, the seeds were surface-sterilized for 5 mins with 0.1 percent $HgCl_2$ and consequently washed numerous times with distilled water to equivocate fungus infection. The washed seeds were kept in the petri dishes holding wet filter paper

and incubated for a period of three days in the dark. The germinated seeds were used for the development of mycorrhizal soil.

Collection of Soil for Experiment: The experimental soil used for the development of mycorrhizal soil was collected from the bank of Sabarmati River, Gandhinagar, Gujarat from a depth of 0-15 cm. The soil was then dehydrated and screened using 2 mm sieve. The soil was mixed strongly for 20 mins in deionized water and physicochemical parameters viz. pH, EC, moisture, organic carbon, water holding capacity, organic matter, available phosphorus, potassium, nitrate, nitrite, exchangeable ammonia were analyzed using standard procedures.

Experimental Design: A greenhouse experiment was carried out for the development of mycorrhizal soil using pot culture technique under the controlled environmental condition with the help of inoculum (VAM) in the form of a combination of soil, spores, root fragments, acquired from Department of Microbiology, Indian Agricultural Research Institute, New Delhi.The greenhouse experiment was performed in 3 kg capability pots perforated at the base. The mixture of collected soil, sand and starter mycorrhizal inoculum (3:1:1) used for the experiment to offer uniformity and permeability to the soil. After that twenty-five, external sanitized sorghum seeds were sown in each pot and their progression was observed for two and a half months. The Hogland solution was provided to the plants to keep free from phosphorous. Around 10 ml of Hogland solution was delivered per pot. The pots were kept in a greenhouse with natural sunlight at temperatures of 27-28°C during the day and 24-26°C during the night. The growth of mycorrhiza (mycorrhizal properties) was dignified for physicochemical changes happening in soil by collecting the rhizospheric soil and roots at the regular intervals of 15 days for the entire period of 75 days. The soil was dehydrated at 105°C for water holding capacity determination. The spore count (per 100 gm. soil) and % colonization by mycorrhizal fungi was also carried out. Sorghum being a fibrous root grass found to grow well for the period of $2^{1/2}$ months in greenhouse condition and delivered an enormous network of fine roots.

Soil Sampling and Analysis: The soil samples from the set up were collected at a regular interval of 15 days in order to determine soil physicochemical properties. The samples were collected in triplicate and preserved in the plastic bags, labelled and analyzed for following parameters: the estimation of pH was (1:2.5 w/v) by digitalized pH meter (Woermann 1973), Electrical conductivity was calculated (1:2.5 w/v) by conductivity meter. Organic carbon and Organic matter were also calculated (Osuji and Adesiyan 2005) with particular reference to total-organic-carbon (TOC), Available Phosphorus was measured (Kovar et al. 2009). Soil moisture, water holding capacity, nitrate, and potassium were also analyzed as per the standard methods.

Description of Bacterial and Fungal Population: The classification for the microbial population started within 5-6 hours of soil collection using serial dilution technique or plate count method (Yee et al. 1998). 1gm of collected soil samples was mixed with 10 mL of sterile water. Once the dilution is completed, the sample was then supplied onto petri plates having an agar growth medium with added nutrients. Afterward, an aliquot of 0.1 mL of dilutions was spread onto agar plates from the suitable dilution tubes and incubated at 37°C. The microbial population was calculated after twenty-four hours. The fungus population was calculated after 48 to 72 hours (Kumar and Fulekar 2018).

$$\text{CFU/ml} = \frac{no\ of\ colonies\ \times dilution\ factor}{Volume\ of\ culture\ plate}$$

Root Colonization: 1 gm. of root segments were cautiously rinsed with distilled water and divided into 1cm long sections. The fragments of root were cleaned with 10 percent potassium hydroxide (KOH) at 90°C for 15 to 30 min and rinsed with distilled water, before acidification through 2 percent Hydrochloric acid (HCl) for 10 min. the roots were stained with 0.01 percent acidic magenta dye (Kormanik et al. 1980).

Statistical Analysis: The physicochemical characterizations of every sample were calculated in triplicates and the obtained values were described as Mean±SD. Correlation factor was determined to ascertain the affiliation between physicochemical properties.

RESULTS AND DISCUSSION

Characterization of Soil: The soil was obtained from Sabarmati river basin for experimental purpose and analyzed for physicochemical analysis. The collected soil was sandy loamy in nature. The pH was found with an average value of 7.83±0.08. Electrical conductivity was found with an average value of 0.36±0.03mS/cm. Soil moisture content with an average value of 12.09±1.08%. The mean water holding capacity of the soil was found 59.25±1.15%. Organic carbon with an average value of 0.36±0.02% and the mean value of organic matter was 0.61±0.03%. Available phosphorus was found with an average value of 0.97±0.04mg/kg Potassium was found with an average concentration of 19.20±0.28mg/kg. The calcium was very high in the collected soil with an average value of 11817±2.65ppm and magnesium was found with an average value of 1270.17±2.75ppm. Nitrate was found with a mean value of 12.62±0.37ppm. Table 1 is

Table 1. Physicochemical Properties of Collected Soil (Sandy loamy)

Physicochemical Properties	Mean±SD
pH	7.83±0.08
EC (mS/cm)	0.36±0.03
Soil moisture (%)	12.09±1.08
Water Holding Capacity (%)	59.25±1.15
Organic Carbon (%)	0.36±0.02
Organic Matter (%)	0.61±0.03
Available Phosphorus (mg/kg)	0.97±0.04
Potassium (mg/kg)	19.20±0.28
Sulphate (mg/kg)	5.08±0.15
Calcium (ppm)	11817.00±2.65
Magnesium (ppm)	1270.17±2.75
Nitrates (ppm)	12.62±0.37
VAM colonization (%)	Nil
Spore count (per 100gm soil)	Nil

showing the physicochemical characterization of soil collected for experimental purpose.

Physicochemical Categorization of Mycorrhizal Inoculum: The mycorrhizal inoculum was acquired from the Department of Microbiology, Indian Agricultural Research Institute, New Delhi and characterized for different parameters (Table 2). The pH was found with an average value of 8.23±0.09. Electrical conductivity was found with an average value of 1.34±0.10mS/cm. Soil moisture content with an average value of 31.20±0.93%. Water holding capacity of the soil was found 41.33±1 .26%. Organic carbon with an average value of 0.61± 0.02%. Organic matter was found 1.05±0.03%. Available phosphorus was found with an average value of 1.24±0.04mg/kg. Potassium was found with an average concentration of 20.37±0.14mg/kg. Nitrate, Nitrite and Exchangeable ammonia were found with an average value of 7.41±0.14mg/kg and 1.91±0.03mg/kg and 35.65±0.30mg/kg respectively.

Table 2. Characterization of Mycorrhizal Inoculum

Physicochemical parameters	Mean±SD
pH	8.23±0.09
EC (mS/cm)	1.34±0.10
Soil moisture (%)	31.20±0.93
Water Holding Capacity (%)	41.33±1.26
Organic Carbon (%)	0.61±0.02
Organic Matter (%)	1.05±0.03
Available Phosphorus (mg/kg)	1.24±0.04
Potassium (mg/kg)	20.37±0.14
Nitrate (mg/kg)	7.41±0.14
Nitrite (mg/kg)	1.91±0.03
Exchangeable Ammonia (mg/kg)	35.65±0.30
VAM colonization (%)	76
Spore count (per 100gm soil)	450

Mycorrhizal soil - Development and Characterization: Mycorrhizal soil has been developed and classified for different physicochemical properties at regular interval of 15 days (Table 3). The pots were kept in the greenhouse for the development of mycorrhizal soil (Figure 7).

Correlation Matrix: It is determined from the Table 4 that significant positive association between the pairs of some physicochemical properties of rhizospheric soil followed as-soil moisture with water holding capacity

Table 3. Variations in different properties of rhizospheric soil during mycorrhizal development

Physicochemical parameters	15 Days Mean±SD	30 Days Mean±SD	45 Days Mean±SD	60 Days Mean±SD	75 Days Mean±SD
pH	8.21±0.02	8.40±0.02	8.26±0.06	8.22±0.07	8.10±0.07
EC (mS/cm)	1.89±0.02	1.80±0.01	2.53±0.04	3.01±0.09	2.40±0.22
Soil moisture (%)	31.67±0.45	41.50±0.60	43.81±0.25	45.92±0.33	46.88±1.49
Water Holding Capacity (%)	34.87±0.35	40.50±1.00	42.46±0.54	40.70±0.44	41.63±0.38
Organic Carbon (%)	0.39±0.02	0.35±0.02	0.55±0.02	0.46±0.02	0.58±0.07
Organic Matter (%)	0.67±0.03	0.59±0.04	0.94±0.04	0.78±0.03	0.87±0.02
Available Phosphorus (mg/kg)	0.95±0.02	0.88±0.09	0.95±0.02	0.97±0.05	1.38±0.47
Potassium (mg/kg)	8.79±0.04	7.48±0.07	10.83±0.14	9.98±0.14	11.11±0.76
Nitrate (mg/kg)	14.43±0.46	9.40±0.16	11.66±0.41	13.42±0.39	14.63±0.51
Nitrite (mg/kg)	8.58±0.30	6.04±0.06	7.84±0.25	9.36±0.45	10.65±0.39
Exchangeable Ammonia (mg/kg)	12.33±0.34	11.20±0.32	15.56±0.36	16.80±0.22	17.55±0.33
VAM colonization (%)	14	29	34	59	76
Spore count (per 100gm soil)	75	190	260	380	560

Table 4. Pearson's correlation coefficient (r) among physicochemical factors of rhizospheric soil during mycorrhizal development

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	1												
B	-0.41	1											
C	-0.17	0.68	1										
D	0.05	0.52	**0.93**	1									
E	-0.70	0.58	0.61	0.59	1								
F	-0.57	0.66	0.56	0.59	**0.95**	1							
G	-0.81	0.21	0.46	0.28	0.72	0.48	1						
H	-0.77	0.70	0.56	0.49	**0.97**	**0.96**	0.65	1					
I	-0.94	0.32	-0.11	-0.36	0.45	0.35	0.61	0.57	1				
J	-0.97	0.55	0.31	0.04	0.68	0.55	0.81	0.76	**0.91**	1			
K	-0.74	0.85	0.73	0.56	0.87	0.82	0.68	**0.92**	0.56	0.82	1		
L	-0.59	0.64	0.85	0.62	0.67	0.51	0.79	0.66	0.37	0.72	**0.86**	1	
M	-0.60	0.60	0.86	0.67	0.74	0.58	0.83	0.71	0.35	0.71	**0.86**	0.99	1

A - pH, B - Electrical conductivity, C - Soil moisture , D - Water holding capacity, E - Organic carbon, F - Organic matter, G - Available phosphorus, H - Potassium, I - Nitrate, J - Nitrite, K - Exchangeable Ammonia, L - VAM colonization, M - Spore count.

(r = 0.93), organic carbon with organic matter (r = 0.95), with potassium (r = 0.97), organic matter with potassium (r = 0.96), potassium with exchangeable ammonia (r = 0.92), nitrate with nitrite (r = 0.91), and VAM colonization is highly positively correlated with spore count (r = 0.99).

Morphological Characteristics of Plant: The morphological properties of the Sorghum plant were also evalu-

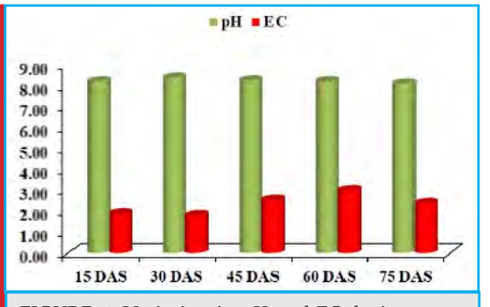

FIGURE 1. Variation in pH and EC during mycorrhizal development

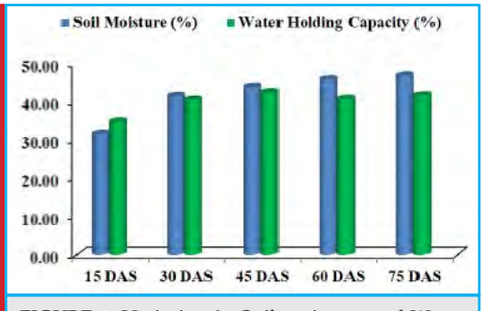

FIGURE 2. Variation in Soil moisture and Water holding capacity during mycorrhizal development

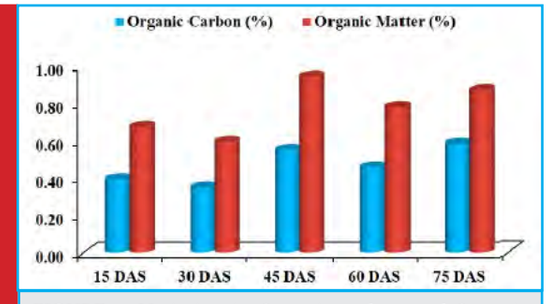

FIGURE 3. Variation in Organic carbon and Organic matter during mycorrhizal development

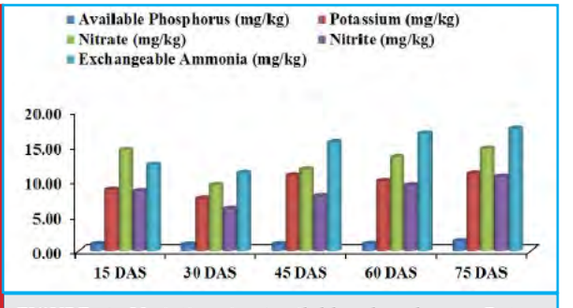

FIGURE 4. Variation in Available phosphorus, Potassium, Nitrate, Nitrite, and Exchangable ammonia during mycorrhizal Development

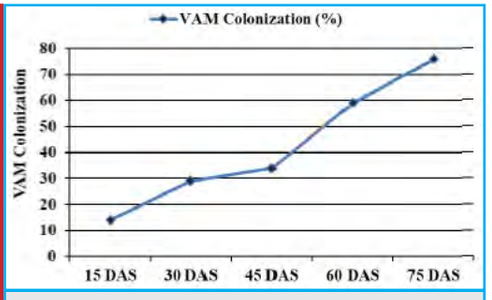

FIGURE 5. VAM Colonization in the soil during mycorrhizal development

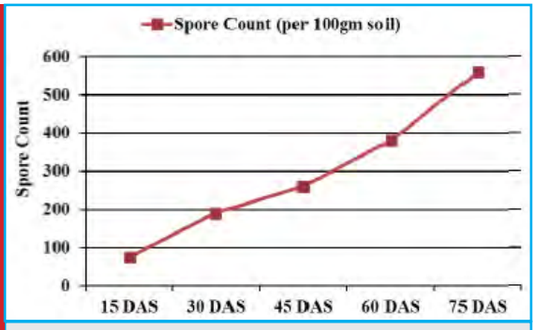

FIGURE 6. Spore Formation in the soil during mycorrhizal development

ated; root length and shoot length were measured at every 15 days during the development of mycorrhizal soil (Table 5).

Estimation of Plant's Biomass: The total biomass of the plant was evaluated by the addition of root dry weight and shoot dry weight (Table 6).

Microbial Status: Mycorrhiza is well-known to collaborate and regulate the bacterial populations and their richness in the soil (Pilon-Smits 2005). Thus the present research work includes the assessment of biotic factors along with physicochemical properties of the soil. The microbial population was evaluated at five intervals (Table 7).

FIGURE 7. Development of mycorrhizal soil in pot culture in Green House using Sorghum Species

Table 5. Characteristics (Root Length and Shoot Length) of Plant

Days	Root Length (cm) Mean±SD	Shoot Length (cm) Mean±SD
15 DAS	3.20 ± 0.24	8.92 ± 0.96
30 DAS	4.80 ± 0.22	17.37 ± 0.66
45 DAS	5.77 ± 0.31	25 ± 0.41
60 DAS	9.20 ± 1.75	30.50 ± 1.08
75 DAS	12.50 ± 0.82	37.97 ± 1.23

The current study mainly focuses on mycorrhizal soil development using *Sorghum bicolor* under the controlled condition of the greenhouse. The physical and chemical properties of soil were also analyzed throughout the progression of mycorrhiza at every 15 days and the results revealed that developed mycorrhizal soil is having enhanced pH, organic carbon and other properties than in the soil collected for the experiment and found to be increasing (Table 3). The pH of the soil remained in the range of 8.2 to 8.1 during the experiment of total two and a half months, while the EC was increasing throughout the experiment (Table 3). The colonization of mycorrhiza is encouraged in well-aired soil so the moisture of the soil was increasing from 31.67% to 46.88%. The soil collected for the experiment was detected to be low in organic carbon 0.36%, which increased during the experiment and ranged from 0.39% to 0.58%. Phosphorus was also observed increasing from 0.95% to 1.38%. It is a critical soil nutrient that AM fungi acquire and transfer to host plants (Cobb et al. 2018). Nitrate and nitrite content did not vary significantly. Increase in carbon during the study as may be contributing to the enhanced progress and fitness of the plants. Vesicular-arbuscular mycorrhiza is sensitive to the moisture of the soil and optimum moisture is essential for vesicular-arbuscular mycorrhiza sporulation (Redhead 1975). The response of vesicular-arbuscular mycorrhiza fungi to soil pH might reliant on the strains and species unifying the native vesicular-arbuscular mycorrhiza flora (Robson 1989). The early research studies reported that Glomus species needs alkaline to neutral soil for their predominance (Mosse et al. 1981). The role of nitrogen is

Table: 6 Dry Biomass (Root, Shoot and Total Biomass) of the Plant

Days	Root Dry Weight (gm.)	Shoot Dry Weight (gm.)	Total Plant Biomass (gm.)
15 DAS	0.025 ± 0.0029	0.884 ± 0.0006	0.909 ± 0.0008
30 DAS	0.054 ± 0.0016	1.184 ± 0.0095	1.238 ± 0.0095
45 DAS	0.097 ± 0.0009	1.943 ± 0.0113	2.040 ± 0.0113
60 DAS	0.1117± 0.0090	2.56 ± 0.0370	2.657 ± 0.0923
75 DAS	0.226 ± 0.0045	3.15 ± 0.0364	3.379 ± 0.0392

Table 7. Microbial Counts during the Development of Mycorrhizal Soil

Microorganism	15 Days	30 Days	45 Days	60 Days	75 Days
Bacterial Count	2.9 X 10^6	3.7 X 10^6	4.9 X 10^6	5.6 X 10^6	6.8 X 10^6
Fungal count	1.6 X 10^4	1.9 X 10^4	2.1 X 10^4	2.6 X 10^4	2.8 X 10^4

very important in inducing the formation of mycorrhiza and functions through stimulating or suppressing the colonization of root and production of spores by arbuscular mycorrhiza fungi (Sylvia and Neal 1990).

Mycorrhizal classification carried out by assessing the colonization of vesicular-arbuscular mycorrhiza (Fig 5) and counting of spore formation at every 15 days (Fig 6). The colonization of host plant's roots was flourished by the 15th day. The establishment of the roots by vesicular-arbuscular mycorrhiza heightened from 14% to 76% from 15th day to 75th day. The colonization was clearly found to be more in the last week and amplified by the end of the experiment. Mickan *et al.*, (2016) reported similar results of amplified colonization for subterranean clover (*Trifolium subterraneum*) grown in water-limited agricultural soils. The most significant period of the existence of vesicular-arbuscular mycorrhiza is spores formation. Its morphological attributes help distinguishing proof and order of VAM into discrete genera (Quilambo 2003).

The findings of the current study have clearly showed that the spore formation was expanded with time (Fig 6). It is clear from our findings that the colonization of root indicated a positive relationship with the number of spores formed in mycorrhizal soil and expected that proliferation in root settlement caused a rise in spore formation throughout the mycorrhiza development. Successive spore formation and colonization of roots may be dependent on the edaphic factors, host plant and microbial population (Fulekar et al. 2017; Khalil et al. 1992).

Conversely, certain investigators described that no substantial association between the density of spores and colonization of VAM (Li et al. 2007). Usually, the spore accessibility affects the colonization of AMF (Muthukumar et al. 2003). The relationships between the mycorrhiza and a bacterial associate may affect other members of the mycorrhizosphere community.

The presence of a significant positive correlation between spore density and root colonization can be ascribed that greater spore density is associated with the rate and magnitude of mycorrhizal development (Table 4). The current research work is in agreement with the earlier findings of Wu et al (2006). The insignificant correlation was noted between spore density and other soil properties like pH, electrical conductivity, organic carbon indicating that these factors had no effect or may poorly reflect spore population density (Hindumathi and Reddy 2011). Various studies have assessed the effect of organic matter on arbuscular mycorrhizae (Gaur and Adholeya 2002; Gryndler et al. 2002) with different results indicating their variable responses on plants and fungi. However, root colonization was significantly negatively correlated with soil pH. The present study suggests that the colonization of roots is prejudiced by soil edaphic features like nutrient status, pH, electrical conductivity, organic carbon and host cultivar vulnerability. Mycorrhizal dependency is a constitutive property of plant species. Rapid colonization of roots during the early stages of the host plant is an essential requirement for good host plant response to mycorrhiza (Hindumathi and Reddy 2011).

Viable plate counts: Microbial population and biochemical processes linked with roots may be affected by arbuscular mycorrhiza fungi colonized roots. Microbial population in the soil can be modified by interacting mycorrhizae, so the bacterial and fungi population was evaluated throughout the mycorrhizal soil inoculum growth. Bacterial CFU enlarged along the period and found 2.9 X 10^6 at 15th day, 3.7 X 10^6 at 30th day, 4.9 X 10^6 at 45th day, 5.6 X 10^6 at 60th day and 6.8 X 10^6 at the 75th day (Table 7). The bacterial species found during the development of mycorrhizal soil are *Streptococcus spp., Bacillus spp., Pseudomonas spp., Alcaligenes spp.* (Table 8). The fungal population was observed with the marginal proliferation 1.6 X 10^4 at 15th day, 1.9 X10^4 at 30th day, 2.1 X 10^4 at 45th day, 2.6 X 10^4 at 60th day and 2.8 X 10^4 at the 75th day (Table 7). The recognized fungal species were Aspergillus niger, Rhizopus spp., Aspergillus flavus Mucor spp. (Table 8).

Role of AMF for Rhizosphere Bioremediation of Heavy Metal Contaminated Soils: During the last decades, the potential of plants has been explored to reduce the heavy metal pollution in soils and AM fungi might possibly play a vital role in such approaches (Chen et al. 2018). Several laboratory studies have been carried out

Table 8. Microbial Species Obtained during Mycorrhizal Soil Development

Bacterial Genera	Fungal Genera
Streptococcus spp.	Aspergillus niger
Bacillus spp.	Rhizopus spp.
Pseudomonas spp.	Aspergillus flavus
Alcaligenes spp.	Mucor spp.

to explore the potential of AM in bioremediation of the soil, however, only few field studies have addressed the applicability of this approach to large scale conditions (Chibuike 2013).

Microorganisms present in the soil play a very important role in the mobilization of metal ions, thus altering their accessibility to plants. Vesicular-arbuscular Mycorrhizal Fungi are usually present in soil and form a significant functional factor of the soil-plant system including anxious soils. Arbuscular Mycorrhizal Fungi might be exaggerated by metal toxicity, but it is revealed that plants developing in soils polluted with metals are populated by Arbuscular Mycorrhizal Fungi (Mathur et al. 2007). Many reports concerning this have quantified spores and estimated root colonization *in situ*. Others have gone further and described metal tolerant AMF in heavy metal polluted soils (Weissenhorn and Leyval 1995). The mycorrhizal fungi can also affect plant tolerance to heavy metals by altering the antioxidant enzyme activities. Azcón et al. (2010) studied the effects of autochthonous microbes on the antioxidant activities of plants developing in a heavy metal contaminated soil. They found that AMF inoculation expressively improved catalase, ascorbate peroxidase, or glutathione reductase activities and helped plants to limit oxidative damage to biomolecules in response to metal stress (Rajkumar et al. 2012). These fungi have the capability of metal chelation, which is provided by organic constituents occur at the edge of the plasma membrane and the separation of poisonous components in vesicles and spores, functioning as a tool that delivers plant lenience to heavy metal (Cornejo et al. 2013).

The rhizoremediation process exploits on the distinctive potentials of flora and the possibilities for transformation of contaminants in the rhizosphere. Hence, scientists are aiming at the rhizospheric zone as of upgraded degradation and plant-microbe communications (Korade and Fulekar 2009; Olson et al. 2003). The utilization of Vesicular-arbuscular mycorrhizal fungi as a clean and cost-effective management strategy may help in the better growth of plants and also in the removal of heavy metal polluted soil.

CONCLUSION

The developed mycorrhizal soil in the current study was further efficiently utilized in greenhouse experiment conducted for the rhizosphere bioremediation of heavy metals. Therefore inoculum was found to have an advantageous relationship of microscopic organisms with root zone in the rhizosphere; consequently subsidizing essentially to the foundation of compelling rhizosphere that can deliver the environment for the existence and development of microbes just as an increment in natural carbon which additional supports to improve deprivation of pollutants in the soil. Arbuscular mycorrhizal fungi endorse many features of plant life, in specific better nutrition, enhanced growth, stress tolerance, and disease resistance. The results also concluded that mycorrhizosphere relations can enhance the growth and health of plants and soil quality. By limited efforts, a fruitful rhizoremediation system could be more useful to many other clean-up technologies.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Role of Teachers' Attitude and Beliefs regarding use of ICT in Indian Classrooms

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ABSTRACT

The aim of this research is to understand the relationship between attitude and beliefs of Indian school teachers towards ICT and ICT usages in the Indian classroom. Sample consists of one hundred and twenty school teachers working in secondary schools of North India. Survey method was used to collect quantitative data. Findings were supported by semi-structured interviews with the purpose of having a deep understanding of major beliefs and motivations of teachers in use of technology. The results revealed that attitude of Indian teachers towards the use of ICT is positive but the use of ICT in Indian classrooms is not sufficient. The major concerns and problems identified by this study in the use of ICT tools by teachers include limited modern and technological infrastructure, rigid time table and fixed curriculum, low technical support, lack of effective training, rigid curriculum and time table, lack of modern methods of evaluation, diploma oriented education and less competencies and motivation on part of teachers in use of ICT. Further, this research suggested that there are no gender differences in the use of ICT by teachers. The study points out the requirement of development of new ways of teacher training which can facilitate and encourage use of ICT effectively in Indian classrooms.

KEY WORDS: TEACHERS' ATTITUDE, TEACHERS' BELIEFS, USE OF ICT, INDIAN CLASSROOMS

INTRODUCTION

Information technologies provide the tools for creating, collecting, storing, using knowledge and for communication and collaboration (Kozma, 2003). Recent researcher also reported that teachers appreciate the role of ICT in classrooms but they are continuously find obstacles in using these technologies into teaching learning process (Balanskat et al, 2006). Today, the importance of education and training in ICT for citizens with the necessary skills to access information and participate in transactions through these technologies is recognised by many

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countries (Kozma, 2008). Research conducted in countries with different culture reports that although there is increase in the availability of ICT tools in schools, there is an indication that teachers are not using ICT as expected (Aldunate and Mehlenbacher, 2010; NESTA, 2012; Nussbau, 2013).

Every country strives to make ICT as integral part of the curriculum but access to technology is not sufficient enough to ensure its efficient use. Despite the international context wherein the importance of ICT-related literacies is universally acknowledged and widely regarded as increasing (Blurton, 1999; Kozma, 2003), there is considerable variation among (and even within) countries with regard to explicit ICT curricula, resources, and teaching approaches, (Kozma, 2008; OECD, 2005; Sturman, and Sizmur, 2011). In addition to problems arising from the variety of approaches in which ICT curricula are conceptualized and delivered, there also arise queries about the nature of the role that teachers, schools and education systems play in supporting the development of ICT related education. Donnelly, (2010) reported that efficient use of ICT in class is a complicated process which needs institutional support and time (Baron and Harrari, 2005). As the incorporation of ICT in teaching learning situations is inevitable, beliefs and attitudes of teachers towards the efficient use of ICT is a central condition for its successful implementation (Ertmer, 2005; Eickelmann, 2011).

India is a emerging country with a big population and since its independence in 1947, education for all people has become the mission of different governments in India.In 2000 India was witnessed a significant development in the field of use of ICT and since then tremendous progress has been made in the field of education with regard to use of ICT tools. The Ministry of Education in India has view that use of ICT in schools would make education more interesting, scientific effective and understandable. During the past 15 years Government of India has spent good amount of money to integrate use of ICT in educational institutions. Various schemes were proposed by Indian government to ensure ICT facilities in all government supported educational institutions with priority to institutions in backward areas and institutions with underprivileged section of the country (MHRD, 2016). India also invested a large amount of money to give opportunities to the teachers to improve their knowledge and skills related to use of ICT tools in the classrooms (IT for Change, 2018).

It is recommended by the National ICT policy that the ICT implementation in school education use free and open technologies, including FOSS (Free and Open Source Software) and OER (Open Educational Resources). Although there is expansion in use of ICT in Indian schools, the research investigating about the level of use of ICT in educational institution is relatively small. Several authors have emphasised on the possible benefits of information and communication technologies (ICT) for improving the quality of education in various countries. ICT is considered as a important tool for building knowledge societies (UNESCO, 2003) and, especially, as a tool in the school education which could help in reconstructing the educational processes and system leading to effective education for all people.A large number of researches emphasised on the need for use of ICT in teaching learning process. Murphy(1995) summarises that problem solving, social growth, independent work, peer teaching, and exploration are the learning outcomes that result from the use of technology in classroom. A research reported that computer-based instructions help people learn more in less time than traditional classroom teaching (Chaudhari, 2015).

Arthy and Gowrishankar, (2015) also concluded that technology can be used as good teaching-aids for example radio and television which not only make the teaching and learning process interesting but will also ensures more learning retention. In recent years various researches focused on the effect of the use of computers in teaching and learning processes (Kirkpatrick and Cuban, 1998; Blok,et al, 2002). Cope and Ward, (2002); Windschitl and Sahl, (2002) also concluded that perception, attitudes, opinions, and assessment of teachers' assumptions are the advantages of the use of ICT in education. Davis, Preston and Sahin, (2009) also reported that preservice teachers' education help them to integrate use of technology in teaching and learning. So teachers are considered as key agent in the effective integration of technology in teaching and learning (Zhao, Tan & Mishra 2001; Teo 2011a).

A good number of researches have been conducted on the importance of teachers' attitude towards the use of ICT and innovations in the education. Many researches show that teachers have positive attitudes about use of ICT (Cure and Ozdener, 2008; Foley & Ojeda, 2008; Karagiorgim & Charalambous, 2006). An early age was found to be a relevant factor for teachers with a positive attitude towards use of ICT (Shaunessy, 2007; Aduwa, 2008) as those in early age have relatively more teaching exposure and experience with ICT and thus feel more engaged and are more comfortable in using it as compared to their older counterparts (Hammond et al., 2008a). Thus, the importance of integrating ICT in teaching and teachers' competence in using technology usually results from formation of a new generation, the 'Net Generation' that is 'the digital natives', referring to young people born between 1982 and 1994 who grew up immersed in technology (Tapscott, 1998; Prensky, 2001a, 2001b; Oblinger & Oblinger, 2005).

Latest researches has been continuously focused on teachers' attitude towards use of ICT. Zhao, Tan and Mishra (2001) asserted that evidence suggests that of teachers' attitudes to be directly associated with use of computers in teaching learning situations. Student progress of learning with ICT will largely depends on teachers' attitudes, and weather they are willing to use technology in teaching (Teo, 2006). Appreciating teachers' attitudes towards use of ICT may result in an understanding of integration of technology and accepting and using technology in classroom. Researches that used data from a survey of 776 information and knowledge workers from a university of U.S., found that participants with negative computer attitudes had less skillss in computer use and were less likely to accept and adapt technology than those who have positive attitudes (Harrison and Rainer, 1992).

Teachers' beliefs and attitudes are fundamentals to successful implementation and using ICT in schools (Badia et al., 2013; Erdogan, 2011; Ertmer, 2005; Kubiatko, 2013; Kusano et al., 2013; Oye et al. 2014; Petko, 2012). A belief is the subjective knowledge of an individual that he considers true and important in context to a specific subject' and as connected an individual's past history, personal values and emotions (Petko, 2012). An attitude can be defined as a complex, multi-dimensional construct comprised to cognitive, affective, and conative components' (Zhang and Aikman, 2007) or as an individual's negative or positive feelings (evaluative affect) about attaining the target behavior' (Fishbein and Ajzen, 1975). Teacher's attitude and beliefs would therefore seem to be crucial with regard to innovations in schools, especially those that combine pedagogies and technology.

Many studies (Atkins & Vasu, 2000; Gbomita, 1997; Moore & Benbasat, 1991; Roblyer & Knezek, 2003; Sugar, Fine and Crawley, 2004) found that teacher's attitude or belief is an important human factor with a significant impact on computer adoption and implementation of technology in classroom. Bullock (2004) also pointed that, attitude of teachers is an important enabling/disabling factor in adoption of technology. Teachers' attitude towards use of computer is the main determinant for computer use in the classroom in future (Myers & Halpin, 2002). A research conducted on pre-service teachers found that there was a significant relationship between use and attitude towards computers (Khine, 2001). The finding were supported by Yuen and Ma (2001) who conducted a research on secondary teachers and found that ICT use in instruction lead to general usefulness, affective attitudes, behavioural control, and pedagogical use are significant in determining the use of ICT. Kumar and Kumar (2003) reported that most of the teachers had a belief that experience in use of ICT positively affects attitudes towards computers. Jackson et al (2001) also revealed that as compared to males, female users hold more negative reactions towards computers and these differences may be a result of usinG computers in different ways.Research also shows that successful use of technology depends on attitudes of teachers in educational settings (Baylor and Ritchie, 2002; Albirini, 2006). Therefore, attitudes towards computers may play a crucial role in accepting and in the actual use of computers.Thus successful utilisation of technology in teaching learning process largely depends on teachers' attitudes towards ICT tools (Kluever, et al., 1994).

The above literature review maps the complex relationships among teachers' beliefs and attitude towards use of ICT. The reviewed studies do not exhibit the Indian context as most of the studies have been conducted on English, Europian and Chinese populations. Use of ICT in education is a growing concept in India and there is a need to study the attitude and beliefs of teachers towards use of ICT in the Indian settings. This study aims to investigate the attitudes and beliefs of secondary school teachers of India. The research questions of this study are as follows

MATERIAL AND METHODS

Research Questions: To study the attitudes and beliefs of Indian school teachers towards use of ICT in teaching and learning situations. To find the gender differences in ICT use. To find out the challenges faced by Indian school teachers in using ICT.

Sample: Sample consist of 150 teachers selected from government and private schools of North India. India has different kinds and levels of schools with reference to use of ICT which can be categorised into four types:

Level A schools: In this type of schools ICT is a distinct feature of their curricular activities. These schools have very good technological infrastructure. The academic plan of these schools is integrated with appropriate use of ICT in academic activities.

Level B schools: This category of schools have only one or two classrooms well-equipped with technology. In these schools there are also some computers in the regular classrooms for use of students and teachers during lessons. The use of ICT is partially included in the academic activities.

Level C schools: In these type of schools there is a well-equipped computer classroom but its use is not compulsory for all teachers. The use of ICT is not included in the academic plan.

Level D schools: The schools with very limited use of ICT in educational tasks. Infrastructure is limited to a computer without network. There no interest and motivation among the teachers for use of ICT.

In India most of government schools fall under level c and d but most good private schools come under level A and B. Teachers selected as sample ranging between 28 and 50 years of age and teaching experience ranges from 5 to 20 years. In this research teachers who are working at level A and level B schools were selected for this research.

Data Collection

A mixed method approach was used to collect the data obtained from the participants. A questionnaire was framed which is designed specifically to address research objectives with regard to teachers' attitude and beliefs towards use of ICT tools inschools in India. The questionnaire was divided into five sections comprising 80 items: personal data (5 items), use of ICT in teaching practice (20 items), attitude towards ICT (25 items), training experience and training needs (20 items), and school equipment (10 items). The questionnaire was based on a five-point Likert scale: 5 = always, 4 = often, 3 = sometimes, 2 = rarely and 1 = never. Quantitative data was supported by open ended semi structured interviews of 15 teachers, teachers were asked to reflect on their major motivational beliefs and attitude towards ICT. All permissions were requested and participants were assured of anonymity. It was guaranteed to the respondents that all information was only used for purpose of research and for statistical treatment. There was no conflict of interests as school teachers' participation was voluntary.

Data analysis

The data collected from participants was analysed using the Statistical Packages for the Social Sciences (SPSS). The analysis includes both descriptive and inferential analysis. Descriptive statistics were used to determine the mean, standard deviation, frequency and percentage. Inferential statistics (t-test) were also used to analyze the research findings and content analysis was used for the semi-structured interviews.

Quantitative data

1- Teachers' attitude and beliefs towards ICT

Quantitative data depicted a picture of attitude of Indian school teachers towards use of ICT. It was revealed from data that most of teachers are enthusiastic and motivated to use ICT tools inside the classroom because they consider that it is useful to seek students' involvement, improve the interaction with students and increase interest and academic performance of both teachers and students.

It is clear from Table 1 that among the total 150 teachers, 16 (10.6 %) have highly unfavorable attitude, 26 (17.3 %) have unfavorable attitude, 38 (25.3 %) have neutral attitude, 52 (34.6 %) have favorable attitude and 18 (12%) have highly favorable attitude towards using ICT in classroom. It is concluded that most of the teachers have favorable attitude towards ICT use.

2- Role of Gender

In order to find out the gender difference between teachers' attitude towards using new technology, t- test was applied on scores of male and female teachers and results are given in table 2.

Table 2 shows that male and female teachers do not differ significantly in their attitudes towards use of ICT in education. These results are supported by the research conducted by (Shapka, & Ferrari, 2003) who studied the computer attitude and outcomes from computer tasks, they also did not find any gender differences. Antonietti and Giorgetti (2006) also reported no gender differences in teachers' beliefs. Further, Rahimi, and Yadollahi, (2011) also discovered no gender differences in computer anxiety or in the teaching experiences. So, it was concluded that there is no effect of gender in use of ICT in education. But there are also some previous studies which are contradictory to these findings as they reported significant differences in computer attitudes by gender (e.g. Margolis & Fisher, 2002; Markauskaite, 2006). Other studies have emphasised that the masculine image in computer use has discouraged females to use technology and this has made them more anxious and less confident (Culley, 1988). This research found no significant relationship for a gender and computer

Table 1. Levels of teachers' attitude towards using ICT

Score range	Male	Female	Total	Percentage	Level
80-139	6	10	16	10.6	Highly Unfavorable
140-199	6	20	26	17.3	Unfavorable
200-259	8	30	38	25.3	Neutral
260-319	12	40	52	34.6	Favorable
320-379	8	10	18	12	Highly Favorable

Table 2. Gender differences in their attitudes towards use of ICT

Gender	N	Mean	SD	t-value
Male	40	244.5	79.8	0.29
Female	110	240.4	66.6	

attitudes as may be in new generation of teachers male and female both have same attitude for ICT use.

Qualitative data

Fifteen interviews were conducted to complement the quantitative information. All interview transcriptions were categorized into two dimensions:

1-Attitude and beliefs for ICT use and the extent to which these teachers use ICT in educational settings.

The study points out that participants have positive attitude and beliefs for ICT use as teaching tools. Teachers believe that pupils like to learn with computers. They are also keen on ICT use in teaching and learning.

"The use of ICT in process of teaching-learning adds significance to education, by improving the teaching effectiveness. It added a new dimension to learning. After the inception of ICT in schools, students found learning in a technology-enhanced environment more stimulating and engaging than in a traditional classroom environment". (Teacher excerpt)

"ICT, motivates me towards learning. It is reliable and provides interactive learning experiences. It facilitates communication and promotes creativity." (Teacher excerpt).

"ICT backed teaching is more used between Class I-X. In terms of subjects being taught, Math, Social Science, Science, English has digitized and visual content which is used for classroom teaching. But for higher classes, mostly the content is designed by the respective subject instructors". (Teacher excerpt). The research suggests that though teachers have favourable attitude and beliefs towards ICT use in education, the use of ICT in Indian classroom is not sufficient.

The majority of private schools are well furnished with ICT infrastructure and have sufficient use of ICT in teaching but Indian government schools have relatively less ICT facilities. In government schools technology is widely used for communication with authorities, at administrative level for filling of e portals of teachers and students but is not used sufficiently in the classroom.The various purposes for which schools are making use of ICT include student attendance, enrolment, academic progress, payment of fee, salary transfer and teacher recruitment were found to be the most common activities related to school management for which schools use ICT. Some other activities cited by the schools are – preparation and sharing of transport roster, library management, vehicle tracking and procurement.

The insufficient use if ICT in teaching in government schools may be attributed to factors like hesitation or lack of time to use ICT in classroom situations. Most of the times government teachers are busy in election and other duties like completion of the target of 100 percent enrolment in schools. They are also occupied in managing various schemes of government like managing midday meal, budgeting of funds etc. and they don't get sufficient time for preparing their presentations. On the other side private schools have much better infrastructure; favorable student teacher ratio and class size than the counterparts in government schools which is favorable for sufficient use of ICT.

2- Challenges in use of ICT in classroom

Various challenges were identified by the teachers in using ICT in classrooms. These challenges are categorized as follows:

- Poor infrastructure and Less access to ICT resources- The study specifies that low access to resources, is a difficult challenge that inhibit teachers from using new technologies into classrooms. Teachers were asked about the challenges they encountered when they use ICT tools in classrooms. Most teachers felt that practical implementation was difficult mainly due to the lack of ICT resources, inadequate institutional support, absence of maintenance staff in the school to support teachers, lack of time in class to use ICT or lack of motivation on part of teachers. The basic barriers in using ICT in teaching reported by Indian teachers are poor infrastructure, hardware hazards and lack of content related software.
- Technical problems –These were found to be another major barrier for teachers. These technical barriers included failing to connect to the Internet, printers not working, buffering of websites and teachers working on old and outdated computers.
- Training Opportunities- There were not enough training opportunities for teachers for using ICT in a classroom environment. Pre service and in service training in ICT is inadequate.

Rashtriya Madhyamik Shiksha Abhiyan (RMSA) of India included the Information and Communication Technology (ICT) as its important component.This programme was launched in 2004 and revised in 2010 to give opportunities to secondary stage students to improve ICT skills and make them learn through computer aided learning process. The scheme is a main reagent to bridge the digital divide of various socio- economic and other geographical barriers amongts students. But in spite of

these schemes there are still various barriors in use of ICT in Indian classrooms.

RESULTS AND DISCUSSION

This study clearly shows that Indian school teachers have positive attitude and beliefs towards use of ICT in classrooms. This study do not support any gender differences in use of ICT as male and female teachers are at equal position in use of ICT. The research also reported that use of ICT in Indian school teaching is inadequate and insufficient. A good number of previous studies also investigated the reasons why teachers do not use computers in teaching (Winnans, and Brown, 1992; Dupagne and Krendl, 1992; Hadleyand Sheingold, 1993) and a list of barriers was found that included lack of experience with ICT; lack of on-site support for teachers using technology; problems in supervising children when using ICT ; less number of ICT specialist teachers to teach students computer skills; lack of computer availability; lack of financial support and lack of time required to effectively integrate technology into the academics. It is needed to provide adequate opportunities to enhance skills of teachers in new technologies in Indian institutions. The teachers should also be motivated to use new technologies in classrooms. Regular professional training opportunites should be given to teachers to improve their interest in teaching with ICT. Findings of the research indicate the need of proper training and motivation of teachers to use ICT and provision of good infrastructure and technical help.

ICT use is not prevelant in government schools and it has been recognised that use of ICT in all government schools will proveto be an effort to bridge the digital and social divide. Further, training opportunities should also be provided to teachers during pre-service education. Effective ICT integration in pre-service teacher training is pivotal in use of ICT in teaching. Prospective teachers should be trained to make use of ICT a regular feature of their teaching routines.This study will also offer priceless information to Indian school administration as well as to educational policy makers regarding the nature of ICT contribution to the teaching learning process. Since the attitude and perceptions of the teachers are critical in determining how effectively an innovation is implemented, it is important to gauge how teachers perceive this innovation and its efficacy as a tool for effective teaching and learning.

The findings of this research have given more consideration to the level of ICT use to improve and encourage more use of ICT in Indian schools. Study also contributes to the existing body of research regarding the use of ICT for educational purposes in emerging countries. The study endorses that future researchers should consider the in-depth qualitative studies including classroom observations and in-depth interviews to study the level of ICT use by teachers.

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Water stress induced physiological and biochemical responses of minor millets and rice at vegetative stage

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ABSTRACT

Abiotic stresses such as heat stress, water stress etc. alter plant growth, metabolism and yield. Among them, water stress is a major one as it occurs severely in major producing areas of the world.It is not only due to the deficit of water but also due to other factors such as high temperatures and severe cold that makes plants not able to absorb enough water from soil to grow well and this is called physiological water stress that leads to a series of disorders in physiological and biochemical processes.Millets are resilient to extreme environmental conditions especially to inadequate water and are rich in nutrients.The current study was undertaken to analyse the effects of water stress on Leaf proline, protein, soluble carbohydrates, chlorophyll content of Minor Millets and Rice genotypes under water stress conditions at 5.5 to 6.5 % SMC (Soil Moisture Content) for Millets and 15-18 % SMC for Rice genotypes at vegetative stage. The photosynthetic pigments (Chlorophyll a, chlorophyll b and total chlorophyll) decreased and the biochemical components (Leaf Proline, Protein, Carbohydrates) increased under water stress. Our study revealed that, among the three crops, Little millet genotype, RLM-37 and Rice genotype, R-RF-127 showed maximum increase in proline, protein and carbohydrate content when compared to control ones. This study suggested that, little millet genotype RLM-37 having water stress tolerant adaptive mechanism and perform better under water stress than Rice genotypes.

KEY WORDS: ABIOTIC STRESS, BIOCHEMICAL ANALYSIS, MINOR MILLET, PHOTOSYNTHETIC PIGMENTS

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INTRODUCTION

Abiotic stresses like water stress activates a series of physiological, morphological, molecular and biochemical changes in plants by affecting growth and productivity negatively. Plants deal with water stress by all these responses. Various morphological mechanisms functioning under water stress situation which includes, water stress escape, water stress avoidance, water stress tolerance and water stress recovery, has also been identified (Kholova et al., 2010, Monneveux et al., 2006 and Blum et al., 2005 Fang et al., 2015). Under water stress, accommodation of dehydrin like proteins was identified in the leaves and roots of water-stressed plants that lead to protect plants from further dehydration losses. The rate and levels of accumulation of proteins, amino acids and sugars may determine the ability of a genotype to withstand the level of water stress. Minor millet is known for its greater level of tolerance against water stress, salinity and diseases. On the darker side, millet have been included in the "Orphan crop" list due to lack in their trade across the world, extra efforts are required in grain processing as well as in the social stigma attached to these crops as "food for the poor". Together these negativities have failed them to seek attention of researchers at all, (Fang et al., 2015, Sharma and Khurana, 2014, Dubey et al., 2018, Sushmitha et al., 2018 Kumari et al., 2019).

Diverse abiotic stresses are the reason for the exceptional tolerance of millets including under water stress. Millets are considered as the climate change compliant crops score high when compared with other grains like Wheat and Rice in terms of high nutritional value and marginal growing conditions. Inspite of the unusual, extraordinary, exceptional nutritional qualities of small millet grains and capacities of millet farming systems, the area under small millet production has been shrinking or declining over the last five decades. Rice is a staple food crop and second largest crop in the world with high Drought Sensitivity Index (Karl, 1983) as it dies below 18 % SMC as it is mainly grown under water-logged condition. The molecular biology of minor millets has been explored to the very less extent and there is much that requires investigation, including the establishment of a genome map and sequenced genome.

MATERIAL AND METHODS

Plant material: The experimental materials of the present investigation comprised of minor millet (Little millet includes RLM-37, BL-4, MM-23, BL-8, BL-15-1, OLM-203 and MM-10, Barnyard millet includes Sawa, VL-29, Melghat-1, Melghat-3 and MM-03) and Rice genotypes (R-RF-127,Moroberekan and MTU-1010) sowing was done in pots separately and maintained in green house at 28±2 ^{o}C.Water stress was induced after 30 days of sowing at the vegetative stage under the green house conditions (Figure 1 & 2). Plants were watered normally once in a day before the stress imposition and the leaf samples were harvested when the soil moisture content in the stress pots as reached below 10% in Millets and below 20% in Rice genotypes in both control and stress condition. The harvested samples were stored immediately in liquid nitrogen at -80^{0} C.

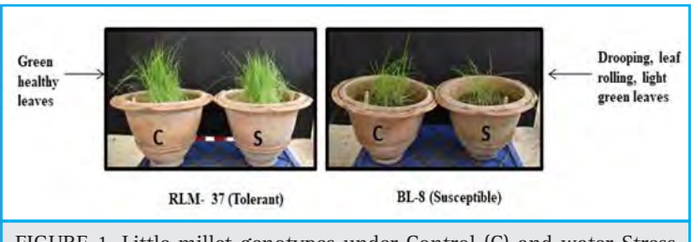

FIGURE 1. Little millet genotypes under Control (C) and water Stress (S) condition at 5.5-6.5 % SMC.

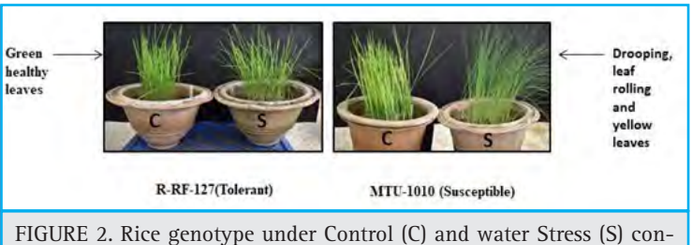

FIGURE 2. Rice genotype under Control (C) and water Stress (S) conditionat 15-18 % SMC.

Biochemical estimation was done by following methods

Leaf proline content was estimated by Acid ninhydrin method as given by Bates et al., (1973). Lowry et al. (1951) method was used for estimation of Leaf protein content, Leaf carbohydrate content was estimated by phenol sulphuric acid method proposed by Krishnaveni et al., (1984). Acetone method was used for estimation of Leaf Chlorophyll content given by Arnon (1949).

Statistical Analysis: The effect of water under stress and control condition in genotypes of Millets and Rice was analysed statistically by CRD by the application of OP-STAT, an online computerized software developed at BHU.

RESULTS AND DISCUSSION

Biochemical characterization of fifteen genotypes of Minor millets and Rice: After 6 days of water stress imposition and at 6% Soil Moisture Content (SMC) for Minor millets and after 4 days of same and at 18% SMC for Rice genotypes. The wide variation for proline, protein, carbohydrates and chlorophyll (Chl a, chl b and total chl) content was recorded in stress tissue as when compared to that of control one's for fifteen genotypes of Minor millets and Rice.

Effect of water stress on leaf Proline content among Minor millets and Rice genotypes: The leaf Proline was estimated by Acid ninhydrin method. Accumulation of proline as an osmolyte under water stress was observed in different Millet and Rice genotypes. The proline content ranged from 0.391 to 1.102 µ mole/g f.wt for stress; whereas in controlled condition it ranged from 0.270 to 0.925 µ mole/g f.wt in Little millet genotypes (Table 1). The Little millet genotype RLM-37(1.102 µ mole/g f.wt) had highest increase in proline content and OLM-203 (0.391 µmole/g f.wt) was recorded with lowest increase in proline content in stress tissue over control. Similarly the proline content ranged from 0.192 to 7.869 µ mole/ tissue under stress; whereas under control condition proline content ranged from 0.015 to 0.204 µ mole/tissue. A significant increase in proline has been observed in response to water stress, favouring osmotic adjustment. When comparing fold increase in proline content under stress when compared with control among eight genotypes BL-15-1 was recorded with (63.460) higher fold increase Sushmitha et al. (2018).

The proline content ranged from 0.146 to 0.903 µ mole/g f.wt for stress; whereas in controlled condition it ranged from 0.111 to 0.520 µmole/g f.wt(Table 1). The Rice genotype, R-RF-127 had highest increase in proline content (0.903 µ mole/g f.wt) and MTU-1010 (0.146 µ mole/g f.wt) was recorded with lowest increase in proline in stress tissue when compared to control.The mean of all the genotypes selected was 0.481µ mole/g f.wtand it ranged from 0.111 to 0.925 µ mole/g f.wtin controlled condition and was increased to 0.826µ mole/g f.wt and it ranged from 0.146 to 1.102µ mole/g f.wtin stress tissue. Among three crops, Little millet genotype, RLM-37(1.102µ mole/g f.wt) was found to have highest increase in proline and Rice genotype, MTU-1010 (0.146µ mole/g f.wt) was found to have lowest increase in proline when compared to control (Table 1). Hence according to our study RLM-37 of Little millet shown to follow tolerant genotype characteristics. There was positive correlation in proline under stress condition. It increased when plants were exposed to stress in all the three crop genotypes. In general, proline content of leaves increased with the decline in irrigation water, suggesting that the production of proline is probably a common response of millet under water stress conditions. The role of proline in adaptation and survival of plants has been well documented by Watanabe et al. (2000) and Saruhan et al. (2006).Osmotic adjustment through accumulation of cellular solutes, such as proline, has been reported as one of the possible means for overcoming osmotic stress caused by the loss of water by Caballero et al.(2005). Teixeira and Pereira (2006) indicated that proline content significantly increased in all potato organs in response to stress condition. This increment was more remarkable in roots and tubers than in the leaves. High levels of proline enable the plant to maintain low water potentials causing the accumulation of compatible osmolytes that makes additional water to be taken up from the environment by the plant, thus buffering the immediate effect of water limit within the organism (Mousa and Abdel-Aziz, 2008).

Lobato et al. (2011) revealed that there was increment in the accumulation of proline and free amino acids in soybean *(Glycine max* cv.Sambaiba) leaves under water limited condition 67 and 388.1%, respectively. On the basis of accumulation of leaf proline content under water stress condition, Little millet genotype, RLM-37 and Finger millet genotype, BR-36 was found to be maximum compared to tolerant genotype GPU 67 hence were identified as potential drought tolerant genotypes by Dubey et al. (2018).

Effect of water stress on leaf Protein content among Minor millets and Rice genotypes: The leaf Protein content was estimated by the method given by Lowryl. Ashraf and Foolad (2005) had reported that higher protein content in tolerant genotypes under water stress condition is due to higher DNA and RNA content, which enhances synthesis and inhibits protein decomposition. Sushmitha et al. (2018) reported that the wide variation for protein content was found in stress tissues for eight

Table 1. Statistical Analysis of biochemical traits (Proline, Protein and Carbohydrates) for Minor millets and Rice genotypes under control and stress condition.

Genotypes	Proline Control (µ mole/gf.wt)	Proline Stress (µ mole/gf.wt)	Protein Control (mg/g f.wt)	Protein Stress (mg/gf.wt)	Carbohydrate Control (mg/g f.wt)	Carbohydrate Stress (mg/g f.wt)
RLM-37	0.397±0.001	1.102±0.001	0.264±0.001	0.558±0.001	0.061±0.001	0.382±0.001
BL-4	0.636±0.000	0.988±0.001	0.330±0.000	0.335±0.001	0.070±0.000	0.135±0.001
MM-23	0.491±0.001	1.000±0.058	0.236±0.001	0.345±0.000	0.078±0.001	0.181±0.001
BL-8	0.925±0.001	1.022±0.001	0.262±0.000	0.314±0.000	0.198±0.001	0.169±0.001
BL-15-1	0.448±0.001	0.495±0.001	0.125±0.000	0.432±0.000	0.495±0.001	0.275±0.001
OLM-203	0.356±0.002	0.391±0.001	0.277±0.001	0.354±0.000	0.070±0.001	0.127±0.001
MM-10	0.270±0.000	0.482±0.001	0.322±0.001	0.391±0.001	0.197±0.001	0.203±0.001
SAWA	0.391±0.001	1.084±0.001	0.265±0.001	0.538±0.000	0.039±0.001	0.178±0.000
VL-29	0.471±0.001	1.000±0.000	0.330±0.001	0.362±0.000	0.091±0.001	0.150±0.001
MELGHAT-1	0.848± 0.001	0.916±0.001	0.280±0.045	0.432±0.001	0.071±0.001	0.140±0.001
MELGHAT-3	0.472± 0.001	1.022±0.000	0.262±0.001	0.319±0.001	0.073±0.000	0.151±0.000
MM-03	0.508±0.000	1.001±0.001	0.278±0.001	0.425±0.001	0.093±0.000	0.177±0.000
R-RF-127	0.378±0.001	0.903±0.001	0.407±0.001	0.538 ±0.000	0.076±0.001	0.378±0.000
Moroberekan	0.520±0.001	0.850±0.001	0.460±0.000	0.511±0.001	0.084±0.001	0.235±0.000
MTU-1010	0.111±0.001	0.146±0.001	0.269±0.001	0.278±0.001	0.082±0.001	0.092±0.000
Mean	0.481	0.826	0.291	0.408	0.118	0.198
Maximum	0.925	1.102	0.46	0.558	0.198	0.382
Minimum	0.111	0.146	0.125	0.278	0.039	0.092
CD(p=0.05)	0.002	0.043	0.034	0.003	0.001	0.002
SE(m)	0.001	0.015	0.012	0.001	0.001	0.001
SE(d)	0.001	0.021	0.016	0.001	0.001	0.001
C.V.	0.243	3.126	6.894	0.474	0.754	0.487

Little millet genotypes (BL-8, MM-23, MM-10, BL-15-1, RLM37, OLM-203, BL-4, JK-8). The protein content ranged from 0.040 to 0.586 mg/tissue under stress condition, whereas 0.027 to 0.080 mg/tissue under control condition. BL-4 (8.746) had the highest fold increase. The protein content ranged from 0.314 to 0.558 mg/g f.wt in stress condition and from 0.236 to 0.432 mg/g f.wtin control in Little millet genotypes (Table 1). The Little millet genotype RLM-37(0.558 mg/g f.wt) had the highest increase in protein content and BL-8 (0.314 mole/g f.wt) was recorded with lowest protein content in stress tissue.

The protein content ranged from 0.278 to 0.538 mg/g f.wt in stress condition and from 0.269 to 0.460 mg/g f.wt in control condition (Table 1). The Rice genotype, R-RF-127 (0.538 mg/g f.wt) had the highest protein in stress and MTU-1010 (0.278 mg/g f.wt) had the lowest protein in stress when compared to control. The mean of all the genotypes selected was 0.408mg/g f.wt and it ranged from 0.278 to 0.558mg/g f.wtin stress tissue and the mean was 0.291mg/g f.wtand it ranged from 0.125 to 0.46 mg/g f.wtin controlled condition. Among the three crops, little millet genotype, RLM-37 (0.558mg/g f.wt) was reported with highest increment in protein and tends to be a tolerant genotype and MTU-1010 (0.278mg/g f.wt) was reported with lowest increase in protein under stress and tends to be a susceptible genotype (Table 1).There was increase in protein content in almost all genotypes of the three crops under stress condition. Hence the biochemical trait protein is positively correlated with both Millets and Rice, under water stress. Little millet genotype, RLM- 37 was found to show maximum protein content compared to tolerant genotype GPU 67 by Dubey et al,(2018).

Effect of water stress on leaf Carbohydrate content among Minor millets and Rice genotypes.

Leaf carbohydrate content was estimated by phenol sulphuric acid method proposed by Krishnaveni et al., (1984). Total carbohydrates in the leaves and seeds

were determined by phenol sulphuric acid method. The observation of carbohydrate content under control and stress condition showed that the carbohydrate content increases significantly with prolongation to water stress. The carbohydrate content ranges from 0.127 to 0.382 mg/ g f.wt in stress condition whereas 0.061 to 0.495 mg/ g f.wt in controlled condition (Table 1). Water stress induced highest increase in carbohydrate content was obtained in Little millet genotype RLM-37 (0.382 mg /g f.wt) and Little millet genotype, OLM-203(0.127 mg /g f.wt) had shown the lowest increase in Carbohydrates content under stresswhen compared to control. Likewise the carbohydrate content ranges from 234.221 to 612.222 mg/tissue under stress condition whereas 153.907 to 302.313 mg/tissue in control condition. BL-15-1 (2.705) had the highest fold increase by Sushmitha et al. (2018).

The carbohydrate content ranges from 0.092 to 0.378 mg/g f.wt in stress condition where as 0.076 to 0.084 mg/g f.wt in controlled condition (Table 1). Rice genotype, R-RF-127 (0.378 mg/ g f.wt) had shown the highest increase in Carbohydrates and MTU-1010 (0.092 mg/ g f.wt) had shown the lowest increase in Carbohydrates under stress.

Water stress induced highest increase in carbohydrate content was recorded in Little millet genotype, RLM-37 (2.571 fold) followed by Finger millet genotype PR-10 14 (2.035 fold) when compared with tolerant genotype GPU-67by Dubey et al. (2018). The mean of all the genotypes selected was 0.198 mg/g f.wt and it ranged from 0.092 to 0.382 mg/g f.wtin stress tissue and the mean was 0.118 mg/g f.wt and it ranged from 0.039 to0.198 mg/g f.wtin controlled condition. Among the three crops, Little millet genotype, RLM-37(0.382mg/g f.wt) was reported with highest increase in carbohydrates content when compared to control and tends to be a tolerant genotype and MTU-1010 (0.092mg/g f.wt) was reported with lowest carbohydrates content(Table 1)and tends to follow susceptible genotype characteristics. It was found to have positive correlation with stress induction. There was increase in carbohydrates content in most of the genotypes of the three crops under stress condition. Hence the biochemical trait, carbohydrate is positively correlated with both, Millets and Rice under water stress. The accumulation of sugars in response to water stress is quite well established (Izanloo et al., 2008; Watanabe et al., 2000).

Soluble sugars may function as an osmo protectant, stabilizing cellular membranes and maintaining turgor pressure. Gene ontology attributes such as proline and soluble sugar accumulations were highly enriched in the water stress-up-regulated genes, suggesting that those metabolic pathways are important in responses to water stress. Indeed, the importance of many of these pathways to water stress tolerance has been empirically supported by transgenic experiments by Umezava et al. (2006). Hermalina et al. (2014) revealed that, total soluble sugar and Proline content in the leaves were significantly ($p<0.05$) increased due to the increment in the level of water stress. The differences in the responses to water stress among the nine selected corn cultivars suggested that each cultivar has different ability to synthesis proline and total soluble sugar with an increase in water stress treatment.

Effect of water stress on Chlorophyll content among Minor millets and Rice genotypes.

Acetone method was used for estimation of Leaf Chlorophyll content given by Arnon. Chlorophyll maintenance is essential for photosynthesis under water stress. The chlorophyll a, chlorophyll b and total chlorophyll content ranges from 0.797 to 2.633 mg/g f.wt, 0.359 to 0.701 mg/g f.wt and 1.146 to 2.418 mg/g f.wt for stressed leaf tissue where as in control condition, it ranges from 0.797 to 2.633 mg/ g f.wt, 0.483 to 1.500 mg/ g f.wt and 1.326 to 4.963 mg/ g f.wtin Little millet genotypes (Table 2). Chl a, chl b and total chl was found to have negative correlation on stress induction. There was decrease in chlorophyll a, b and total chlorophyll content in almost all the genotypes of the three crops under stress condition (Table 2). Among the Minor millet genotypes, Little millet genotype, OLM-203 had the lowest decrease in Chlorophyll a content (0.764mg/g f.wt), MM-10 in Chlorophyll b content (0.359mg/g f.wt) and OLM-203 in total chlorophyll content(1.146 mg/g f.wt) and Little millet genotype, RLM-37 had the highest decrease in Chlorophyll a content (1.359 mg/g f.wt), Chlorophyll b content(0.701 mg/g f.wt) and Total chlorophyll content (2.418 mg/g f.wt) under water stress as when compared to control.

Sushmitha et al. (2018) revealed that, a wide variation for chlorophyll content was found in stress tissues for eight Little millet genotypes (MM-23, BL-8, RLM-37, OLM-203, MM-10, BL-15-1, BL-4, JK-8). Chlorophyll a, Chlorophyll b and Total Chlorophyll content ranged from 0.783 to 2.441 mg/tissue, 0.403 to 1.332 mg/tissue, 1.330 to 3.811 mg/tissue respectively for stress leaf tissue whereas under control condition it ranged from 1.223 to 3.075 mg/tissue, 0.597 to 3.006 mg/tissue, 1.819 to 6.047 mg/tissue respectively. The genotype MM-10 had the highest fold reduction of 2.011 mg/tissue in the total chlorophyll content, where as in case of chlorophyll a, the genotype MM-23 showed highest fold reduction of 2.00 and in chlorophyll b, the genotype JK-8 showed the highest fold decrease of 3.00.

In rice genotypes, chlorophyll a, chlorophyll b and total chlorophyll content ranges from 0.400 to 0.933mg/g f.wt, 0.203 to 0.434 mg/g f.wt and 1.133 to 1.877 mg/g f.wt for stressed leaf tissue where as in

Table 2. Statistical Analysis of photosynthetic pigments (Chlorophyll a, chlorophyll b and Total chlorophyll) for Minor millets and Rice genotypes under control and stress condition.

Genotypes	Chlorophyll a Control (mg/gf.wt)	Chlorophyll a Stress (mg/gf.wt)	Chlorophyll b Control (mg/gf.wt)	Chlorophyll b Stress (mg/gf.wt)	Total Chl control (mg/gf.wt)	Total Chl Stress (mg/gf.wt)
RLM-37	2.633±0.001	1.359±0.001	1.500±0.000	0.701±0.001	4.963±0.001	2.418±0.001
BL-4	1.498±0.000	1.264±0.001	0.702±0.001	0.473±0.001	2.499±0.001	1.426±0.000
MM-23	1.338±0.001	0.951±0.000	0.568±0.001	0.554±0.001	1.893±0.001	1.519±0.001
BL-8	1.290±0.000	1.150±0.000	0.690±0.000	0.596±0.004	1.852±0.000	1.747±0.000
BL-15-1	1.076±0.001	0.980±0.006	0.656±0.000	0.461±0.001	1.946±0.000	1.530±0.006
OLM-203	0.797± 0.001	0.764±0.001	0.483± 0.001	0.382±0.000	1.326±0.000	1.146±0.001
MM-10	1.274±0.001	0.872±0.001	0.513±0.001	0.359±0.001	1.491±0.001	1.230±0.000
SAWA	2.600±0.000	1.370±0.001	1.168±0.000	0.648±0.001	4.048±0.001	2.298±0.000
VL-29	2.231±0.001	1.296±0.004	0.928±0.001	0.516±0.001	2.225±0.000	1.956±0.001
MELGHAT-1	1.578±0.000	1.287±0.001	0.811±0.001	0.629±0.001	3.254±0.001	1.854±0.000
MELGHAT-3	1.578±0.000	1.271±0.000	0.786±0.008	0.600±0.006	2.545±0.000	2.049±0.001
MM-03	1.442±0.001	0.958±0.001	0.652±0.001	0.428±0.001	2.093±0.000	1.387±0.000
R-RF-127	1.263±0.001	0.933±0.001	0.880±0.000	0.434±0.001	3.077±0.000	1.877±0.001
Moroberekan	0.500±0.000	0.441±0.001	0.567±0.001	0.343±0.004	2.027±0.000	1.296±0.001
MTU-1010	0.400±0.001	0.400±0.000	0.313±0.001	0.203±0.001	1.528±0.001	1.133±0.001
Mean	1.337	1.010	0.747	0.488	2.451	1.657
Maximum	2.633	1.359	1.500	0.701	4.963	2.418
Minimum	0.400	0.400	0.313	0.203	1.326	1.133
CD(p=0.05)	0.002	0.005	0.006	0.006	0.001	0.005
SE(m)	0.001	0.002	0.002	0.002	0.000	0.002
SE(d)	0.001	0.003	0.003	0.003	0.001	0.002
C.V.	0.063	0.319	0.473	0.737	0.035	0.164

NOTE: Each mean indicates: Mean of three independent replicates at each time. µ mole/ g f.wt : micro mole per gram fresh weight, mg/ g f.wt : milligram per gram fresh weight.

control condition it ranged from 0.400 to 1.263mg/ g f.wt, 0.313 to 0.880 mg/g f.wt and 1.528 to 3.077 mg/g f.wt respectively (Table 2). Among the Rice genotypes, Moroberekan had the lowest decrease in Chlorophyll a content (0.441 mg/ g f.wt), MTU-1010 in Chlorophyll b content (0.203 mg/g f.wt) and MTU-1010 in total chlorophyll content(1.133 mg/g f.wt)and R-RF-127(0.933 mg/g f.wt), had the highest decrease in Chlorophyll a content, Chlorophyll b content (0.434 mg/g f.wt) and Total chlorophyll content (1.877 mg/g f.wt) under water stress. Manirannan et al. (2007) found a depression in CHL a and b and TC in *Helianthus annuus L.* under water stress. The chlorophyll was decreased from well watered (control) condition to severe water stress (13 DID) and there was plant pigment increment by the age of maturity in plant by Paul et al. (2013). At the highest stressed condition with respect to its control set, CR dhan 40 showed best performance for chlorophyll content by Kumari et al. (2019).

Dubey et al. (2018) concluded that Reduced fold decrease in chlorophyll a content was recorded in finger millet genotype, GPU 67 (1.129 Fold) followed by BR 36 (1.265 fold). The mean of all the genotypes selected was 1.010 mg/g f.wt and it ranged from 0.4 to 1.359 mg/g f.wtin stress tissue and the mean was 1.337 mg/g f.wt and it ranged from 0.4 to 2.633 mg/g f.wtin controlled condition. Among the three crops, Rice genotype, MTU-1010 (0.4mg/g f.wt) was recorded with lowest reduction in chlorophyll a content and Little millet genotype, RLM-37 (1.359 mg/g f.wt) was reported with highest reduction in chlorophyll a content under water stress.

Out of seven genotypes under study, minimum decrease was reported in Little Millet genotype OLM-203 (0.994 fold) followed by finger millet genotype GPU-67 (1.254 fold) by Dubey et al., (2018). The mean of all the genotypes selected was 0.488 mg/g f.wt and it ranged from 0.203 to 0.701 mg/g f.wt in stress tissue and the mean was 0.747 mg/g f.wt and it ranged from 0.313 to 1.500 mg/g f.wt in controlled condition. Among the three crops, Rice genotype,MTU-1010 (0.203 mg/g f.wt) was recorded with lowest decrease in chlorophyll b content and Little millet genotype, RLM-37 (0.701 mg/g f.wt) was recorded with highest decrease in chlorophyll b and under stress.

Dubey et al. (2018) reported that, the minimum decrease were recorded in finger millet GPU-67 (1.173 fold) followed by PR-10 14 (1.440 fold) and BR-36 (1.709 fold). The mean of all the genotypes selected was 1.657 mg/g f.wt and it ranged from 1.133 to 2.418 mg/g f.wtin stress tissue and it increased to the mean 2.451 mg/g f.wt and it ranged from 1.326 to 4.963 mg/g f.wtin controlled condition. Among the three crops, Rice genotype, MTU-1010 (1.133mg/g f.wt) was recorded with lowest decrease and Little millet genotype, RLM-37(2.418mg/g f.wt) was recorded with highest decrease in total chlorophyll under stress (Table 2).

Among the three crops, Rice genotype, MTU-1010was recorded with lowest reduction in chlorophyll a, chlorophyll b and total chlorophyll content as already in control itself it was having less chlorophyll content so it is showing less chlorophyll content and Little millet genotype, RLM-37 was reported with highest reduction in chlorophyll content under water stress but found to be tolerant genotype by morphological, physiological and majority of aspects.

CONCLUSION

Plants in water stress time adapt themselves by making some changes in their physiological and biochemical features. Accumulations of soluble carbohydrates, proline and protein increased under water stress. Chlorophyll content are more resistant to water stress.Our current study reveals that, after 6 days of water stress imposition and at 6% Soil moisture content for Minor milletand after 4 days of same at 18% SMC for Rice genotypes.Among Minor millets and Rice genotypes, RLM-37 was found to be show maximum increase in proline, carbohydrate and protein which indicates their comparable potential for water stress tolerance. This can be taken as a base for water stress tolerance response of the crop, which may be useful for further validation studies of genes for water stress tolerance in millet and other crop plants.

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Support Vector Machine and Particle Swarm Optimization Based Classification of Ovarian Tumour

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ABSTRACT

Ovarian tumour is the most widely recognized reason for death among gynecological malignancies. There are various sorts of clinical and nonclinical highlights that are utilized to examine and break down the contrasts among kindhearted and dangerous ovarian tumors. Computer Aided Diagnosis (CAD) frameworks of high precision are being created as an underlying test for ovarian tumor order rather than biopsy, which is the present highest quality level indicative test. The system uses the K-means clustering for segmentation and the classification methodology is done by the KSVM (Kernel Support Vector Machine) and PSO (Particle Swarm Optimization) classification approach. This automatic framework consists of four steps: preprocessing, segmentation, feature extraction and feature selection, classification, finally, the parameter values of the KSVM (Kernel Support Vector Machine) classifier are dynamically optimized using the PSO (Particle Swarm Optimization) optimization algorithm. It is a bio-inspired optimization algorithm, and PSO optimizers to get the best out of the classification accuracy. An efficient ovarian tumour segmentation, feature extraction and selection by using PSO and classification is offered in this work by combining different Kernel SVM Classifier which provides accurately identify and classify the ovarian tumor in MR image.

KEY WORDS: OVARIAN TUMOR DETECTION, MAGNETIC RESONANCE IMAGING (MRI), PSO (PARTICLE SWARM OPTIMIZATION) OPTIMIZER, KSVM (KERNEL SUPPORT VECTOR MACHINE)

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INTRODUCTION

Cancer is made up of irregular cells that grow although body does not want them. When cancer cells is in the body change and grow out of control. In mostly tumours, the abnormal cells mature to form a mass. If cancer cells grows in the body long enough and also nearby areas. They extent to other parts of the body or metastasis. Ovarian tumour is very dangerous killer for women which are not specific sign indications of cancer and typically it identify in the last stage. All the cases are being diagnosed at final stage because of poor identification practices (Pathak, 2015). In the ovarian tumour mass prediction plays key role, it can be diagnosed from the ultrasound image that tumour mass is benign lesion or malignant lesion or metastatic. The MR image in medical application and other several fields is enormous. It has a number of benefits medical imaging modalities over other. There are different mode of inputs are obtainable for diagnostics like Computed Tomography, Ultrasound imaging, Magnetic Resonance Imaging, Positron Emission Tomography. From all this input methods the proposed system are focused on ultrasound imaging for the reason that it has some advantages like it is non-invasive, competency of forming real time images, accurate, portable and not hurtful to human being (Acharya 2013). Ovarian malignancy is a striking general wellbeing concern, which, disregarding its rare rate, remains the deadliest type of gynecological harm. As indicated by the WHO, every year an evaluated aggregate of two lakh and fifty thousand instances of ovarian malignant growth will be analyzed and a lakh and fifty thousand patients will capitulate to this sickness, speaking to the seventh most basic type of malignant growth and the eighth driving reason for disease related demise among ladies overall at all (Michael-Antony Lisio, 2019). So, in this proposed system have used medical images which are low cost in nature and easily accessible.

MATERIAL AND METHODS

In this proposed method, the process has taken in test part and training part as shown in Fig.1.Improvement of an efficient diagnosing the Ovarian cancer in the good time may help surgeons. MatLab has used to develop the proposed method. The input image or data to the proposed system is an MR Ovarian image. This system consists of four stages namely i) Pre-processing ii) Segmentation iii) Feature Extraction and Feature Selection using Particle swarm optimization (PSO) and vi) Kernel SVM classification (Quadratic SVM kernel, Linear SVM Kernel, Polygonal SVM Kernel, Radial basis function (RBF) SVM kernel). The input MR Ovarian image is pre-processed in both the test and training parts. Preprocessing is executed to boost the image value for further processing. For all that purpose, the first stage taken is removal of noise. Noise is removed by using adaptive median filtering method to remove the noise element and increase the image intensity. The next stage going on in preprocessing step is normalizing the background (Galdames, 2012). This has been completed by a threshold based edge detection with canny technique. The following stage is segmentation. Now, a novel K means clustering algorithm is proposed to segment the tumor (Raj Kumar, 2013).

Different label formation is acquired from the clustered output. After the labeled output, region of interest (ROI) which tumor part is segmented. Since the ovarian tumor segmented outputs, the features are taken out (Islam, 2013) by using texture extraction technique named by way of gray level co-occurrence matrix (GLCM) method. Up to now, all the procedure would be common for both the part such as training and testing part. The extracted features gray level co-occurrence matrix (GLCM) will be made as training feature matrix and keep back as feature vectors in the training part. In this proposed system shown in Figure 1, the Ovarian MRI have taken to assess the upgrading of the proposed method. The datasets are consisted of 50 Ovarian MRI images, out of which 87% of the MR ovarian images are used for training part, and the whole thing of the 50 images are used for testing part. To pick the greatest feature vector or feature

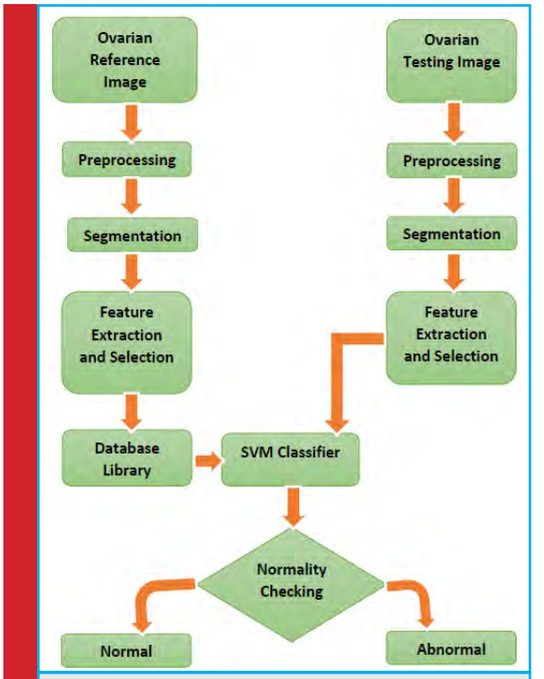

FIGURE 1. Proposed Ovarian tumor detection block diagram

selection from the trained feature matrix an optimization technique known as Particle swarm optimization (PSO) is proposed. This will increase the performance of classification. In the testing part, all complete processes such as preprocessing, clustering, and segmentation are brought about as same as the training part by proposed method. After, the segmented output, it is needed for the classifier to detect the Ovarian MRI as normal or abnormal. As a result of this Kernel SVM classification, the classification performance is enriched.

Pre-processing of Ovarian MRI

Ovarian MRIs are corrupted through the process of imaging because of image communication and image digitization by noise. However, there are lots of filters which have used for filtering the images, more or less of them corrupt the miniature information of the image and nearly conventional filters will process the image smoothing and therefore, toughen the edges of the image (Pan, M.S, Tang, J.T, Yang, X.L, 2011).

From now, the proposed pre-processing stages namely De-noising image computed with the equ. (1). Where, Let W_{xy} signifies the set of coordinates in a rectangular sub image window of size k × l centered at point (x,y). The second step in preprocessing is to eliminate normalize the background at the preprocessing phase itself, meanwhile it may upset the segmentation outcome. At this time, canny edge detection method is used to identify edge from MRI ovarian equ. (2).

$$\hat{f}(x,y)=\text{Median}\{gr(u,v)\}\ (u,v)\epsilon\ Wx,y \tag{1}$$

$$'I'(X,Y) = c(m,n) * I(x,y) = \sum_{m=-N}^{N}\sum_{n=-N}^{N} c(m,n) * I(x-m,y-n) \tag{2}$$

where c (m,n)-Convolution kernel, I(x, y) - Original image, 'I'(X,Y) - Filtered image,$2N$ +1- convolution kernel size.

Segmentation

When the image is preprocessed by filtering, the noise is removed. This will be often beneficial to extract the pixels which are related. The maximum area of linked pixel is called connected component which are partition the image into segments (Ulagamuthalvi .V, 2017).

The number of clusters k with principal cluster centroid was selected r_i=1,2,.m. Separation of the input data points into k clusters with assigning each statistic point Qj to the neighboring cluster centroid r_i using the designated distance measure,

$$Dij = |Qj - ri| \tag{3}$$

where Q = {q1, q2, . . .qn} is the input data. Govern a cluster assignment matrix Si representing the separation of the data with the Binary bias value of the jth data to the ith cluster as it were S = $|s_{ij}|$, where s_{ij} in {0,1}for all i, j

$$\sum_{i=1}^{k} sij = 1\ for\ all\ j \quad and \quad 0 < \sum_{j-1}^{n} uij < n\ for\ all\ 1 \tag{4}$$

Recomputed the centroid using the association values by

$$ri = \frac{\sum_{j-1}^{n} sij * Qj}{\sum_{ij-1}^{n} sij} \quad for\ all\ i \tag{5}$$

Separation of the input data points into k clusters with assigning every one data point x_j to the neighboring cluster centroid r_i using the designated distance measure and The k-means clustering technique optimizes function E_w (s, t) then

$$Ew(s,t) = \sum_{i-1}^{n}\sum_{j-1}^{n} |Qj - ri|^2 \tag{6}$$

Feature Extraction: The transformation of an image into its set of features is registered by feature extraction. It is very challenging to train the classifier with all the feature extracted MR image. Hereafter it is essential to select the appropriate features from the feature extracted MR image. Numerous methods have proposed for feature extraction, feature based on wavelet transform, Gabor features, principal component analysis (PCA), minimum noise fraction transform, decision boundary feature extraction, discriminant analysis and nonparametric weighted feature Y. Zhang et al [16]. Here the texture features are analyzed by using gray level co-occurrence matrix (GLCM) procedure.

The co-occurrence matrix P (i, j | t, d) compute the co-occurrence of pixels with grey values i and j at a confident distance t and in a confident direction d. Constructed on the number of strong suit pixels in all variation, The gray level co-occurrence matrix (GLCM) technique is a method of eliminating second order values constancy elements. However, the performance of a certain GLCM built on aspect, on upper of the location the constancy features; based on the quantity of gray levels applied. The following representations are: μ be the mean of P. μx, μy, μx and μy had the means and standard deviations of Px and Py. G has the co-occurrence matrix size. Now the amount of columns and rows of co-occurrence matrix is identical. The following GLCM features are detached in research work: Mean, Standard Deviation, entropy, Variance, Skewness, Kurtosis, Inverse distinction moment (IDM), Contrast, Correlation, Energy, homogeneity. Let i and j are the coefficients of GLCM, $M_{i,j}$ is the element in the GLCM at the coordinates i and j and N is the dimension of the GLCM.

Particle swarm optimization (PSO): In the feature extraction phase, numbers of more texture patterns are

takeout which leads to computations requisite for classification is improved. Hereafter it is essential to choose the features that are fit for classification.Particle Swarm Optimization (PSO) is a recent swarm intelligent algorithm after GA and Ant Colony Algorithm. It has been a significant part of transformative calculation, generally utilized in neural system preparing, non-direct programming, multi-target enhancement and other areas. For the PSO, every particle has its individual position and speed, and there is wellness esteem which choice by the wellness work. PSO calculation utilizes the speed position model.There are N-measurement search space, M particles, the particle position is Yi = {Yi1 , Yi2 ,..... Yin} ,speed is Vi , we change the present speed and position as follows: ω - idleness weight, w1, w2 - increasing speed factor, r1, r2 -irregular number somewhere in the range of 0 and 1.

$$Vi(t+1)=\omega Vi(t0+w1r1*Pibest(t)-Yi(t)+w2r2*(Pgbest(t)-Yi(t) \quad (7)$$

$$Yi(t+1)=Yi(t)+Vi(t+1) \quad (8)$$

From the capacity and we can see that the particles can pass judgment on the flight speed and position without anyone else's input understanding, then they additionally affected by social. Searching for the harmony among character and social may locate the better arrangement, improve the inquiry execution.The presentation of dormancy factor ω is to adjust the worldwide and neighborhood search capacity, huge idleness weight because solid worldwide hunt capacity. c1 c2 alter the progression σ, when molecule fly to the worldwide best particles and individual best particles. Excessively little, the molecule might be far from the objective zone; Too huge, the particle will all of a sudden traveled to the objective region, over the ideal arrangement. At present, there is no particular criteria lead us how to pick the dormancy weight and increasing speed. Individuals regularly utilize the experience esteem, c1=c2=2, w=0.923.CLERC concluded that c1=c2=2.05 and a few scientists think about that c1=c2 ought to have various qualities.

Particle Swarm Algorithm: Training set = {initial training samples};
Arbitrarily generate number of candidate discriminatory patterns P*i* ;
Find the pattern to the search grid in the order of the generation;
Generate number of particle agents randomly uniformly in the search grid, where each particle agent is located in one grid.
WHILE (iteration < Max no of iteration)
/** Use PCO algorithm to credit the pheromone to the pattern and attributes **/
FOR j = 1 to (no of contestant patterns)
Update the pheromone of the pattern designated by the particle agents; or else, reduction the pheromone in
the pattern;
FOR k = 1 to no of features in each pattern
Update the pheromone of all feature trails by accumulative pheromone in the trails followed by agent_i (proportionate to the fitness value) and reducing pheromone in the other trails
END FOR_LOOP(k)
END FOR_LOOP(j)
/** Use the enhanced PSO algorithm to modify the movement of the particle agent in this iteration. */
FOR l = 1 to (no of particle agents)
Update the pattern and feature pheromones using equation (7) and (8).
IF pattern fitness value of present position < their neighbors' pattern fitness significance
Move to the neighbor's pattern;
IF the fitness value of the feature(s) in the previous position is (or are) greater than the novel position
Substitute those lower fitness features with the higher fitness feature from previous position;
ELSE
Leave of absence the features of the novel pattern as it is.
ELSE
Stopover in the existing pattern position;
END FOR_LOOP (l)
END WHILE

Classification: Support vector machine (SVM) classifier is a powerful and clear-cut learning technique. It is categorized into two types: linear SVM and non-linear vector SVM. In Linear SVMs, training points have support vectors which describe the hyper-plane. In Non-linear SVMs, arranged a splitting hyper-plane in the typical space and classify peaks in that space. The kernel plays the important role on dot invention in the feature selection (Zhang, 2012). It has great speculation properties. It can be effectively prepared, and performs well on nonlinear data. It performs well with numerous highlights and furthermore with less number of preparing tests. It searches for the hyperplane as a choice surface which isolates the 2 classes with greatest edge. Subsequently, the isolating hyperplane will be situated opposite to the briefest line isolating the raised bodies of the preparation highlights for each class, and it will be found halfway along this line. So as to group the nonlinear information, portions can be used to outline input information to a high-dimensional space. Polynomial kernel, Linear kernel of order 1, 2, and 3, and the RBF portion are generally utilized.

Kernel SVM classifier (Quadratic SVM kernel, Linear SVM Kernel, Polygonal SVM Kernel, Radial basis function (RBF) SVM kernel) gets the input from the features extracted via the Gray Level Co-occurrence Matrix technique. In this work, normal and ovarian image is chosen. SVM classification is used for classification the image into abnormal and normal in which subject is denoted through vectors (C. Cortes and V. Vapnik, 1995).

Linear Kernel SVM classifier contains in defining the function.

$$f(x) = sign(\langle w,x\rangle + b) \quad (9)$$

The kernel is a function which simulates the prediction of the initial data in a feature vector using higher

dimension Φ: $K^n \rightarrow S$. In this new space the data are measured as linearly separable. To put on this, the dot product $\langle x_i, x_j \rangle$ is substituted by the function:

$$K(x, xi) = \langle \phi(x), \phi(xi) \rangle \quad (10)$$

Then and there the new function used to classify the data equation (11)

$$f(x) = sign\left(\sum_{i=1}^{Ns} Yi * \alpha i * K(x, xi) + b\right) \quad (11)$$

Kernels are generally used equ (12) and (13)

$$\text{Polynomial kernel: } K(x,xi)=((x.xi)+1)*P \quad (12)$$

$$\text{RBF kernel: } K(x,xi)= exp^{-|x-xi|2/2\sigma 2} \quad (13)$$

RESULTS AND DISCUSSION

One instance of intense ovarian malignancy and benign tumor was collected. Non-enhanced and Noisy Ovarian tumor Magnetic Resonance Image (512 × 512) are used in the detection of all data of malignant and benign shown in Figure 2(a). The MR ovarian image in original form by a tumor and various operations of proposed algorithm is shown in Fig. 2. Four phases were there in the proposed method. A First step involves of suppression of noise using by median filtering which increase the efficiency of the image shown in Figure 2(c). The second step, normalized by global thresholding technique shown in Fig. 2(b). In, the third step k-means clustering procedure used to extract the tumor region shown in Figure 2(e). In, the fourth step gray level co-occurrence matrix (GLCM) used to extract thirteen texture for ovarian. Therefore, the resulting features in the Kernel SVM classifier are trained, that automatically check whether the MR Ovarian image is normal or abnormal (Malignant, Benign) shown in Figure 2(f). Four classifiers namely Quadratic SVM kernel, Linear SVM Kernel, Polygonal SVM Kernel, Radial basis function (RBF) SVM kernel have been developed to classify normal and abnormal ovarian. The spatial area based surface highlights GLCM are taken out from Ovarian MRI and are put away as highlight spaces. These element spaces are given to contribution of preparing the Kernel SVM. The classifier exactness is improved because of the streamlining of highlights choice utilizing PSO in which figuring the new position of each particle is registering clearly from the blend of its particular best position and the global most prominent position. The PSO figuring beats all of the estimations under scrutiny on various benchmark limits. It is for the most part propelled the accuracy in location of ovarian tumor and request of ovarian generous and threatening development.

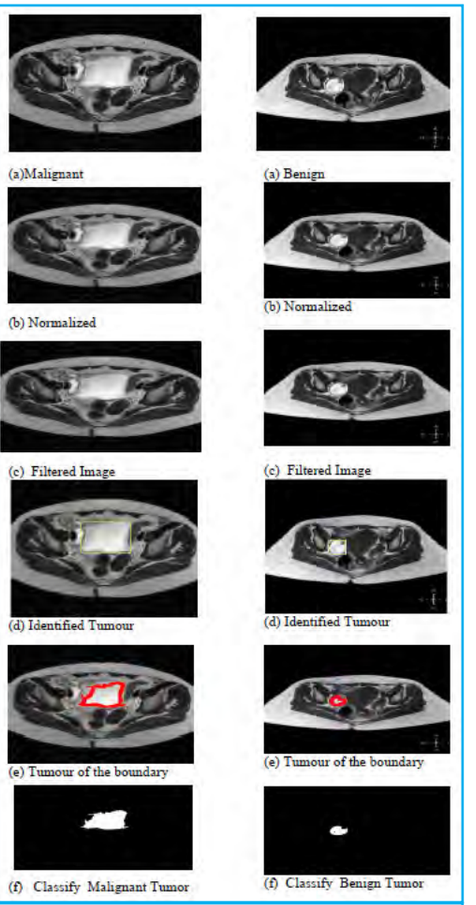

FIGURE 2. Output of Ovarian MR Image for (a) Malignant and Benign Tumour (b) Normalized Image (c) Filtering (d) & (e) Tumour Identify and Boundary Detection (f) Classify Tumour

CONCLUSION

Automatic computer aided detection system helps in detecting ovarian cancer has been developed in proposed system. This system aids to surgeon to identify malignant, benign on Ovarian MRI images and diagnosis accurate computable results. An efficient ovarian tumor segmentation, feature extraction and selection by using PSO and classification is proposed in this work by combining different Kernel SVM Classifier. The preprocessing of ovarian MRI gives encouraging outcomes. The spatial domain based texture features GLCM are taken out from Ovarian MRI and are stored as feature spaces. These feature spaces are provided to input of training the Kernel SVM. The classifier accuracy is enhanced due to the optimization of features selection using PSO in which calculation the new position of every particle is computing straightforwardly from the mix of its

specific best position and the global greatest position. The PSO calculation beats every one of the calculations under investigation on numerous benchmark capacities. It generally helped to advance the precision in location and order of ovarian benign and malignant growth.

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Morphology and Seasonal variation of fowl tape worm, *Raillietina tetragona* (Molin, 1858) in Purba Bardhaman, West Bengal, India

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ABSTRACT

The tape worm, *Raillietina tetragona* (Molin 1858) is a common parasite of domestic fowl, *Gallus domesticus* in West Bengal. This cestode parasite plays a significant role in growth and reproduction of fowl. In the current study, Light microscopic (LM) examination showed that the present cestode has a minute pin like scolex or head (18.98µm – 19.76 µm) and a long flat body divided into immature, mature and gravid proglottids. Head bears a rostellum (2.94 -3.11µm), four acetabula (6.79µm - 7.4µm) and followed by a neck (15.1µm – 16.27µm). Seasonal variation of *R. tetragona* from *Gallus domesticus* in Purba Bardhaman, West Bengal, India has been studied through two years span (January, 2016 to December, 2017). Monthly variations of infection of *Raillietina tetragona* showed comparatively higher prevalence in hot summer months (58.33 - 70 %) and humid rainy season (80-95.24%) and lower prevalence during winter season (0 - 10%). Mean intensity of *Raillietina tetragona* was also higher in summer (1-2.5) and rainy season (3.36-10.2). The present study on the morphology and seasonal prevalence of *Raillietina tetragona* in Purba Bardhaman region of West Bengal showed that more awareness should be focused towards upgrading and maintenance of the local free ranging chicken breeds.

KEY WORDS: *RAILLIETINA TETRAGONA, GALLUS DOMESTICUS*, LIGHT MICROSCOPIC OBSERVATIONS, PREVALENCE, PURBA BARDHAMAN, INDIA

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INTRODUCTION

Domestic fowl, *Gallus domesticus* (Linnaeus 1758) (Galiformes: Phasianidae), is supposed to have derived from the South East Asian and wild Indian red forest chicken (Permin and Ranvig, 2001). These fowls are with high nutritional value. Sometimes these are domesticated traditionally under free-range management systems in villages where little or no supplementary foodstuff is provided and the poultry farmers are unaware of any veterinary care, which finally leads to the parasitic infections to the chicken (Gary and Richard 2012). According to Rohde (1994), there are some other factors like temperature, humidity, rainfall and parasite maturation which influence the parasitic infection in domestic fowl. Thus environment plays a significant function in the seasonal variability of these cestode parasites. During the present study *G. domesticus* were collected from Purba Bardhaman and surrounding rural areas while some other gastrointestinal cestode parasites were also recovered from the domestic fowl gut and *R. tetragona* was found to be the most prevalent species among all. This is also supported by the findings of Khan et al. (2016).

In the present survey morphological observations were made using light microscope (LM) and measurements of its cephalic regions were taken. Other morphological characters like shape of the scolex; rostellum and suckers, number of eggs in the egg capsules and shape of the eggs were also made.However, reports on seasonal prevalence of *Raillietina tetragona* in domestic fowl of Purba Bardhaman region of West Bengal could not be noticed in the accessible reports. The two year survey was carried out to determine the prevalence of this cestode parasite in *G. domesticus* occurring in and around Purba Bardhaman, West Bengal, India.

MATERIAL AND METHODS

Freshly dissected out guts of the domestic fowl, *Gallus domesticus* were purchased from the local markets of Bardhaman, West Bengal, brought to the laboratory, as soon as possible, and examined for the presence of *Raillietina tetragona* in each week of every month for a period of consecutive two year study period from January 2016 to December 2017. Live worms were washed in normal saline (0.85% NaCl) and freed from the adhering host materials, fixed and preserved in 70% alcohol. For identification and morphological observations, the adult cestodes were washed in normal saline, dried and flattened on filter paper, fixed in 70% alcohol and observed with the help of compound light microscope.

The prevalence of infection and mean intensity were calculated from the recorded data with the help of the following formulas

$$\text{Prevalence of infection (In percentage)} = \frac{\text{no. of infected } \textit{Gallus domesticus}}{\text{total number of } \textit{Gallus domesticus} \text{ examined(Infected + uninfected)}} \text{X } 100$$

$$\text{Mean intensity} = \frac{\textit{Total no. of Raillietina} \text{ sp. collected from infected intestine}}{\text{No. of infected } \textit{Gallus domesticus} \text{ in the sample}}$$

RESULTS

General morphology of the cestode

Light microscopic (LM) study showed that the body of *R. tetragona* was typically divided into scolex, followed by a short unsegmented region, the neck, succeeded by a chain of proglottids consisting of immature, mature and gravid segments. Scolex is small pin like and provided with four well-formed hemispherical acetabula, armed with hooks and an armed disc shaped rostellum (**Plate 1**). In the mature proglottids the genital pores are unilateral (**Plate 3**) and in the gravid proglottids (**Plate 4**) eggs are found in egg capsules, each containing six to twelve eggs.

The current LM observation showed that the scolex of *Raillietina tetragona* was about **18.98µm – 19.76µm** in width, neck was about 15.1**µm- 16.27µm** and rostellum was 2.94**µm** – 3.11**µm** in width and armed with one row of hooks. Four acetabula were measured about 7.08µm, 6.95**µm**, 6.79µm and 7.4**µm** in length and 4.12**µm**, 4.03**µm**, 3.17**µm** and 3.71**µm** in width respectively. Mature proglottids (**Plate 2**) were rectangular in shape (65.68**µm - 68.96µm**) and gravid proglottids (60.85**µm**-61.44**µm**) were more or less square containing numerous egg capsules (**Plate 5**) which were more or less round or oval in shape and measured about 0.85**µm**- 1.09**µm**.

During the two year study period from January 2016 to December 2017, out of 382 fowl guts examined, 230 were found infested with *R. tetragona* and totally 907 parasites were collected. As *R. tetragona* was isolated all through the year from *Gallus domesticus*, characterization and seasonal prevalence were done for this species only. Monthly variations in respect to percentage of prevalence andthe mean intensityof *R. tetragona* infection have been presented in **Table 1** and total number of parasite present in *Gallus domesticus* in each season for the two year study period has been shown in **Figure 1** .The result showed that average prevalence percentage was 51.91%. The study revealed that the higher percentage of prevalence of *R. tetragona* occurred in summer season showing higher peak in May (61.58% -70%) and it showed its highest values during rainy season and the maximum value of percentage of prevalence was recorded in the month of July (93.33% - 95.24%). From September to November it tends to lower down and the lowest prevalence occurred in winter (0 -10%). The mean

Table 1. Monthly variation in % of prevalence and Mean intensity of infection of *Raillietina tetragona* infecting *Gallus domesticus*

Month of collection	Number of gut examined		Infected host		Parasite present		Prevalence %		Mean Intensity	
	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
January -16	8	10	0	0	0	0	0	0	0	0
February -16	8	14	1	2	1	3	12.5	14.28	1	1.5
March -16	12	16	2	2	2	4	16.67	12.5	1	2
April -16	12	10	7	6	12	10	58.33	60	1.71	1.66
May-16	20	13	14	8	25	20	70	61.58	1.79	2.5
June-16	20	17	16	15	54	67	80	88.24	3.36	4.46
July-16	15	21	14	20	120	204	93.33	95.24	8.57	10.2
August -16	19	23	16	21	95	119	84.21	91.3	5.93	5.66
September -16	25	15	18	11	50	52	72	73.33	2.78	4.72
October-16	24	28	20	23	22	26	83.33	82.14	1.1	1.13
November -16	15	16	7	5	9	10	46.67	31.25	1.28	2
December -16	10	11	1	1	1	1	10	9.09	1	1
Total duration: 2 years	total no. of sample: 382		Total number of infected host: 230		Total number of parasite present: 907		Mean percentage of prevalence: 51.91%			
Period of study: January 2016 to December 2017										

intensity of infection also declined in the winter season (0-1) during the two year study period.

DISCUSSION

Raillietina tetragona has a cosmopolitan distribution occurring in pigeon, chicken and guinea fowl. This tapeworm completes its development in two hosts. Birds are the definitive hosts and ants, mostly of the species *Tetramorium*, and housefly of the species *Pheidole* and *Musca* act as the intermediate hosts (Horsfall 1938; Soulsby 1982; Su 1986). Light Microscopic observations revealed that *Raillietina tetragona* was different from the other *Raillietina spp*. in respect to the shape of scolex, rostellum and acetabula. The hooks of the rostellum were placed in one row (Butboonchoo et al., 2016). This type of study was also carried by some scientists earlier (Hofstad et al. 1984; Sawada 1964 and 1965) but scanty literature is available. Lalchhandama (2009) observed the morphological structures of *R. echinobothrida from* fowl gut and Mu et al. (2009) worked on R. echinobothrida and *R. tetragonal* from the gut of domestic

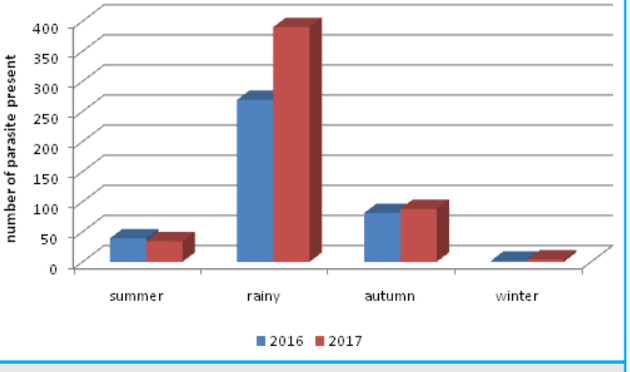

FIGURE 1. Seasonal prevalence (No. of parasite) of *Raillietina tetragona* present in *Gallus domesticus* during two year study period from January 2016 to December 2017

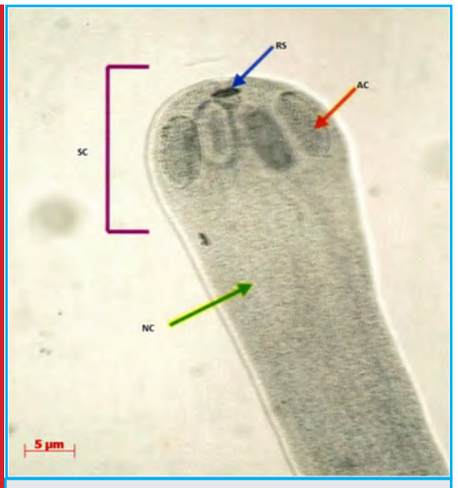

PLATE 1. Light Microscopic view of anterior portion of *Raillietina tetragona* showing Scolex (SC), Rostellum (RS), Acetabulum (AC) and Neck (NC)

chicken. In the recent year, Butboonchoo et al. (2016) distinctly differentiated four different species of *Raillietina spp*. including *Raillietina tetragona* on the basis of Light Microscopic and Scanning Electron Microscopic observations. The present study showed similar findings.

Seasonal variability of *R. tetragona* in domestic fowl, *Gallus domesticus*, was checked throughout the year and comparatively higher percentage of prevalence and mean intensity of this parasite were observed in the summer and rainy season. Seasonal variability of cestodes were showed that changes in prevalence and infection rates could depend upon host, parasite and also upon the geographical location (Biswal et al. 2013). Oniye et al. (2001) in Nigeria reported a high prevalence of

PLATE 2. Light Microscopic view of Mature proglottids of *Raillietina tetragona*

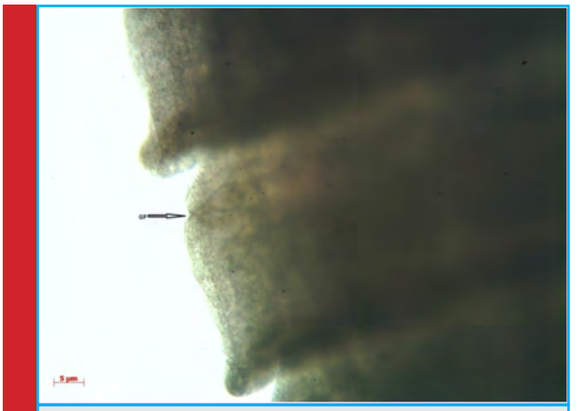

PLATE 3. Light Microscopic view of Mature proglottid of *Raillietina tetragona* showing genital pore (GP)

Raillietina tetragona in the month of June and August. Fakae et al. (1991) from Eastern Nigeria reported 72.5 % prevalence of *R. tetragona* in *Gallus gallus* during dry season (November to April). These finding were supported by the previous results as reported by Frontovo, (2000), Fakae et al. (1991) and Oniye et al. (2001).

Seasonal prevalence of *R. tetragona* also varied with geographical location and other climatic conditions. According to Adang et al. (2008) *R. tetragona* was found to be the most common cestode parasite in domestic pigeon, occurring in 11 months of the annual cycle, showing highest prevalence in rainy season in Zaria, Northern Nigeria. Salam et al. (2010) and Sheikh et al. (2016) reported that the prevalence of *R. cesticillus* from *Gallus gallus domesticus* in Kashmir is highest in summer followed by autumn, spring and winter months respectively. Butt et al. (2014) reported that the average percentage of prevalence of *Raillietina cesticillus* in *G. domesticus* was 83.5% during July to November, 2013 in Hyderabad, Sindh, Pakistan. Accord-

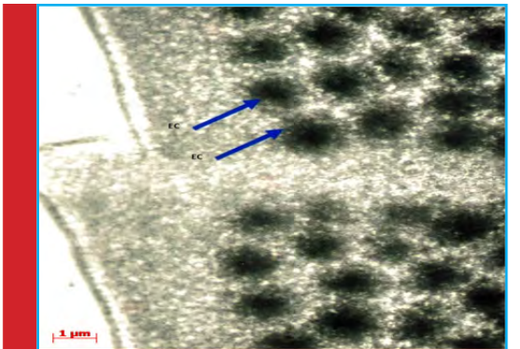

PLATE 4. Light Microscopic view of Gravid proglottids of *Raillietina tetragona* showing Egg Capsules (EC)

PLATE 5. Light Microscopic view of Egg Capsules of *Raillietina tetragona* isolated from gravid proglottids

ing to the observations of Sheikh et al. (2016) the high prevalence of *Raillietina sp*. infection was seen during warm summer followed by autumn, spring and winter months. Except winter, the high prevalence of cestode infection throughout the year is a strong evidence of unscientific management and control in domestic fowls. As a result an infection creating environment originates continuously. However, Shukla et al. (2012) reported that the maximum prevalence of *Raillietina sp*. in *G. domesticus* from Ahmednagar district, Maharashtra, India was found in winter followed by rainy season and minimum prevalence was noted in summer. According to Patil and Bhamare (2018), a high prevalence of *Raillietina sp*. in *G. domesticus* from Nashik district of Maharashtra, India occurred in winter season followed by summer season and low in rainy season. A possible explanation of this report is the availability of the intermediate host and oncosphere stages of *Raillietina sp*. increases during cold climatic condition. The oncosphere stages are taken by the intermediate hosts and grow into cysticercoids which become adult in the definitive host during summer season.

In our survey greater number of mature cestodes were obtained during summer and rainy seasons whereas in autumn mostly immature stages were obtained. High rate in the percentage of prevalence and mean intensity of *R. tetragona* in the summer and rainy season may be a result of the abundance of infective stage and the stage bearing intermediate hosts in the survey zone during these seasons. It seems that moisture and temperature favour the development of intermediate hosts, the development of eggs in soil as well as proliferate the reproductive rate of parasites (Møller, 2010). Low prevalence of *R. tetragona* in winter season may be the result of elimination of multiple infections. This condition may occur due to intraspecific antagonistic reaction.

In fact, reports from other researchers around the world in different times showed that *Raillietina spp*. has a very high prevalence of infection in birds especially in indigenous chickens. (Ahmed and Sinha, 1993; Puttalakshmamma et al. 2008; Nnadi et al. 2010; Sreedevi et al. 2016; da Silva et al. 2016). According to a recent report of Jajere et al. (2018) who found the guinea fowls have been heavily infected with *Raillietina spp*. and among the various species, *Raillietina tetragona* was most abundant having 72.8% prevalence of infection. When the infective stages of intestinal parasites pass out to the surroundings, they have to get over the environmental hazards before they reach their suitable hosts for further development (Gillett, 1974). So, favourable environmental conditions are also necessary for the effective transmission of the parasites (Smith, 1990) and seasonal variations in the social conduct of the host and their accumulation can also describe for seasonal dynamics of parasitic infections (Hosseini et al. 2004). The time period for obtaining table size, in case of indigenous breed, is longer than exotic breeds which are usually feed on artificial supplementary diets. So another reason of higher infection is may be due to improper management, the inadequacy of food grains for the local breeds and they have to feed on insects, mites and worms which may be the intermediate hosts for the cestode parasite and has been shown to improve susceptibility to parasitism in the system (Shukla and Mishra, 2013).

Moreover, earlier studies have shown that the immune systems of humans, rodents and birds got weaker by rough climatic conditions, under-nutrition or investment in reproduction (Lloyd 1995; Nelson et al. 2002) and due to low immunity level, hosts become more vulnerable to infections (Hillgarth and Wingfield, 1997). A longer breeding period of the host bird provides a selective advantage to the parasites (Dunn and Winkler 2010; Møller et al. 2010). Reports have shown that during the breeding season of host birds, antibody production and cell mediated immunity rate is getting weaker which is the reason for higher rates of parasitic infection (Hillgarth and Wingfield, 1997; Moreno et al. 2001). According to Sheikh et al. (2016) the host birds have shown a moderate resistance against the cestode parasites with the advancement of age because of the improved immune status in grown-ups than in young ones. Chicks hatch out during late spring. During summer and autumn they are in their young age when they get exposed to parasite infection. The young and the juvenile forms are susceptible to the parasite because they are immunologically weak. This may also affect the seasonal variability of *Raillietina tetragona*. Till now

there are many surveys on *Raillietina spp*. reported from different parts of the world but it is still needs to execute more studies to follow the changing dynamics of helminth infection in domestic poultry management.

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Compliance with ethical standards: Though our Institution does not have such ethics committee, domestic fowl has been slaughtered for the present work following the guiding principle of CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals) established by the Act of the Indian Parliament.

Conflict of interest: All the authors have declared that in the present work there is no conflict of interest.

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Evaluation of the Potential of *Gymnema sylvestre* on Some Biochemical, Pancreatic Histoarchitecture and Hematologic Parameters of Alloxan Induced Diabetic Wistar Rats

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ABSTRACT

Now day's diabetes has been the endemic in the current society and the history of relying on the herbal is quite long. The present research work explains the efficacy of the ethanolic extract of *Gymnema sylvestre* (500mg/kg.b.wt) on various parameters like fasting glucose, amylase, lipase, some hematologic parameters. Restoration in the concentration of serum amylase, lipase, and plasma glucose admits positive results. Remodeling of the histology correlates with the Biochemical parameters. Hematological parameters in the present study in the diabetic condition is not much influenced. The result obtained from all those parameters were significant to explain its importance in controlling the diabetes symptom and associated complications. The statistical analysis was done using Prism Figure pad Values represented are mean ± SD (n=5). Significant level was calculated by Tukey multiple range tests compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value $p<0.05$

KEY WORDS: DIABETES, HISTOARCHITECTURE, PANCREAS, HEMATOLOGY, ENZYME

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INTRODUCTION

Diabetes mellitus is characterized by chronic hyperglycemia with disturbance of carbohydrate, fat and protein metabolism resulting from dysregulated insulin secretion, insulin action or both. Diabetes causes heavy annual economic burden to the patient, and its treatment is carried out with several other serious symptom like weight gain, obesity and increased toxicity to the various organ. Practicing of modern medicine to bring glucose to the normal level increases chance to obesity and resultant low grade inflammation pertaining to insulin resistance (Anne et al., 2015, Saltiel and Olefsky, 2017, Ruiz et al., 2019, Hooda et al., 2019).

Type 2 diabetes mellitus is characterized by peripheral insulin resistance and relative insulin deficiency, which may range from predominant insulin resistance and relative insulin deficiency to predominant insulin secretory defect with insulin resistance (Patel and Mishra (2011). Diabetes mellitus is caused by the abnormality of carbohydrate metabolism which is linked to low blood insulin level insensitivity of target organs to insulin (Maiti *et al.,* 2004). Diabetes complications are the major causes of mortality morbidity in the western countries (Leahy, 2005). All those complication is caused due to excessive presence of glucose in the blood and this leads to glycosylation of major proteins causing misbehaved homeostatic regulation (Dotz et al., 2018).

The condition of altered glucose regulation is caused due to altered insulin formation, hormonal imbalance that controls glucose homeostasis leads to difficulty in achieving good control (Azari et al., 2017).Pancreas is an elongated gland, consisting of head, body and tail. It is a myxocrine gland, of which exocrine part produced digestive enzymes whereas endocrine gland produces hormones, through islet of Langerhans, (Longnecker and Daniel (2014). Secretary cells are acinus, or tubuloacinus cell. Beta cells are at the centre of the Langerhans while alpha cell are at the periphery of the beta cells (Korc et al., 1981,1978, Chen et al., 2017).

Exocrine gland is interconnected with network of ductile system (Ballian and Brunicardi, 2007). Acinar cell are the simple cuboidal epithelial cell (Cesmebasi et al., 2017). Blood vessel network in the pancreas is uniquely designed. In the islet of Langerhans, β cell receives the blood first through arterioles, and arterioles drain its blood firstly in to the centre through fenestrated capillaries where β cell reside, (Covantev et al., 2019).

Failure in pancreatic β cells occurs very early red. Study led out by (Gastendelli et al., 2013) found that person diagnosed with IGT, by the time he had already lost >80% of his pancreatic β cells function. In an another study it was mentioned deficiency in the β cell mass in type II Diabetes and this was due to stress mediated apoptosis of cells (Chandirasegaran et al., 2017). It was also found that obese individuals had 60% less β cell relative to the non diabetic obese (Butler et al., 2003).

MATERIALS AND METHODS

For the present research work healthy wistar rats (*Rattus norvegicus*) were selected and provided anambient physical and physiological condition as per the standard protocol and all the experimental protocol was carried based on the guideline adopted by Mahavir Cancer Sansthan ethical committee, Phulwarisariff Patna.

Feeding: The laboratory rats were fed on laboratory prepared enriched bread constitutes wheat flour, jaggery, powdered milk and gram flour. For providing vitamin supplement they were fed with carrot, sprouted gram and sprouted moong bean.

Induction of diabetes

Alloxan was used as a diabetogenic material. It is [2,4,5,6-tetraoxypyrimidine,5-6 dioxyuran] a pyamidine derivative of uric acid. Formerly it was discovered by Brugnallete in 1818 and then by wohler in 1838. Its diabetogenic nature came into existence when Dunn et al in 1943 mentioned necrosis in Central Islet cell, since it is being regarded as a diabetogenic material for the animal model. Diabetes was induced by repeated dose of Alloxan monohydrate 100 mg/kg. b.wt in cold citrate buffer bearing pH 4.5.

Plant materials: Leaves of *Gymnema sylvestre*

Sample Collection: After the treatment of the extract for 10, 20, and 30 days respectively the pancreatic tissues

Details of grouping and treatment given to rats for *Gymnema sylvestre*

Cage no.	Treatment	Average weight	No. of rats in each cage	Selected dose Mg/kg/body weight
1.	Normal/control	180-200gm	5	Olive Oil
2.	Alloxan treated	180-200gm	5	100mg/kg.b.wt
3.	10 days treated *G. sylvestre*	180-200gm	5	500mg/kg.b.wt
4.	20 days treated *G. sylvestre*	180-200gm	5	500mg/kg.b.wt
5.	30 days treated *G. sylvestre*	180-200gm	5	500mg/kg.b.wt

were isolated for Histopathology. Whole blood was used for CBC parameters and serum was used for Amylase and lipase and plasma was used for fasting glucose.

Chemicals and reagents: All the reagents were prepared in the laboratory using high grade chemical. Glucose estimation was done by GOD POD method, hemoglobin by Drabkin, Amylase by Direct substrate method, Lipase by using Turbidometric UV method etc, and the tissues were dyed with Hematoxylene and Eosine.

Statistical analysis

In the present investigation the results are expressed as Mean±SD for five animals in each group. The data was analysed by repeated measure analysis of variance (ANOVA) followed by Tukey multiple range test compared with the entire column. Prism Graph pad 3.0 was used.

p value of <0.05 was considered significant.

$$\bar{X} = (\sum_{i=1}^{n} Xi)/n$$

$$SD = \sqrt{\frac{\sum |x - \bar{x}|^2}{n}}$$

Where,

∑ Summation, X - Individual value in the data set

$\bar{X}$ Arithmetic mean, n - Number of the data in the set

RESULTS AND DISCUSSION

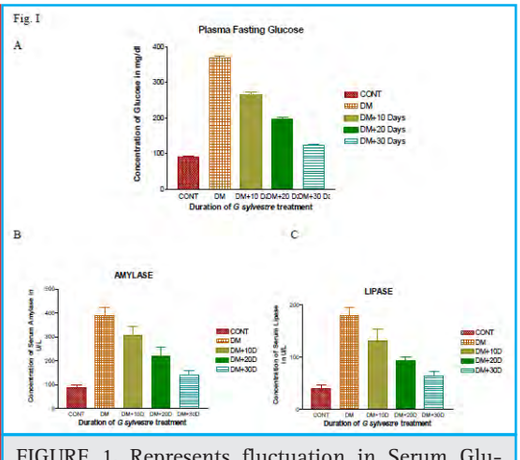


FIGURE 1. Represents fluctuation in Serum Glucose, Amylase, and Lipase in the classified groups. The diabetic rats showed significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range tests compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value p<0.05, A- represents Serum Glucose level, B- represents serum Amylase and C- represents: Serum Lipase in *Gymnema sylvestre* treated diabetic wistar rats

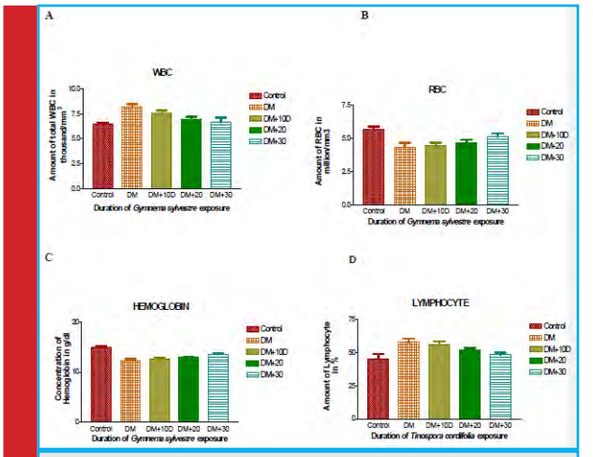


FIGURE 2. Represents fluctuation in Total WBC, RBC, Hemoglobin and Lymphocyte in the classified groups. The diabetic rats showed significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range test compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value p<0.05, A- represents Total WBC level in *Tinospora cordifolia* treated group, B- represents Total RBC, C- represents Hemoglobin D- Represents Lymphocyte in *Gymnema sylvestre* groups.

Rats with Normal and Diabetic Histoarchitecture

Photomicrograph I A, and B are normal rats histoarchitecture having normal cellular arrangement islet of Langerhans (IL) and Acinar cell (Ac) with well developed vascular and ductile system. Cells are compact and well mannered. The Alloxan treated Diabetic rats shows atrophy, tissue degeneration , poor cellularity of islets of langerhans (IL) and damaged islets cells along with hyperchromicity in diabetic control group. Widening of sinusoids(s), necrosis(N) and degenerated Acinar cells (Ac). Diabetic control: (C): Necrosis of exocrine, lymphocytic infiltration, depletion of intercellular duct. (D). Necrosis of islet of Langerhans, intercellular as well as intracellular duct depletion, necrosis of the main duct. (E): Showing ductule congestion which is one of the common cause results from depletion of ductile system in the pancreas.

Rats treated *Gymnema sylvestre* extract Stained with Hematoxyline and Eosin

Photomicrograph II. Showing the treatment group *G. sylvestre* treated group for 10, 20, and 30 days respectively. variable and more intercellular spaces along with deranged ductile system, however concentration of β cell is quite little (F) comparatively *Gymnema sylvestre*

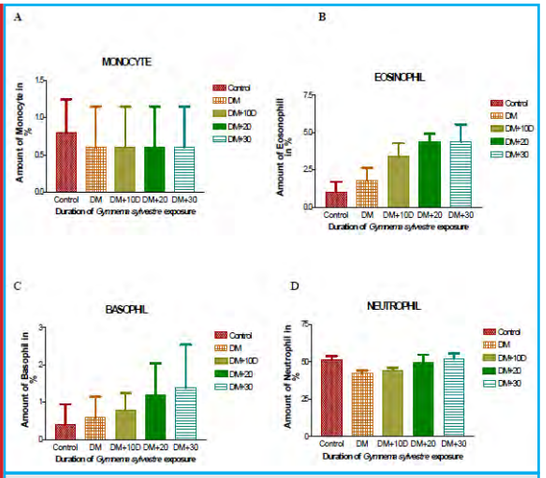


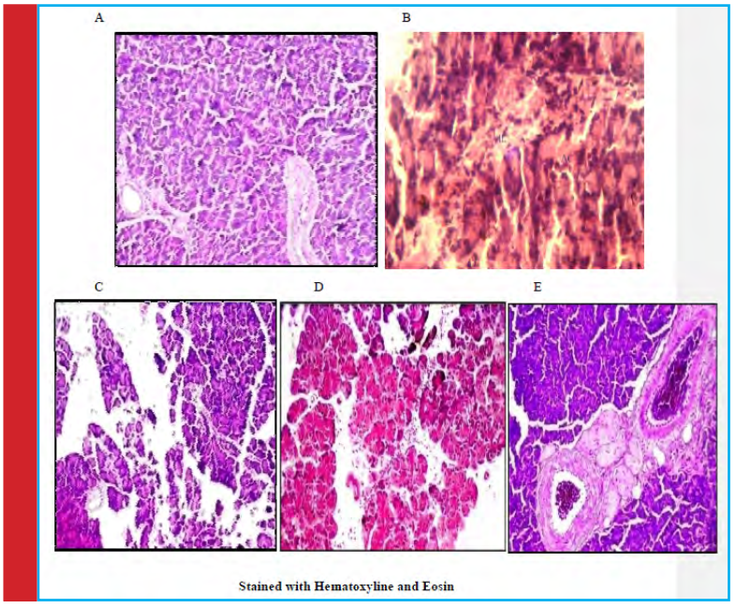
FIGURE 3. Represents fluctuation in Monocyte, Eosinophil, Basophil and Neutrophil in the classified groups. The diabetic rats showed significant recovery after treatment with GSE extract for the subsequent days. Values are mean ± SD (n=5). Significant level was calculated by Tukey multiple range test compared from the entire column after the ANOVA. The level of significance represented here for diabetic VS treated group having p value $p<0.05$, A- represents Monocyte level ,B- represents Eosinophil, C- represents Basophil and D- Represents Neutrophil in *Gymnema-sylvestre* groups.

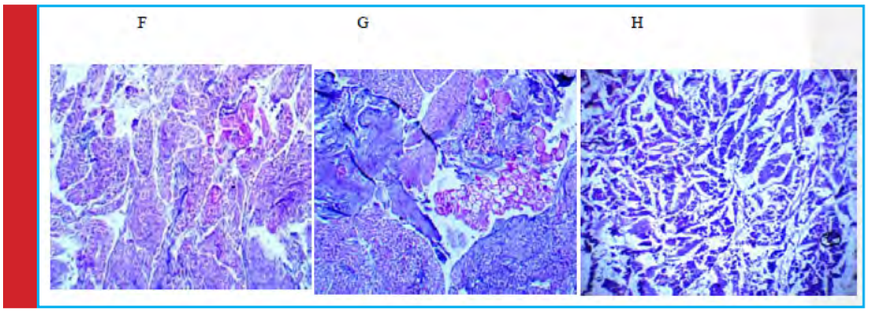
extract has better and much more reduced spaces and ductile system is well developed in 10 days of treatment (F) but as dose prolonged for 30 days the scenario of β cell scanty resolved to a lot of extent (G) & (H)

DISCUSSION

The concentration of plasma glucose plays important role in the diabetes responsible for entire dysregularities and associated complications. 75.54% increase in fasting plasma glucose was registered in diabetic subject however herbal treatment restored the value to to 74% after treatment upto 30th days as compared to normal control (Fig. I). Decrease in blood glucose level registered its significance Histoarchitectural remodeling of pancreas, reduction of Glycation and Its associated complications (Myung-Hwa Kang, et al., 2014). Amylase and lipase pronounces the overall pancreatic exocrine health. Comparatively lipase is more specific to pancreatic as compared to Amylase. The alleviated level of both amylase and lipase in diabetic groups was noticed by 4.50 times that of the normal control while after treatment it was reduced to nearly 1.60 times to that of control (Fig. I).

Significant recovery was noticed in both the cases and the results obtained registers great importance of the ethanolic extract for control of elevated pancreatic digestive enzyme in diabetes which parallels with the findings of (Thakur et al., 2016, Sellami et al., 2017). Total leucocyte count in diabetic condition was 1.27 times whereas after ethanolic extract administration for 30 days it returned to about 1.02 times that of the normal. (Fig. II), the present outcome satisfies the findings of (Gilad Twig, et al., 2013). The other possible reason might be the chronic low grade inflammation associated with insulin resistance and type 2 Diabetes; however recovery in presence of herbal extract was significant. Total erythrocyte values are lying in the normal range





but towards the lower extreme (Fig. II). The reason behind decrease in the RBC content may be its membranal modification, reduced Na+K+ATPase activity and high lipid peroxidation of the RBC membrane. In an study carried out by (Buys et al., 2013) states decrease in RBC's average life span. Decrease in the erythrocyte count was displayed by the decreased hemoglobin concentration in diabetic and slight restoration was obtained after the drug administration (Fig. II).

From the above discussion co-relation between hemoglobin and total erythrocyte count was well established. The diabetic rat lymphocyte concentration was 1.28 times to that of normal but with the extract of *Gymnema sylvestre* it was only 1.02 times to that of the normal (Fig. II). And this slightly increase in the lymphocyte is not a big deal because this is a general phenomenon of the hyperglycemia that individual are more prone to microbial infection. However, after the extract administration as the glucose concentration went down the normalization of lymphocyte also attained. Eosinophil, Monocyte, Basophils, Neutrophils count in the differential section doesn't draw any peculiar impact of effect of diabetes in the hematological changes except in lymphocyte which was result of opportunistic condition related to infection (Fig. III). The results are Mean ± SD with $p<0.05$, were calculated using Tukey multiple ranges test, after ANOVA and compared with the entire column.

Present study is also based on the potential of herbal extract and its impact on the diabetic pancreas. A, and B are normal rats histoarchitecture having normal cellular arrangement islet of Langerhans (IL) and Acinar cell (Ac) with well developed vascular and ductile system (Gorczyca et al., 2017). Cells are compact and well mannered, the result agrees with other reports (Aggarwal 2010, Chakraborty et al., 2012). Regeneration and restoration of pancreatic β cell has been the matter of concern. In the current study reduction in the pancreatic β cell mass was noticed along with inflammation, congestion, scattered β cells, neutrophillic excavation showing ruptured blood vessel has been noticed in diabetic subjects (Fig. 1, C, D, and E). Showing the treatment group *Gymnema sylvestre* treated group for 10, 20, and 30 days respectively. variable and more intercellular spaces along with deranged ductile system, however concentration of βcell is quite little (F) *Gymnema sylvestre* extract shows significant and much more reduced spaces, congestion (Aralelimath, and Bhise 2012) and ductile system is well developed in 10 days of treatment (I) but as dose prolonged for 30 days the scenario of β cell scanty resolved to a lot of extent (H) & (K) which co-relates with (Ahmed A B et al., 2010).

CONCLUSION

The findings in the present study opens several avenues especially in the pancreatic regeneration and dedifferentiation of other cells despite of β-cells, however further clinical trial on humans are required to reach any specific conclusion because the results based on route of administration and intestinal absorption may vary the outcomes. Overall we can say that relying on the herbal formulation from early will certainly reduce the diabetic complication and its prevalence.

Conflict of interest

The authors declare there is no any conflict of interest.

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Geo-Accumulation Index of Heavy Metals in Pond Water Sediment of Raipur

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ABSTRACT

Problem-based learning (PBL) has been implemented to replace classical teaching method with college system in Under-Sediment can accumulate trace elements in the environment. An investigative study was carried out to determine the heavy metal in the pond water sediment (n=10) samples of Raipur city Chhattisgarh. The samples were digested and analyzed for extractable metal i.e. Cd, Cr, Cu, and Pb. The extent of elemental pollution was evaluated using with the enrichment factor (EF) and geoaccumulation index (*I*geo). The assessment of heavy metal was derived using the geo-accumulation index (Igeo). This study revealed that the sediment is predominantly by Cr < Pb ≈ Cd < Cu < Zn. The data reveal elevated concentrations of Cu (6.4 - 15.3 mg kg^{-1}), Cu(8.5-14.2 mg kg^{-1}) Pb (0.8 -3.9 mg kg^{-1}) and Cr (6.5-17.2 mg kg^{-1}) and Zn(6.4 – 21.2 mg kg^{-1}). Except for Cr, other heavy metals have been accumulated (EF>0.5). The highest EFs of Cu, Cd, Pb and Zn are 0.6, 0.7, 0.86 and 0.86, respectively. The Igeo and EF revealed that location 9 and location 10 were extremely enriched with all heavy metals. All location posed high ecological risk (0.5), except location1, which had moderate ecological risk. The outputs from this study are expected to provide the background levels of pollutants and help develop regional sediment quality guideline values in the present studied area.

KEY WORDS: HEAVY METALS, GEO-ACCUMULATION INDEX, POND SEDIMENT

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INTRODUCTION

Metals are important contaminants in marine environments that can be derived from natural and anthropogenic activities. Assessing metal contamination in marine sediments using ecological risk assessment approaches is important for aquatic site managers because of their abundance, toxicity, bioavailability, persistence and potential ecological risk. Sediment is often perceived as a scavenger of trace elements due to its ability to transport and accumulate trace elements. Determining the spatial distribution of trace elements in sediment is essential to provide basic information for the identification of hotspot areas and to prioritize site mitigation strategies (Li et al. 2012, Shafie et al 2013, Hossaina et al 2019, Zhang et al 2019).

Heavy metals may enter into aquatic ecosystems from anthropogenic sources, such as industrial waste water discharges, sewage waste water, fossil fuel combustion and atmospheric deposition (Linnik et al., 2000; Campbell et al., 2001; Lwanga et al., 2003). Distinguishing between anthropogenic and natural sources of elements in sediment is imperative because it is capable of determining the degree of pollution, safeguarding the health status of the aquatic system, and facilitating effective management of the coastal environment (Shafie et al. 2013). Thus, indexes such as the enrichment factor (EF) and geo-accumulation index (Igeo) are used as indicators to identify and quantity the degree of elemental pollution and to assess the intensity of anthropogenic contaminants accumulated in sediment (Barbieri 2016). Geo-accumulation indexing approach, I_{geo} is used to quantify the degree of anthropogenic contamination and compare different metals that appear in different ranges of concentration in the sludge (Muller 1969).

$$Igeo = \ln (Cn/1.5 \times Bn) \quad (1)$$

Where Cn= measured concentration, mg kg-1and Bn = geochemical background value, mg kg^{-1}.

In eqn. 1, average values were used and 1.5 is the factor used for lithologic variations of trace elements. The geo-accumulation index compares the measured concentration of the element in the fine-grained sludge fraction Cn with the geochemical background value Bn. Average values of soil samples of the study region (which is taken as reference point) are considered as Bn values. The index of geo-accumulation consists of seven grades, whereby the highest grade reflects 100-fold enrichment above background values (Praveena et al., 2008). Förstner et al. 1993 listed geo-accumulation classes and the corresponding contamination intensity for different indices Table 1.

In present study, the total concentration of heavy metal (i.e. As, Cd, Cr, Cu, and Pb), loads in soils samples collected is investigated. This study will helps in finding the contamination level of Heavy metals in various pond sediment in Raipur city. This study focused specifcally on risk assessment of sediment metal contaminants. Details about sediment metal analysis have been described in greater detail by Zhang et al. (2019).

Table 1. Geo-accumulation index classification

Sediment Igeo Contamination	Geoaccumulation class intensity	Index, I_{geo}
> 5	6	Very strong
> 4 - 5	5	Strong to very strong
> 3 - 4	4	Strong
> 2 - 3	3	Moderate to strong
> 1 - 2	2	Moderate
> 0 - 1	1	Uncontaminated to moderate
> 0	0	Practically uncontaminated

MATERIAL AND METHODS

Collection of sample: The samples were collected from different pond sediment of Raipur city. The samples were dried, grinded to a fine powder with mortar and passed through a sieve of 0.1 mm size.

Chemical and reagents: The AR grade (E. Merck) chemicals were used for digestion of the soil and plant samples. The ICP multi-element standard (E. Merck) and the European standard 13346:2000 EN 13346:46 were used for the quality control of the data.

Preparation of sample: The sludge samples air dried weighed and placed in a dehydrator at approximately 80°C for 48-72 hours depending on sample size. The samples were ground to a fine powder with mortar and passed through a sieve of < 0.1 mm mesh size. The weighed amount (0.5 g) of the sample was digested with 5 ml HNO_3 + 2 ml $HClO_4$ + 1 ml HF in the closed microwave oven as prescribed in the literature.

Analysis of sample: The Varian Liberty AX Sequential ICP-AES, were used for analysis of the trace metals in the sediment samples.

RESULTS AND DISCUSSION

Contents of heavy metals: The content of elements i.e. Cr, Cu, Pb, Cd, and Zn they ranged from Cr (6.4 - 15.3 mg kg^{-1}), Cu(8.5-14.2 mg kg^{-1}) Pb (0.8 -3.9 mg kg^{-1}), Cd (6.5-17.2 mg kg^{-1}) and Zn (6.4- 21.2 mg kg^{-1}). with

mean value 8.1, 8.8, 1.5, 8.9, and 7.4 mg kg^{-1}, respectively. Chromium concentration ranges from 6.4 - 15.3 mg kg-1with a mean value of 8.1 mg kg^{-1}. A moderately high positive correlation with Zn, Pb and Cu was established and its concentration falls also in moderately contaminated. Cr is a low-mobility element. Wastes and sewage waters unrestrainedly disposed from steel and textile industry facilities are the source of Cr in the study area. High doses of Cr cause liver and kidney damages and chromate dusts are known to be carcinogenic (Jumbe & Nandini, 2012).

Cadmium concentration ranges from 6.5-17.2 mgkg-1 with mean value 8.9 metal mg kg^{-1}. It is used as an anticorrosive, electroplated on steel; cadmium sulfide and selenide are commonly used as pigments in plastics, batteries and in various electronic components. It is also used with inorganic fertilizers produced from phosphate ores and when these products are no more servisable, they are thrown into the dump as waste. During decomposition, the Cd component is leached into the surrounding soil and over time gets accumulated in the soil. Cadmium is extremely toxic and the primary use of soil high in Cd in form of manure for the cultivation of vegetables and other food crops could cause adverse health effect to consumers such as renal disease and cancer (Gorenc et al., 2004). Moreover, when ingested by humans, cadmium accumulates in the intestine, liver and kidney and chronic exposure of Cd causes proximal tubular disease and osteomalacia (Pascual et al., 2004).

Therefore, the soils from this dumpsite are not suitable for agricultural purposes. Concentration in Copper varied from Cu(8.5-14.2 mg kg^{-1}) with an average value of 48.8 mg/kg. A moderately high positive correlation with Cd was established. Copper is widely used in electrical wiring, roofing, various alloys, pigments, cooking utensils, piping and in the chemical industries. Copper compounds are used in fungicides, algicides, insecticides, wood preservation, electroplating, dye manufacture, engraving, lithography, petroleum refining and pyrotechnics. It is also added to fertilizers and animal feeds as a nutrient to support plant and animal growth. Lead contamination ranges from 0.8 -3.9 mg kg^{-1} with mean value 1.5 mg kg^{-1}. Lead enters to human or animal metabolism either via food chain or by intake of soil dust. Gasoline vehicles are the main source of lead pollution. Lead is non essential for plants and animals and is toxic by ingestion-being a cumulative poison (MacFarlane & Burchett, 2002). Lead toxicity leads to anaemia both by impairment of haemobiosynthesis and acceleration of red blood cell destruction. In addition, Pb reduces sperm count, damages kidney, liver, blood vessels, nervous system and other tissues in human (Anglin-Brown et al., 1995).

Other uses of lead is in the production of lead acid batteries, solder, alloys, cable sheathing, pigments, ammunition, glass and plastic stabilizers. Tetraethyl and tetramethyl lead are important due to their extensive use as antiknock compounds in petrol (Mielke et al., 1991; McAllister et al., 2005). Zn concentrations ranges from 6.4- 21.2 mg kg-1 with mean value 7.4 mg kg^{-1}. Zinc exists as a variety of water-soluble salts. These are highly persistent in water with half-life greater than 200 days. Zinc and its salts have acute toxicity to aquatic life. Zinc acts as a catalytic or structural component in numerous enzymes, involved in energy metabolism and in transcription and translation. Zinc finds its industrial application as a coating on other base materials and acting as anti-corrosive substances (galvanising). It is also used in the manufacturing of brass, lightweight structures of aircrafts, batteries, paints and in textile industries (Gupta et al 2014).

Table 1. Geoaccumulation index (Igeo) of heavy metals in SOIL

Location	Igeo Cr	Igeo Cu	Igeo Pb	Igeo Cd	Igeo Zn
1	0.87	0.37	0.02	1.07	0.67
2	0.62	0.64	0.8	0.93	0.68
3	0.28	0.85	0.86	0.74	0.87
4	0.16	0.45	0.87	0.74	0.98
5	0.18	0.54	0..8	0.65	0.96
6	0.27	0.74	0.78	0.78	0.98
7	0.87	0.75	0.68	0.91	0.787
8	0.74	0.52	0.92	0.87	0.92
9	0.87	0.7	0.86	1.08	0.94
Mean	0.540	0.618	0.724	0.863	0.865
Max	0.87	0.85	0.92	1.08	0.98
Min	0.16	0.37	0.02	0.65	0.67

Geoaccumulation Index: The index of geoaccumulation (Igeo) was assessed based on the values proposed by Müller 1969 and their Igeo values estimated is found in the following increasing order: Cr ≈ Cu < Pb <As <Cd.

According to the Muller scale (Muller, 1981), the calculated results of Igeo values (Table 1, Figure 1) indicate that Zn (24%) and Cd (24%) can be considered as a strong pollutant at all study locations (Igeo >0.85. Pb contributes (20 %) with Igeo value 0.72. Cr and Cu both shows very less degree of pollution and the contamination level. The order of contribution of various heavy metals on the basis of Igeo follows: Zn = Cd > Pb > Cu>Cr. Data reveal except location 1 at all locations are from moderately to strong polluted through Zn and Cd. Although the nature of the Igeo calculation, which involves the logarithmic function and a background multiplication factor of 1.5, is somewhat different from other pollution calculation methods discussed in this study (Looi 2019). It is found that ore

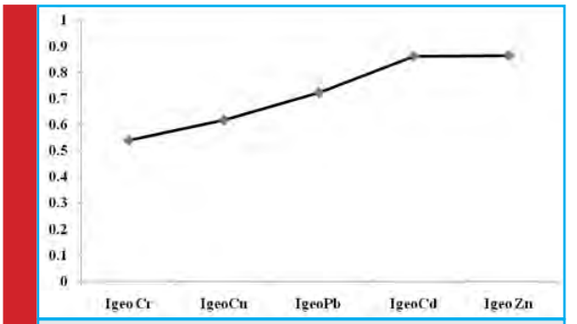

FIGURE 1. Igeo of heavy metals in sediment

extraction and processing and metallurgical industries stand atop the most polluting sources (Hossain et al, 2019, Zhang et al, 2019).

CONCLUSION

This study showed that most sediments samples exhibited low variation of metal distribution indicating similar and limited pollution sources. Heavy metal pollution is a nefarious issue with implications for life. In this study, geo-accumulation index were used for determining the environmental quality of sediment in terms of heavy metal accumulation. The sediment samples were suffering from moderately contaminate with the studied heavy metals according to Igeo values. The result revealed the following trend in their order of geo-accumulation in the sludge: Cr ≈ Cu << Pb < Cd < Zn. In near future, the whole aquatic and terrestrial environment may contaminate with the toxic elements. High concentrations of these trace metals may present potential health risk for the human populations residing in the vicinity of the studied area.

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Reverse Transcriptase Polymerase Chain Reaction: A Promising tool for Rapid Identification of *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis remains a major cause of morbidity & mortality globally. Rapid diagnosis of MTB is important for prevention of tuberculosis. Sputum smear microscopy does not differentiate between viable and dead bacilli. Ribosomal RNA (rRNA) based methods are one of the important tools for rapid detection of viable MTB from patients samples. The aim of the study was to detect MTB in Sputum samples of follow up patients of MTB by Reverse Transcriptase RT PCR and to analyze the results of RT PCR with smear microscopy. 211 follow up sputum Samples were received through the Revised National Tuberculosis Control Programme (RNTCP). RNA was extracted from culture isolates and then processed by Reverse Transcriptase RT PCR targeting 16SrRNA gene. Direct smear microscopy of all sputa were done prior to processing. The RT PCR assay showed overall 59.87% accuracy. Out of a total 211 samples, 66 (31.2 %) were positive and 145 (68.7) were negative for Reverse transcriptase RT PCR. Of 66 RT positive samples, 38 (57.5 %) were smear positive and 28 (42.4 %) were smear negative. Of 145 RT negative samples, 33 (15.6%) were smear positive and 112 (53%) were smear negative. RT PCR could detect viable MTB in smear negative samples with 57.88% sensitivity (CI 95%, 44.79% to 69.66%) and with 77.24% specificity (69.55% to 83.79%).To conclude,Reverse transcriptase RT PCR may prove to be a promising tool for early detection of *Mycobacterium tuberculosis*.

KEY WORDS: FOLLOW UP, MTB, SMEAR MICROSCOPY, REVERSE TRANSCRIPTASE, RT PCR, 16SRRNA

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INTRODUCTION

Tuberculosis (TB) remains a major cause of morbidity and mortality globally. Rapid diagnosis of TB is vital for prevention of further transmission of tuberculosis (PMDT 2017). Smear microscopy is a rapid, simple and inexpensive technique which is highly specific for the diagnosis of active tuberculosis. However, sputum smear microscopy has considerable limitations. The sensitivity is grossly compromised when the bacterial load is less than 10,000 organisms/ml sputum sample. Moreover, it does not differentiate between viable and dead bacilli (Honeyborne *et al.* 2011). Ribosomal RNA (rRNA) based methods are one of the important tools for rapid detection of viable MTB from patients samples. rRNA constitutes 80% of total RNA and is the most conserved region which is structurally more stable and have longer half - life, (Belasco *et al.* 1986, Desikan 2013, PMDT 2017 and WHO 2018). In the present study, in addition to the smear microscopy, rRNA based reverse transcriptase real time PCR targeting 16SrRNA gene has been used as a technique for rapid detection of *Mycobacterium tuberculosis*.

MATERIALS AND METHODS

A total of 211 sputum samples previously diagnosed as pulmonary tuberculosis were examined in the study. The study was approved by Institutional Ethics Committee (IEC), BMHRC. Smears of all sputum samples were prepared and stained with Ziehl–Neelsen stain to observe the presence of acid fast bacilli by bright field microscopy under the 100X objective. Grading of smears was performed as per the criteria defined by RNTCP (RNTCP 2018). The sputum samples were digested and decontaminated by N-acetyl-L cysteine-sodium hydroxide-Citrate method as per the guidelines by RNTCP (RNTCP 2018).

The sputum samples were cultured on LJ medium since culture is a gold standard. Extraction of RNA from the decontaminated sediments was performed by a commercially available RNA extraction kit (Nucleopore RNA isolation kit, Genetix Biotech Asia Pvt.ltd) as per the manufacturer's protocol. The synthesis of cDNA was carried out with a commercially available kit (High Capacity cDNA reverse transcription kit) as per the manufacturer's protocol . In brief, reaction mixture was prepared by adding 10x RT PCR Buffer-2ul, 25x Dntp Mix-0.8ul, 10x RT Random Primer-2ul, Multiscribe reverse transcriptase-1ul, RNAase Inhibitor-1ul, Nuclease Free Water was added to make the final reaction volume of 10ul. The PCR was carried out with the following cycling conditions: 370C for 120 minutes and then 850C for 5 minutes for 45 cycles.

Real Time PCR

The detection of MTB using 16SrRNA gene in sputum samples was analyzed by Light Cycler 2.0 (Roche Diagnostics, Meylan, France) using a commercially available kit (fast start Essential DNA Probe master mix, Roche Diagnostics, Meylan, France). Primers and probes were synthesized commercially as published in the previous literature (Juan *et al.* 2012) MTBC 16S forward (5'-GGGATGCATGTCTTGTGGTG-3') and MTBC 16S reverse (5'-CCGTCGTCGCCTTGGTAG-3') primers, which amplify 100 bp fragment of the 16SrRNA gene, and a 21 bp Taqman probe (5'-CGGGCTCATCCC ACACCGCTA-3') labeled at the 5' end with 6-carboxyfluorescein (6-FAM), and at the 3' end with the quencher N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA) (Invitrogen, Carlsbad, CA, USA) were used for RT PCR. The master mix was prepared by adding probe master-10ul, Forward primer-1ul, Reverse primer-1ul, Probe-1.6ul and Nuclease Free Water to make the final volume of 15ul. The Real Time PCR was run with the following cycling conditions, at 50^{0}C for 2 min to denature the DNA template, 95^{0}C for 10 min to activate the Taq polymerase, followed by 40 cycles at 95^{0}C for 15s.

RESULTS AND DISCUSSION

Out of a total of 211 samples, 140 (66.3%) were smear negative and 71 (33.6%) were smear Positive. Out of 211 samples, 66 (31.2 %) were positive and 145 (68.7%) were negative for MTB by reverse transcriptase RT PCR. Of 66 RT PCR positive samples, 38 (57.5 %) were smear positive and 28 (42.4 %) were smear negative. Of 145 RT PCR negative samples, 33 (22.7%) were smear positive and 112 (77.2%) were smear negative (Table 1). In our study 16S rRNA based RT PCR had 57.88% sensitivity (CI 95%, 44.79% to 69.66%) 77.24% specificity (CI 95%, 69.55% to 83.79%), and overall 71.09% accu-

Table 1. RT PCR and Microscopy (n=211)

	RT PCR Positive		RT PCR Negative		Total	
	No.	%	No.	%	No.	%
Smear Positive	38	57.5	33	22.7	71	33.6
Smear Negative	28	42.4	112	77.2	140	66.35
Total	66	31.2	145	68.7	211	

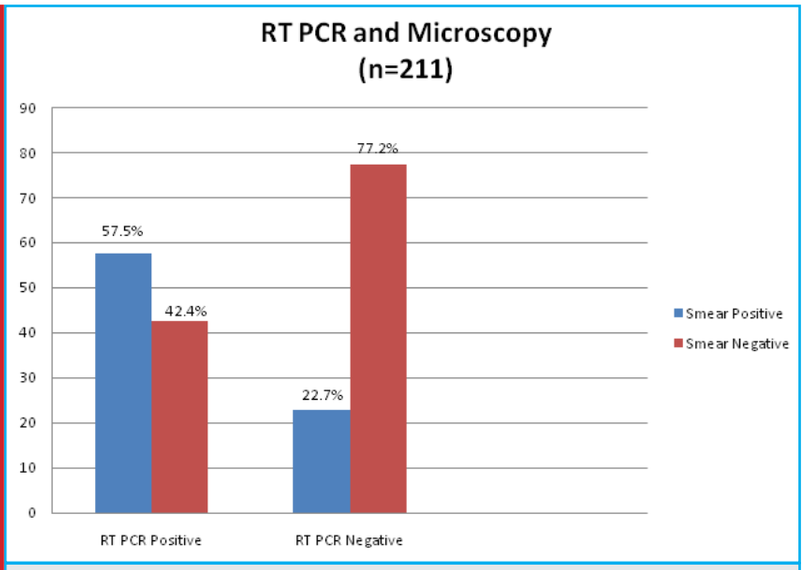

FIGURE 1. RT PCR and Microscopy

Table 2. Performance parameters of RT PCR with respect to Smear Microscopy

Method	Result	RT PCR Positive (n=211)	RT PCR Negative (n=211)	Sensitivity (%)	Specificity (%)	Positive Predictive value (%)	Negative Predictive value (%)	Accuracy (%)
Smear Microscopy	Positive	38	33	57.5	77.2	53.5	80	71.09
	Negative	28	112					

racy (CI 95%, 64.47% to 77.11%) for detection of MTB. (Table 1, Figure 1 and table 2). However smear microscopy had 49.30% sensitivity (CI 95%, 37.22% to 61.44%), 84.29% specificity (CI 95%, 77.18% to 89.88%), PPV 61.40% (CI 95%, 50.35% to 71.40%), NPV 76.62% (72.05% to 80.65%) and overall 72.51% accuracy. While both tests are rapid tests, performance characteristics of16S rRNA based real time PCR are significantly higher than that of smear microscopy.

In our study it was found that there were 3 samples which were culture positive but RT PCR negative. This may be due to the presence of PCR inhibitors in the sputum samples, mismatch of primer pairing or the presence of fragmented tubercle bacilli that might have resulted in suboptimal quality of RNA. The sensitivity of rRNA based RT PCR was found to be more over the culture in our study, indicated by 12 samples that were culture negative but RT PCR positive. Given the high sensitivity and specificity, molecular assays are widely accepted as a promising diagnostic tool for the detection of MTB (Woese 1987). rRNA constitutes 80% of the total RNA and is structurally more stable than messenger RNA(mRNA). Moreover, rRNA is present at 1000 - 10000 times more in copy numbers than genomic DNA, therefore, rRNA, particularly 16S rRNA, is a good target for the detection of MTB, (Woese 1987) from the clinical samples.

The detection of 16S rRNA is a reflection of the metabolic state of the total population of bacteria, therefore, the measurement of 16S rRNA can be used as an indicator of viability (Hellyer *et al.* 1999). The average turnaround time of our study was found to be three days. 16SrRNA based real time PCR therefore appears to be a promising tool for rapid identification of *Mycobacterium tuberculosis*. The challenge would be to make 16S rRNA based Real Time PCR available and accessible to the population that needs TB diagnosis but can afford it to least. Further studies and innovations to make it a cost effective test are the need of the hour.

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Significance of Accuracy Levels in Cancer Prediction using Machine Learning Techniques

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ABSTRACT

Across the world, any cancer becomes a calamity for a person who is suffering from it, mainly women are facing a real challenge when it comes to breast cancer. Breast cancer can be diagnosed at an early stage to overcome the consequences at a later stage. In the field of Computer Science, Machine Learning (ML) techniques are competent enough to diagnose the stages of cancer. ML techniques work upon the data which are collected from hospitals of suspected patients. There are various ML techniques which can build a model in order to diagnose cancer on the basis of finding accuracy level. In this paper, we have discussed the significance of accuracy level for predicting the cancer. In previous works, it has been observed that 100% accuracy is found on data analysis by some researchers. Although 100% accuracy must have given perfect prediction but it is observed that prediction was not so, sometimes it gives incorrect prediction also. So, prediction technique is scaled up with inclusion of more parameters precision, recall, F1- measure, Receiver Operating Characteristics (ROC) area and Area Under Curve (AUC) score.

KEY WORDS: ACCURACY, AUC, CANCER, F1-MEASURE, MACHINE LEARNING, PRECISION, RECALL, ROC

INTRODUCTION

Human body is made up of billions of cells and when cells start growing, becomes lumps and later on develops into tumor. Tumor is of two types: Malignant and Benign. Malignant is dangerous that causes another tumor and consequently cancer. Benign tumor usually does not cause cancer in short span of time. In this paper, there is a discussion of breast cancer which is due to internal or external disbalance of hormonal activities.

There are some traditional methods to diagnose and prognose the cancer but sometimes these methods take

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long time to diagnose. A Machine Learning is a field of Artificial Intelligence (AI) in computer science which can implement many computational intelligent techniques for the fast and accurate prediction of cancer. ML techniques can precisely predict different type of tumors. It motivated us to work on cancer prediction technique and working to find alternative parameters for better prediction.

This paper comprises of four sections. In section 1, Introduction of cancer has been described. Section 2 summarizes the previous related work. In section 3, study on various machine learning techniques have been presented. Section 4 presents the various vital parameters including accuracy for breast cancer prediction. In section 5, breast cancer prediction performance metrics is discussed. In section 6, experiments and results are shown and in the last section, conclusion and discussion are explained.

Ahmad et al. (2013) compared 3 ML techniques viz. Decision Tree (C4.5), ANN and SVM on Iranian Centre for Breast Cancer datasets of 1189 patient and found the accuracy as 93.6%, 94.7% and 95.7%. Ali et al. (2019) proposed a prediction model using "big data" to explore feature selection and cross-validation in omics file datasets. Further described anti-drug drug response modelling and predicting their phenotypic responses. Asri et al. (2016) compared different ML algorithms: SVM, C4.5, NB and kNN for WBCD dataset which has 699 instances and 11 integer-valued attributes. Among all algorithms, SVM gave the highest accuracy 97.13% with lowest error rate conducted in WEKA data mining tool.

Bevilacqua et al. (2006) used IDEST novel approach based on ANN for WBCD datasets and found 98.6% accuracy. Boughorbel et al. (2017) focused on another metric known as Matthews Correlation Coefficient (MCC) to handle imbalance data using SVM and Bayes classifier. Burt et al. (2018) diagnosed a breast cancer with deep learning network using a system Computer-aided detection and diagnosis (CAD). It mainly looks for images captured by MRI, X-rays and compared with human expertise like radiologists, clinicians etc. Bychkov et al. (2018) took a sample of 420 images of colorectal cancer and apply deep learning outperformed AUC 0.69 which is better than AUC 0.58 and AUC 0.57 preformed by human expert and whole -slide level respectively.

Chaurasia et al. (2014) explained data mining techniques to predict cancer when they applied RepTree (C4.5), RBF Network and Simple Logistics on 286 samples and found accuracy as 71.32%, 73.77% and 74.47% respectively. Coudray et al. (2018) used a model deep convolutional neural network (inception v3) on whole-slide images obtained from The Cancer Genome Atlas (TCGA) and obtained an improved AUC score from 0.733 to 0.856 in the detection of cancer subtype.

Elgedawy et al. (2017) applied 3 machine learning techniques: Naïve Bayes, SVM and RF. Out of them RF is the most appropriate and useful algorithm to give the best accuracy as 99.42% where SVM and NB produced 98.8% and 98.24% accuracy respectively. Huang MW et al. (2017) used SVM ensemble classifier along with boosting method and RBF kernel based SVM to predict the accuracy in cancer dataset. In case of small-scale dataset, GA+RBF SVM ensembled with boosting method and produced 98.28% accuracy whereas in large dataset, RBF SVM ensembled with boosting method produced 99.52% accuracy.

Nguyen et al. (2013) shown experiments on two datasets WBCD (diagnosis) & WBCP (prognosis) from Wisconsin Breast Cancer Dataset and claimed for 99.8% and 99.7% accuracy.Pirooznia et al. (2007) compared many ML techniques and found 100% accuracy when they applied SVM-RFE on 84 sample which is a less size of data.

Sahu et al. (2012) proposed a novel approach using PSO along with SVM and k-NN ML techniques applied on the size of 87 sample and found 100% accuracy. Sivakami et al. (2015) proposed a hybrid technique DT-SVM to forecast cancer prediction for the dataset of 699 instances of WBCD repository and obtained accuracy as 91%.

Steiner et al. (2018) shown a impact of deep learning assistance on lymph of breast cancer and found micro metastases in the images with a range from 0.02, 0.002, 0.018 and 0.0005. Lower the value of micro metastases like 0.0005, higher the accuracy in lymph node of breast cancer. Xiao et al. (2018) discussed a multi-model ensemble method based on deep learning to find the accuracy and effective of different classifier. The data were supplied in the form of gene expression. This method was tested on three public RNA-seq data sets.

MACHINE LEARNING TECHNIQUES

Machine Learning (ML) is a part of AI. ML is used to infer the knowledge from the behavior of data. There are many areas where ML can be applied. In this paper we are discussing the cancer related issues. So, ML uses the techniques to generalize the biological sample of a given datasets. Following popularly used ML techniques have been introduced briefly.

Decision Tree

A Decision Tree (AL-SALIHY et al. 2017, Yue et al. 2018, Ponnuraja et al. 2017) is a binary classifier used to take the decision on attributes of the dataset. It looks like the tree but it is an inverted tree.

Random Forest

A Random Forest (Okun et al. 2007, Nahid et al. 2017, Ghongade et al. 2018) is another classifier, based on

decision tree, which is a next step when multiple decision tree resides together.

Support Vector Machine

Support Vector Machine (SVM) is a very effective classifier that classify the feature's outcome in two categories with a hyperplane having distance between the samples. (Huang MW et al. 2017, Sweilam et al. 2010, Sewak et al. 2007)

k-Nearest Neighbor (kNN)

kNN (Pawlovsky 2017, Rodriguez et al. 2018, Meneses et al. 2019, Al-Hadidi et al. 2016) is an essential classifier which makes a group of similar patterns with dataset sample. In general, the value of k is randomly put from 1 to 5 or extends up to 11 depends on how many neighbors are needed for grouping. There are various distance measurement mathematical equations such as Euclidean, Manhattan, Minkowski, Chebyshev, Cosine Similarity, Cosine Distance to find the distance between the neighbor.

Naïve Bayes Classifier

This classifier is based on Bayes' theorem (Maysanjaya et al. 2018, Rashmi et al. 2015, Soria et al. 2008) which takes into consideration of independent feature of the data. Dependent feature creates more correlation effect in prediction.

SIGNIFICANCE OF LEVEL OF ACCURACY FOR BREAST CANCER PREDICTION

There are many ML techniques for cancer diagnosis and prognosis. Many researchers have calculated the level of accuracy in percentage using different ML techniques such as Random Forest, SVM, Naïve Bayes, Decision Tree (Nguyen et al. 2013, Ahmad et al. 2013, Chaurasia et al. 2014, Sivakami et al. 2015, Elgedawy et al. 2017) for cancer prediction as shown below in table 1 for breast cancer datasets.

Table 1. ML Techniques' Accuracy level for prediction of Breast Cancer

Type of Cancer: Breast Cancer			
S. No.	**ML Technique**	**Sample**	**Accuracy**
1	Random Forest	699	99.82%
2	DT-SVM	699	91%
3	Random Forest	699	99.24%
	SVM		98.8%
	Naïve Bayes		98.24%
4	Decision Tree (C4.5)	1189	93.6%
	ANN		94.7%
	SVM		95.7%
5	RepTree (C4.5)	286	71.32%
	Radial Basis Function Network		73.77%
	Simple Logistic		74.47%

Table 2. Level of accuracy of ML Techniques for small data size

S. No.	ML Technique	Types of Cancer	Sample	Accuracy
1	SVM-RFE	Breast Cancer	84	100%
2	PSO-KNN	Breast Cancer	97	100%
	PSO-SVM			

On the basis of above results, it is not easy to say that a particular Machine Learning technique is fit suitably for the diagnosis of breast cancer for a particular dataset on the basis of level of accuracy only because 100% accuracy comes in underfit condition where drawn conclusion of cancer prediction can't be correct.

Following table 2 (Pirooznia et al. 2007, Rajeshwari et al. 2011, Sahu et al. 2012, Gunavathi et al. 2014) shows 100% accuracy level using some other ensemble Machine Learning techniques on selected datasets where 100% accuracy is not predicting cancer correctly.

Table 3. Performance parameter metrics for dataset *BCWD11

Algorithm	Confusion Matrix Components				Performance Parameters (*BCWD11)					
	True Positive (TP)	True Negative (TN)	False Positive (FP)	False Negative (FN)	Accuracy	Precision	Recall	F-Measure	MCC	ROC Area
Naïve Bayes	436	235	22	6	95.99%	96.2%	96%	96%	0.914	98.6%
SVM (SMO)	445	231	13	10	96.7%	96.7%	96.7%	96.7%	0.927	96.5%
KNN (IBK)	443	222	15	19	95.13%	95.1%	95.1%	95.1%	0.892	94.5%
Decision Tree (J48)	438	223	20	18	94.56%	94.6%	94.6%	94.6%	0.880	95.5%
Random Forest	444	230	14	11	96.42%	96.4%	96.4%	96.4%	0.921	99%

Table 4. Performance parameter metrics for dataset **WBCD32

Algorithm	Confusion Matrix Components				Performance Parameters (**WBCD32)					
	True Positive (TP)	True Negative (TN)	False Positive (FP)	False Negative (FN)	Accuracy	Precision	Recall	F-Measure	MCC	ROC Area
Naïve Bayes	190	337	22	20	92.61%	92.6%	92.6%	92.6%	0.842	97.6%
SVM (SMO)	201	356	11	1	97.89%	97.9%	97.9%	97.9%	0.955	97.3%
KNN (IBK)	200	347	12	10	96.13%	96.1%	96.1%	96.1%	0.917	95.6%
Decision Tree (J48)	194	335	18	22	92.97%	93%	93%	93%	0.85	92.3%
Random Forest	196	350	16	7	95.95%	96%	96%	95.9%	0.913	99.1%

BREAST CANCER PREDICTION METRICS

The basis of performance parameter metrics is confusion matrix and then metrics such as accuracy, precision, recall, f-measure, Mathew's Correlation Coefficient (MCC), is calculated (Kourou et al. 2015, Baker 2003, Yang et al. 2017, Tilaki 2013).

CONFUSION MATRIX

A Confusion matrix is a summary of prediction results on a classification problem. In it, the number of correct and incorrect predictions are summarized with count values and broken down by each class, the concept is shown in table 5.

On the basis of above table, various metrics parameter has been defined below.Accuracy (Barlow et al. 2004, Tharwat 2018) is the number of correct predictions divided by total number of predictions made. Mathematically accuracy (Acc) is given by the following formula

$$Acc = \frac{TP + TN}{TP + TN + FP + FN}$$

Sometimes accuracy yields the same value with respect to multiple classifiers and this makes more complication with Error Rate (ERR) or misclassification rate (Jensen et al. 2010, Barlow et al. 2004, Tharwat 2018). Error Rate can be calculated as below

$$ERR = 1 - Acc = 1 - \frac{TP + TN}{TP + TN + FP + FN}$$

Table 5. Confusion Matrix

		Actual	
		Valid	Not Valid
Predicted	Accept (Recurrence)	True Positive (TP)	False Positive (FP)
	Reject (No Recurrence)	False Negative (FN)	True Negative (TN)

Sometimes because of accuracy paradox, accuracy is not sufficient to find the best model. Improving the accuracy by reducing the error is not appropriate. Therefore, ROC Curve and AUC score are better option to use for prediction instead of using the only parameter accuracy.

In this paper other additional parameter such as ROC and AUC metrics have been calculated to find the actual best suitable fit model.

ROC AND AREA UNDER CURVE (AUC)

ROC (Baker 2003, Yang et al. 2017, Tilaki 2013) curve demonstrates the tradeoff between the true positive fraction and false positive fraction to evaluate the positivity. AUC (Yang et al. 2017, Tilaki 2013) is a measure of the model's performance which is based on the ROC curve. This curve plots two parameters: True Positive Rate & False Positive Rate. Both the parameters are defined below.

True Positive Rate (TPR), termed as sensitivity also, is the fraction of positives correctly classified divided by total positives and is defined as below

$$\text{True Positive Rate, } TPR = \frac{TP}{TP+FN}$$

False Positive Rate (FPR) is the fraction of negative incorrectly classified divided by total negatives and is defined as below

$$\text{False Positive Rate, } FPR = \frac{FP}{FP+TN}$$

AUC is an effective and combined measure of TPR and FPR that describes the inherent validity of diagnostic tests (Kouruo et al. 2015). In below figure 1, FPR and TPR are represented by x-axis and y-axis respectively. The ROC indicates the curve of value ranging from 0 to 1 whereas AUC shows the area under curve. Dashed-line partitions True values and False value. True values lie above the dashed line and False values lie below the line.

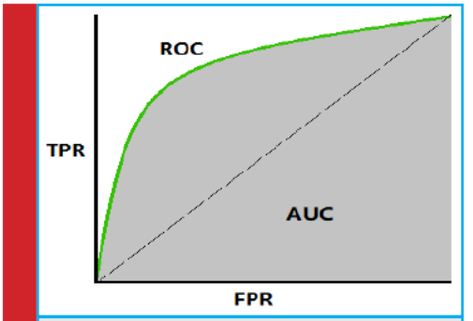

FIGURE 1. Indicating ROC Curve and AUC

RESULTS AND DISCUSSION

The experimental setup is designed under the environment of Windows 10 operating System, Python 3.x version and smart IDE Spyder which is a unit of Anaconda distribution. The dataset has been used from Wisconsin Breast Cancer Dataset having 10 major features in *BCWD11 and 30 major features in **WBCD32 (Kumar et al. 2019). The respective performance measurement metrics is calculated on the basis of confusion matrix given in table 3 & table 4 respectively.

On the basis of TPR and FPR, the ROC curve is plotted for all machine learning classifiers to obtain AUC score. The following graph is showing the AUC score in figure 3 below.

On the basis of fact that higher the value of AUC near to 1, the model is considered to be the best. In figure 2, it is observed that the best model for said dataset is Random Forest classifier, has the highest value of AUC i.e. 0.97.In similar fashion, another experiment has been performed for **WBCD32 and performance of Random Forest classifier is again observed as the best model for AUC score 0.98 shown in figure 3 below.

This paper discussed the confusion matrix and performance parameters useful for prediction. Parameters are accuracy, precision, F-measure, MCC and ROC area.

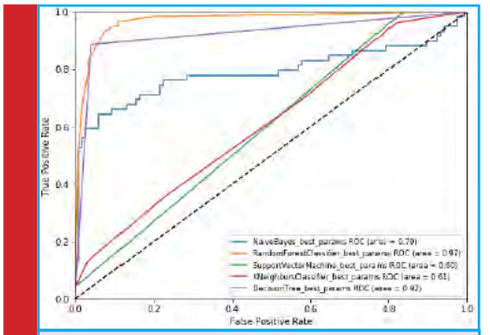

FIGURE 2. AUC score for *BCWD11

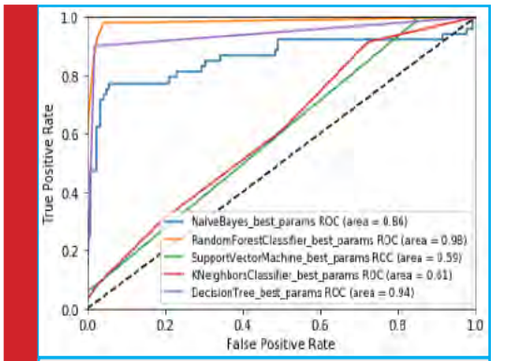

FIGURE 3. AUC score for **WBCD32

Based on all the parameters, the outcome of this paper was to find out the AUC score whereas in previous works, only accuracy was calculated.

We focused to locate the best fit model for selected breast cancer dataset. In general, it is observed that only accuracy is used for cancer prediction. But accuracy does not give perfect prediction. It is identified that AUC score is significant to consider for correct prediction of breast cancer instead of the only parameter 'accuracy'. Random Forest classifier found fit perfectly for the used dataset among all other four classifiers viz Naïve Bayes, SVM, kNN and Decision Tree. There are also few more considerable measurement metrics such as Youden's index (YI), Discriminant power (DP), Balanced classification rate (BCR), Optimization precision (OP), Jaccard (or Tanimoto similarity coefficient), and many more. In future, we intend to do feature engineering for better prediction of breast cancer.

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Utilization of Agro-industrial By-products for Production of Lipase Using Mix Culture Batch Process

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ABSTRACT

Lipase or triacylglycerol acyl ester hydrolases belong to serine hydrolase family also called as carboxylic acid esterases, whose number is EC 3.1.1.3 according to enzyme-commission that helps to break the bond by reaction with water. Microbial lipase production is preferable than plants and animals because of the more rapid growth of microbes, ease to genetic manipulation, requires low-cost media, stability and their specific properties. Currently, microbial lipase has many applications in the industrial field. Agro-industrial by-products are used in the biotechnology field because it contains carbon, nitrogen, minerals and other nutrients, and they are of low cost. Production of lipase by micro-organism is carried out with the help of agriculture by-product using them as substrate.In mix culture experiment in case of each substrate, maximum lipase activity was obtained using mustard oil cake (10.199458 U/ml), sesame oil cake (10.6731 U/ml), linseed oil cake (9.947174941 U/ml), and soybean oil cake (9.5716 U/ml). Overall mix culture experiment, sesame oil cake gave highest lipase activity.

KEY WORDS: AGRICULTURE RESIDUES; MUSTARD OIL CAKE, LINSEED OIL CAKE, SESAME OIL CAKE, AND SOYBEAN OIL CAKE; OPTIMIZATION; LIPASE; *MIX CULTURE*; SUBMERGED FERMENTATION

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INTRODUCTION

Lipase or triacylglycerol acyl ester hydrolases belong to serine hydrolase family also called as carboxylic acid esterases, whose number is EC 3.1.1.3 according to enzyme-commission that helps to break the bond by reaction with water. Protease, amidase, glucosidase, nitrilases, epoxide enzyme also belongs to a hydrolase family (Patrick Fickers et al., 2011) (Gupta et al., 2004); (Hasan et al., 2006); (Hasan et al., 2009); (Jaeger & Eggert, 2002); (Salihu et al., 2012). Conventionally, lipase enzyme was obtained from animal sources. Lipase enzyme was firstly discovered by Claud Bernard in 1856 in pancreatic juice, which converts the insoluble oil-fats to the soluble product (Hasan et al., 2006). Pancreatic lipase is present with many other enzymes such as trypsin, which gives bitter taste with undesirable effect. Lipase enzyme easily extracted from plants but the process in fermenter performed is complicated and increase the production cost. So plant lipase has not used for commercial application. Microbial lipase production is preferable than plants and animals because of the more rapid growth of microbes, ease to genetic manipulation, requires low-cost media, stability and their specific properties (Rocha, Padez, & Morais, 1998). First lipase production was carried out by *Bacillus prodigiosus*, *Bacillus pyocyaneus*, *and Bacillus fluorescent* in 1901. Maximum lipase production by the microorganism is based on different strains and characteristics, specificity, stability, performance, and mechanism of action (Bornscheuer et al., 2013) (Sharma et al., 2011, Geoffry and Archer 2018).

So the problems of lipase production by animals and plants overcome by microbial lipase. Microbial lipase attracted more attention due to low production cost and has economic importance. Now a day, the demand for lipase is fulfilled from micro-organism like bacteria, yeast, fungi, and actinomycetes, which produce vast diversity of extracellular lipase (Sharma et al., 2011). Fermentation process for the lipase production by SmF and SSF in the last few years. Submerged fermentation and solid state fermentation technique are the most common and conventional process used for lipase production which has an advantage over the other processes (Sun & Xu, 2008). Agro-industrial by-products are used in the biotechnology field because it contains carbon, nitrogen, minerals and other nutrients, and they are of low cost. Production of lipase by micro-organism is carried out with the help of agriculture by-product using them as substrate. Waste as substrate contains a high amount of nutrients and minerals that are essential for the growth of microorganism. Sometimes substrates act as an inducer for microbial growth. The substrate that has an essential lipid component required for the production of lipase is called an ideal substrate. Otherwise, assemble essential component then provides the value-added supplements for the growth of microorganism (Pandey et al., 1999) (Mitchell et al., 2000) (Martínez-Herrera et al., 2006). Much agriculture residual are converted to renewable product for using as substrate for lipase production using microorganism. Lipids are present in oil-cake after extraction of oil in industries (Singhania et al., 2008 Hasan et al 2018).

Currently, microbial lipase has many applications in the industrial field. Esterification and transesterification reaction demand is increasing day by day because the lipase enzyme has performed the mechanism in non-aqueous media condition. (Mendes et al., 2012) (Hasan et al., 2006) (Sun et al., 2013) (Kapoor & Gupta, 2012). Microbial lipase has broad application in another biotechnological field such as leather, textile, pulp and paper, cosmetics, and fat- oil industries.

MATERIALS AND METHODS

Culture procuration

Bacillus licheniformis MTCC 3244 and *Bacillus coagulans* MTCC 10305 was procured from Microbial Culture Collection and gene bank (MTCC), Institute of Microbial Technology, Chandigarh, India. Bothstock cultures were maintained on media composition containing (g/l) Yeast extract, 2; Beef extract, 1; Peptone, 5; NaCl, 5; Agar, 15.

Substrates collection & pretreatment

Mustard oil cake, linseed oil cake, sesame oil cake and soybean oil cake were collected from the local market at Kalyanpur, Kanpur (U.P.). Cakes were dried in a hot air oven at 75˚C for 2 to 3 days and were ground in a mixer. Cakes were stained by strainer (0.5mm) to obtain fine powder form. The powder form of cakes was stored in airtight containers. Powder form cakes were then used as substrate.

Lipase production by submerged fermentation

Lipase production using selected strain *Bacillus licheni-fomis* MTCC3244 and *Bacillus coagulans* 10305 was carried out by submerged fermentation. A semi-synthetic liquid medium containing (%) Glucose, 1; Peptone, 1; Yeast extract, 0.5; MgSO4.7H2O, 0.05; KCl, 0.05; FeSO4.7H2O, 0.001; Olive oil, 0.3%; Substrate (cake oil), 2.5 (Gutarra et al., 2009) was adjust the different pH then sterilized by autoclaving at 121˚C,15 psi for 15 min. Flasks were inoculated with of 0.25% *Bacillus licheni-fomis* MTCC3244 and *Bacillus coagulans* 10305 was added 20 ml of production medium (in Erlenmeyer flask of 100 ml volume). The Erlenmeyer flasks were incubated at different pH, temperature (˚C) and inoculum concentra-

Table 1. Lipase producing microorganisms with the production process and lipase activity

S. No.	Micro-organism	Lipase activity	References
1.	*Yarrowia lipolytica YlLip2*	42900	(Yu, Wen, & Tan, 2010)
2.	*Candida cylindracea* CBS786, *Candida rugosa* CBS2275, *Yerrowia lipolytica* W29 (ATCC20460)	30 U/L/h 20 U/L/h 7 U/L/h	(Gonçalves, Oliveira et al., 2012)
3.	*Pseudomonas aeruginosa*	204.12 U/mg	(Zouaoui & Bouziane, 2012)
5.	Bacteria SSB1N	0.1128 µg/ml/min	(N. A. Hasan et al., 2018)
6.	*Serratia marcescens* ECU1010	640 U/g	(Long, Xu, & Pan, 2007)
7.	*Garbage lipase enzyme*	57.43 U/ml	(Selvakumar & Sivashanmugam, 2017)
8.	*Pseudomonas aeruginosa*	60 U/ml	(Saravanan et al., 2007)
9.	*Fusarium solani* NFCCI 4084	7.8 U/ml	(Geoffry & Achur, 2018)
10..	Thermomyces lanuginosus (GSLMBKU-10, GSLMBKU-13, GSLMBKU-14)	205.80 µg/ml, 225.30 µg/ml, 165.23 µg/ml	(Sreelatha et al., 2017)
11.	*Aspergillus niger J-1*	1.46 IU/ml In SmF, 4.8 IU/ml in SSF	(Falony et al., 2006)
12.	*Aspergillus niger* AS-02	49.37 U/g	(Salihu et al., 2016)
13.	*Yarrowia lipolytica* (CECT 1240)	57.9 U/cm^3	(Domínguez et al.,2003)
14.	Candida cylindracea (NRRL Y-17506)	9.231 IU/ml	(D'Annibale et al., 2006a)
15.	*Bacillus licheniformis* 016	1870 U/L	(Baltaci et al., 2018)
16.	*E.coli* BL21 (DE3)	206 U/ml	(Chai et al., 2018)
17.	*Penicillium simplicissimum*	44.8 U/g	(Godoy et al., 2009)
18.	*Penicillium simplicissimum*	30 Ugd/s	(Asenjo & Andrews, 2008)
20.	*Bacillus subtilis*	4.96 U/ml	(Suci et al., 2018)
21.	*Penicillum restrictum*	30.3 U/g	(Gombert, Pinto et al., 1999)
22.	*Aspergillus oryzae* NCIM 1212, *Aspergillus japonicas* MTCC 1975	18.9 U/g 23 U/g	(Jain & Naik, 2018)
23.	*Bacillus stratosphericus* PSP8	47 U/ml	(Ismail et al., 2018)
24.	*Yarrowia lipolytica*	68.03 U/g	(da S. Pereira et al., 2019).
25.	*Bacillus coagulans*	78069 U/g	(Alkan et al., 2007)
26.	*Rhodotorula glutinis* HL25	75.2 U/l	(Taskin et al., 2016)
27.	*Penicillium* gracilenta CBMAI 1583	1.62 U/ml	(Turati et al., 2019)
28.	*Bacillus cereus*	117.3±20 U/ml	(Vasiee et al., 2016)
29.	*Penicillium* P58 and P74	139.2 U lipase/g 140.7 U lipase/g	(Rigo et al., 2010)

tion (%) on rotary shaker at 100 rpm. Each 1 ml samples were then centrifuged for 5 min at 12000 rpm. The supernatant was collected. The absorbance of the culture was measured by spectrophotometer at wavelength 410 nm. Maximum lipase activity was achieved by different parameters pH (4, 5, 6, 7, 8, 9), temperature (35˚C, 40˚C, 45˚C, 50˚C) and inoculum concentration (5%, 10%, 15%). Medium optimization studies were carried out by studying one-factor-at-a-time and all factors were kept same.

Lipase activity determination

The activity of extracellular lipase was measured by an assay to measure the amount of para-nitophenol (PNP) formed from p-nitrophenol acetate (pNPA). In the assay, 2.87 ml of 100 mM potassium phosphate buffer (KPB) at pH 7 was added to the 100 µl culture supernatant in the test tube. After preincubation at 30˚C for 3 minute, the reaction was started by quick mixing the solution with 30 µl of 100 mM p-NPA solution in DMSO. After 10 min-

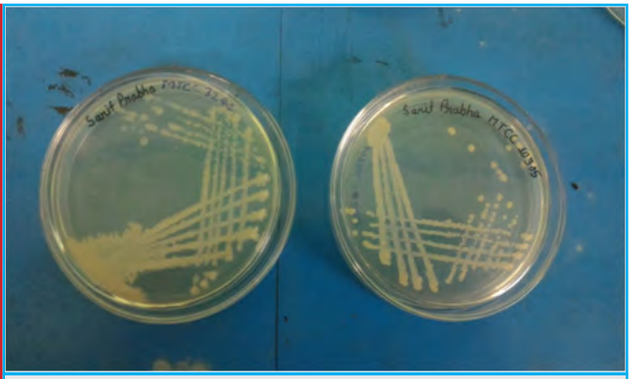

FIGURE 1. Growth of *Bacillus licheniformis* MTCC 3244 and *Bacillus coagulans* MTCC 10305

pH	OD at different time interval (410nm)			
	24 Hr	Activity of lipase (U/ml)	48Hr	Activity of lipase (U/ml)
4	0.449	2.3118	0.321	1.65275
5	0.623	3.2077	0.354	1.8227
6	0.899	4.62875	0.867	4.464
7	1.063	5.473	0.661	3.40335
8	1.807	9.304	1.928	9.9269
9	1.562	8.04245	1.632	8.40285

FIGURE 2. Lipase activity of mix culture at different pH using mustard oil cake as substrate

pH	OD at different time interval (410nm)			
	24 Hr	Activity of lipase (U/ml)	48Hr	Activity of lipase (U/ml)
4	0.612	3.15105	0.773	3.98
5	0.66	3.3982	0.801	4.1242
6	0.673	3.46515	0.837	4.30955
7	1.362	7.01265	1.342	6.9097
8	1.617	8.32535	1.892	9.74155
9	1.492	7.682	1.532	7.88795

FIGURE 3. Lipase activity of mix culture at different pH using linseed oil cake as substrate

pH	OD at different time interval (410nm)			
	24 Hr	Activity of lipase (U/ml)	48Hr	Activity of lipase (U/ml)
4	0.484	2.492	0.632	3.25405
5	0.205	1.0555	0.493	2.53835
6	0.684	3.5218	1.324	6.817
7	1.857	9.5613	1.699	8.7478
8	2.013	10.3646	1.681	8.65515
9	1.108	5.70485	0.521	2.6825

FIGURE 4. Lipase activity of Mix cultureat different pH using sesame oil cake as substrate

pH	OD at different time interval (410nm)			
	24 Hr	Activity of lipase (U/ml)	48Hr	Activity of lipase (U/ml)
4	0.484	2.492	0.213	1.0967
5	0.708	3.64535	0.504	2.595
6	0.728	3.74835	0.739	3.80495
7	0.968	4.98405	1.312	6.75525
8	1.859	9.5716	1.727	8.892
9	1.521	7.83135	1.632	8.40285

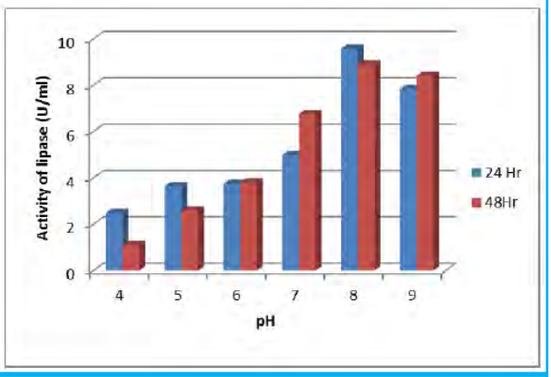

FIGURE 5. Lipase activity of Mix cultureat different pH using soybean oil cake as substrate

ute, change in absorbance at 410 nm was recorded with a spectrophotometer. Amount of PNP formed was calculated by using standard prepared at different dilutions of PNP. One lipase unit was defined as amount of lipase enzyme required to convert 1µmole of pNPA to PNP per minute under above condition (Long, Xu, & Pan, 2007).

RESULTS & DISCUSSION

Culture procuration

Optimization of process parameter by using the one-factor-at-a-time (OFAT) method pH Lipase activity of

Temp. (℃)	OD at different time interval (410nm)			
	24 Hr	Activity of lipase (U/ml)	48Hr	Activity of lipase (U/ml)
35	1.171	6.02925	1.095	5.63795
40	1.308	6.73465	1.265	6.51325
45	1.155	5.94685	1.065	5.48345
50	0.408	2.1007	0.372	1.91535

FIGURE 6. Lipase activity of mix culture at different temperature using mustard oil cake as substrate

Temp. (℃)	OD at different time interval (410nm)			
	24 Hr	Activity of lipase (U/ml)	48Hr	Activity of lipase (U/ml)
35	1.166	6.0035	1.189	6.1219
40	1.495	7.69745	1.313	6.76035
45	1.428	7.3525	1.244	6.4051
50	0.318	1.6373	0.262	1.349

FIGURE 7. Lipase activity of mix culture at different temperature using linseed oil cake as substrate

Temp. (°C)	OD at different time interval (410nm)			
	24 Hr	Activity of lipase (U/ml)	48Hr	Activity of lipase (U/ml)
35	1.233	6.34845	1.196	6.15795
40	2.073	10.6731	1.881	9.6849
45	1.38	7.10535	1.244	6.4051
50	0.441	2.2706	0.416	2.1419

FIGURE 8. Lipase activity of Mix cultureat different temperature using sesame oil cake as substrate

mix culture at different pH using mustard oil cake as substrate

As shown in table 2, maximum lipase activity was observed at pH 8 that was 9.9269 U/mlafter 48 hr.

Lipase activity of mix culture at different pH using linseed oil cake as substrate

As shown in table 3, Maximum lipase activity was observed at pH 8 that was 9.74155 U/mlafter 48 hr.

Lipase activity of Mix cultureat different pH using sesame oil cake as substrate

As shown in table 4, Maximum lipase activity was observed at pH 8 that was 10.3646 U/mlafter 24 hr.

Lipase activity of Mix cultureat different pH using soybean oil cake as substrate

As shown in table 5, Maximum lipase activity was observed at pH 8 that was 9.5716 U/mlafter 24 hr.

Temperature

Lipase activity of mix culture at different temperature using mustard oil cake as substrate

As shown in table 6, Maximum lipase activity was observed at 40˚C that was 6.73465 U/mlafter 24 hr.

Lipase activity of mix culture at different temperature using linseed oil cake as substrate

As shown in table 7, Maximum lipase activity was observed at 40˚C that was 7.69745 U/mlafter 24 hr.

Lipase activity of Mix cultureat different temperature using sesame oil cake as substrate

As shown in table 8, Maximum lipase activity was observed at 40˚C that was 10.6731 U/mlafter 24 hr.

Lipase activity of Mix cultureat different temperature using soybean oil cake as substrate

Temp. (°C)	OD at different time interval (410nm)			
	24 Hr	Activity of lipase (U/ml)	48Hr	Activity of lipase (U/ml)
35	1.169	6.01895	1.144	5.89025
40	1.607	8.2741	1.586	8.166
45	1.549	7.9755	1.323	6.81185
50	0.149	0.76715	0.291	1.4983

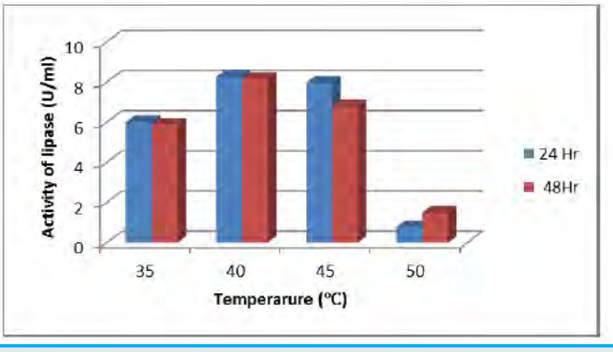

FIGURE 9. Lipase activity of Mix cultureat different temperature using soybean oil cake as substrate

Inoculum concentration	OD at 24 hr (410)	
	24hr	Activity of lipase (U/ml)
5%	1.743	8.97408174
10%	1.981	10.19945836
15%	1.856	9.555878204

FIGURE 10. Lipase activity of mix culture at different inoculum concentration using mustard oil cake as substrate

Inoculum concentration	OD at 24 hr (410)	
	24hr	Activity of lipase (U/ml)
5%	1.721	8.860811632
10%	1.932	9.947174941
15%	1.831	9.427162172

FIGURE 11. Lipase activity of mix culture at different inoculum concentration using linseed oil cake as substrate

As shown in table 9, Maximum lipase activity was observed at 40˚C that was 8.2741 U/mlafter 24 hr.

Inoculum concentration: Lipase activity of mix culture at different inoculum concentration using mustard oil cake as substrate

As shown in the table 10, Maximum lipase activity is observed at 10% that was 10.19945836 U/mlafter 24 hr.

Lipase activity of mix culture at different inoculum concentration using linseed oil cake as substrate

As shown in the table 11, Maximum lipase activity was observed at 10% that was 9.947174941 U/mlafter 24 hr.

Lipase activity of mix culture at different inoculum concentration using sesame oil cake as substrate

As shown in the table 12, Maximum lipase activity was observed at 10% that was 9.62281054 U/mlafter 24 hr.

Lipase activity of Mix cultureat different inoculum concentration using soybean oil cake as substrate

As shown in table 13, Maximum lipase activity is observed at 10% 7.686921421 U/mlafter 24 hr.

CONCLUSION

Both strains *Bacillus licheniformis* MTCC 3244 and *Bacillus coagulans* MTCC 10305 showed the lipase activity. In mix culture experiment in case of each substrate, maximum lipase activity was obtained using mustard oil cake (10.199458 U/ml) at pH 8 maintaining at temperature 40˚C with 10% inoculum concentration after 24 hr, sesame oil cake (10.6731 U/ml) at pH 8 maintaining at temperature 40˚C with 0.5% inoculum concentration after 24 hr, linseed oil cake (9.947174941 U/ml) at pH 8 maintain-

Inoculum concentration	OD at 24 hr (410)	
	24hr	Activity of lipase (U/ml)
5%	1.634	8.412879841
10%	1.869	9.62281054
15%	1.703	8.768136089

FIGURE 12. Lipase activity of mix culture at different inoculum concentration using sesame oil cake as substrate

Inoculum concentration	OD at 24 hr (410)	
	24hr	Activity of lipase (U/ml)
5%	1.321	6.801355122
10%	1.493	7.686921421
15%	1.398	7.1978005

FIGURE 13. Lipase activity of Mix cultureat different inoculum concentration using soybean oil cake as substrate

ing at temperature 40˚C with 10% inoculum concentration after 24 hr, and soybean oil cake (9.5716 U/ml) at pH 8 maintaining at temperature 30˚C with 0.5% inoculum concentration after 24 hr. Overall mix culture experiment, sesame oil cake gave highest lipase activity.

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Green Diesel Production from Waste Cooking Oil: Performance Computation and Combustion Analysis at Different speeds in Single Cylinder CI Engine

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ABSTRACT

By considering economic, environmental and availability aspect, waste cooking oil was selected as a feedstock for production of green diesel. Hydro processing technique was used to obtain green diesel and Na2CO3 is used as a catalyst. The fuel properties such as specific gravity, kinematic viscosity, net calorific value and flash point were measured. The performance characteristics of green diesel were investigated in single cylinder compression ignition engine. Indicated thermal efficiency, break thermal efficiency, break specific fuel consumption (BSFC) and fuel consumption in kg/hour were investigated at different rpm and different load. The maximum indicated thermal efficiency was 87.96% at 1450 rpm and 8.97 kg load whereas maximum break thermal efficiency was 38.14% at 1410 rpm and 16.68 kg load. Minimum BSFC was 0.25 kg/kWh at 1410 rpm and 16.68 kg load whereas minimum fuel consumption was 0.42 kg/hour at 1490 rpm and 0.86 kg load. Physics of combustion of green diesel was analyzed in CI engine and compared with petro diesel. It was found that green diesel has shorter ignition delay which is an indications for good combustion. Green diesel has the high cylinder pressure peak as compared to petro-diesel. The peak pressure in case of green diesel was 72.8 bar at 376° crank angle and for petro-diesel was 72.2 bar at 376° crank angle. The combustion information was obtained from the pressure sensor located in the engine cylinder head

KEY WORDS: WASTE COOKING OIL, GREEN DIESEL, COMBUSTION, IGNITION DELAY

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INTRODUCTION

Everybody has a desire to live in comfort and pollution free environment. To achieve comfort, human being introduced machinery in modern world such as engine. Generally engines run on fossil fuel which generates harmful emission and are ultimate cause of pollution and global warming (Ramya et al. 2012). In rapidly growing global energy consumption, diesel engine play the main role (Shameer et al. 2018). Although emission of machinery cannot be eliminated completely but a significant reduction is possible by use of green fuel or bio fuels. Green fuels have one more advantage that is these are renewable. Bio fuels have been introduced as a substitute for fossil fuels (Yu et al. 2013). (Deshpande and Kulkarni 2012) derived green fuel from *Jatropha* oil. Animal fats (TAG) and modified vegetable oils in nature can be used for the production of chemical products such as biofuels (Suarez et al .2007). Due to chemical and thermal instability and high viscosity, vegetable oils forms carbon deposits inside the combustion chamber of diesel engines and hence not used directly in engine (Biswas and Sharma 2013). (Buzetzki et al. 2011) used zeolite catalysts with NaY and clinoptilolite to investigate the cracking of vegetable oils (sunflower, rapeseed, jatropha, waste frying oils and soybean). Most of researcher investigated the influence of biodiesel on engine performance, combustion and emissions (Ozener et al. 2014, Zhang et al. 2013, Qi et al. 2010) but very few on green diesel. Biodiesel has few disadvantages like higher viscosity, higher production cost, restriction on feedstock use, low energy density and higher freezing point. (Agarwal 2007, Knothe et al. 2005). Crude palm oil increases the deposit formation in cylinder as compared to petro diesel (Pipitone and Costanza 2018).

The different technologies used a variety of feedstock including lignocelluloses biomass (such as wood and crop residues, dedicated herbaceous or tree energy crops), grains, sugar crops, vegetable oil (soybean, canola/rapeseed, etc.), animal fat (beef tallow, pork lard), and waste cooking greases. These all technologies targeted hydrocarbon products that are similar to petroleum diesel (Milbrandt et al. 2009). (An et al. 2012) computed the combustion and emissions of waste cooking based biodiesel at partial load conditions. (Silitonga et al. 2013) investigated the performance and exhaust emissions of *Ceiba pentandra* blend biodiesel in diesel engine. Two common techniques are used to produce diesel-type fuel from biomass which are hydro processing and transesterification of triglycerides. Transesterification is used to obtain biodiesel whereas hydro processing is used to synthesize green diesel (Boyás et al. 2012). The cost of feed stock affects the total cost of biofuel significantly. Waste cooking oil (WCO) is more economically viable then vegetable oils, also it is presumed that cooking oil becomes carcinogenic after heating for a long period and it is harmful for health, that is why WCO was selected as a feedstock.

MATERIAL AND METHODS

This experimental work is divided into three parts. The first part of the experiment involves production of green diesel, second part is related to the measurement of key properties of green diesel and third part is associated with performance computation and combustion analysis.

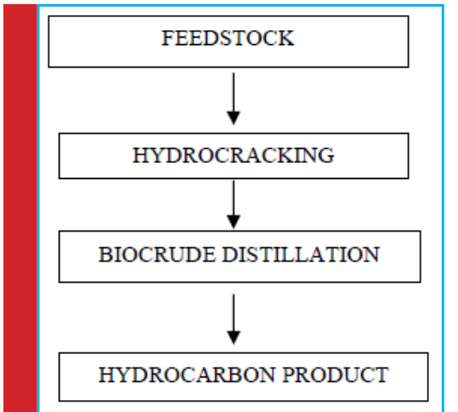

FIGURE 1. Flow chart of steps involved in the production of Green Diesel

Production of green diesel: For production of green diesel, waste cooking oil was selected as a feedstock where as Na_2CO_3 is used as a catalyst. In the present study green diesel was produced by hydro processing of waste cooking oil. Hydroprocessing completed in two phases (hydrocracking and biocrude distillation). The steps involves in the production of green diesel are shown below in figure 1 with the help of flowchart.

In first step, 1.5 liters waste cooking oil was taken with the help of measuring tube. The quantity of sample has been decided on the basis of capacity of the cylinder of HTHP reactor (2 liters). Na_2Co_3 (anhydrous) was mixed in the sample of waste cooking oil (WCO) as a catalyst. Sample was filled into the reactor, and hydrogen (H_2) was supplied up to 5.5 bar pressure through the inlet vent. Selection of catalyst and optimum pressure of H_2 was predefined on the basis of production optimization. About 87 %(by weight) bio crude could be obtained after hydro processing while the balance 8 % escapes as non-condensable gases and 4-5% water at the bottom of the collecting flask. The resultant bio crude was investigated with respect to appropriate standards and distilled as per ASTM D2892 and ASTM D5236 using the TBP distillation unit, (Rakopoulos et al. 2006).

FIGURE 2. Different distillates of biocrude oil

Table 1. Approximate molecular size of crude oil fractions

Fraction	No. of carbons
Gases	1-4
Gasoline	7-11
Aviation fuel/kerosene	9-16
Diesel	12-22
Residue	>40

Different hydrocarbon chain lengths have progressively higher boiling points, so they can be separated by distillation. A fractional distillation tower will heat, vaporize, and then condense into different products. In the present experimental investigation, 1200 ml (1100 gm) bio-crude was taken for distillation. After distillation about 110 ml gasoline, 25 ml aviation/kerosene and 240 ml diesel fraction is being produced. Diesel fuel fraction has been produced at 180-380°C temperature range, aviation fuel between 140-180°C and gasoline fuel fraction at 35- 140°C. Different distillates of biocrude oil have different color and different number of carbon. Color of different biocrude oils after biocrude distillation is shown in figure 2. where as carbon atom range shown in Table 1.

Fuel properties of green diesel: Key fuel properties such as specific gravity, kinematic viscosity, cetane number and flash point were measured. (Pham et al.2018) introduced a correlation to predict kinematic viscosity and density of biodiesel. The principle involves the oscillating U-tube which is a technique to determine the density of liquids and gases based on electronic measurement of the frequency of oscillation, from which the density value is calculated. From density, specific gravity of fuel can be calculated. The engine frequency of this container is influenced by the sample's mass. The direction of oscillation is normal to the level of the two branches. The oscillator's eigen frequency is only influenced by the part of the sample that is actually involved in the oscillation. These properties were measured as per ASTM D975.Instrument used for measurement of flash point and fire point follows the ASTM D92 – 12b stand-

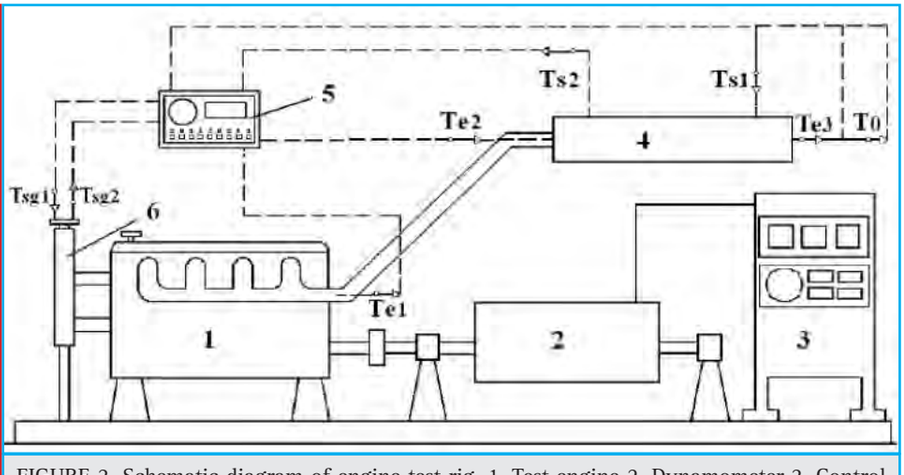

FIGURE 3. Schematic diagram of engine test rig. 1. Test engine 2. Dynamometer 3. Control board 4. Exhaust calorimeter 5. Temperature indicator 6. Cooling tower

ard. The kinematic viscosity of the oil was determined as per ASTM D 446 –12 using kinematic viscosity bath. The device was manufactured by Lawler Manufacturing Corporation, USA, with Model 86-17D

Performance computation and combustion analysis: Engine test rig was used for basic measurements, which usually should be undertaken to evaluate the performance of an engine on almost all tests, like engine speed, break power, indicated power, fuel consumption. The test engine and dynamometer were controlled by a microprocessor system equipped with data acquisition and logging. Sensors were fitted to the engine and dynamometer to measure relevant parameters and send the data to the control system. The sensors measured engine load, engine speed, inlet air temperature, exhaust gas temperature, lubrication oil temperature, fuel consumption and the cooling water temperature.

RESULTS AND DISCUSSION

The fuel properties help us to explain the behavior of fuel. So, it is necessary to measure the fuel properties. The important fuel properties such as specific gravity, kinematic viscosity, net calorific value and flash point etc. were measured in laboratory. These fuel properties for green diesel are shown in the table 2

Table 2. Green diesel fuel properties

Fuel properties	Magnitude
Specfic gravity	0.78
Kinematic viscosity (centi-stoke)	3.0823
Flash point (°C)	114
Cetane Number	84
Net calorific value (KJ/KgK)	38823

From the computational data in table 2 it is concluded that green diesel has low specific gravity and high cetane number. Kinematic viscosity about 3.0 centistoke which is satisfactory with past research. The net calorific value is 38823 kJ/kg K which has considerable variation with calorific value calculated by Kennedy et al.(2015). The cause of variation may be due to different feedstock or different production method or may be any device error or due to limited experiment. To overcome this, more experiment need to be performed. The performance parameters: Include indicated thermal efficiency, brake thermal efficiency, specific fuel consumption (kg/kWh), and fuel consumption were calculated at different rpm and at different load condition. Performance data of green diesel observed by this experiment is shown in the table 3 and table 4.

From table 3 it is observed that if load is increased and engine speed is decreased then initially indicated thermal efficiency and break thermal efficiency both increases but after that they start decreasing. It can be noticed that indicated thermal efficiency is maximum at 1450 rpm and 8.97 kg load whereas maximum break thermal efficiency is 38.14 % at 1410 rpm and 16.68 kg load. BthEff increases with load because if load increases power also increases.

Table 3. Indicated and brake thermal efficiency using green diesel

Speed (rpm)	Load	IThEff (%)	BthEff (%)
1490	0.86	83.82	5.18
1470	4.90	85.76	21.29
1450	8.97	87.96	33.97
1430	12.84	74.23	35.98
1410	16.68	67.98	38.14
1390	20.62	51.37	31.20

Table 4. SFC & Fuel consumption for green diesel

Speed (rpm)	Load (kg)	SFC (kg/kWh)	Fuel (kg/h)
1490	0.86	1.79	0.42
1470	4.90	0.43	0.56
1450	8.97	0.27	0.69
1430	12.84	0.26	0.97
1410	16.68	0.25	1.48
1390	20.62	0.30	1.67

Specific fuel consumption in kg/kWh decrease with increase in load but it starts increasing when load is increased up to 20.62 kg. This is due to initial increase in brake power with load but after certain value of load, brake power also decreases. The fuel consumed in kg/h increases with load. This is because power requirements increases with load and ultimately consumption of fuel increases

In combustion analysis, the behavior of fuel – air mixture inside the cylinder is analyzed to find out the physics behind the performance and emissions characteristics of the fuel. During the combustion of green diesel, the variation in pressure, temperature, net heat release, and fraction of fuel mass burned were investigated with crank angle and then compared to petro diesel. Combustion information was obtained from the pressure sensor located in the engine cylinder head. The in-cylinder pressure plot reflects slightly late combustion and larger ignition delay for the green diesel fuels when compared to petro-diesel fuel. In the initial phase of combustion, the pressure rise for green diesel was less because it does not contain smaller length hydrocarbons that can mix quickly with air. Diesel fuel is composed of a wide range of hydrocarbons with varying chain lengths in the range of C-8 to C-21. Green diesel consists of primarily C-11 to C-17 alkenes (Yoon and Lee

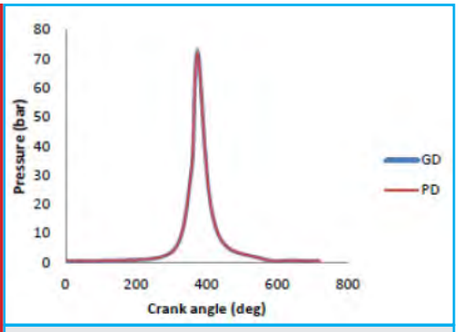

FIGURE 4. In cylinder pressure variation with crank angle

Figure 7 shows the plots of the net heat release rate for both fuels. The heat release rate curve for both the fuels follow a similar pattern. Both have a pre-mixed combustion phase and a diffusion combustion phase. The green diesel fuels have shorter ignition delays (Dolanimi et al. 2015) and higher heat release rates as compared to petro diesel. The previous literature reported the shorter ignition delays experienced by green diesel fuels and may be due to thermal cracking which tends to break up longer chain hydrocarbons to form smaller hydrocarbons and gaseous compounds which are liable to ignite much faster than their longer chain hydrocarbon counterparts. (Yoon and, Lee 2011). But in present

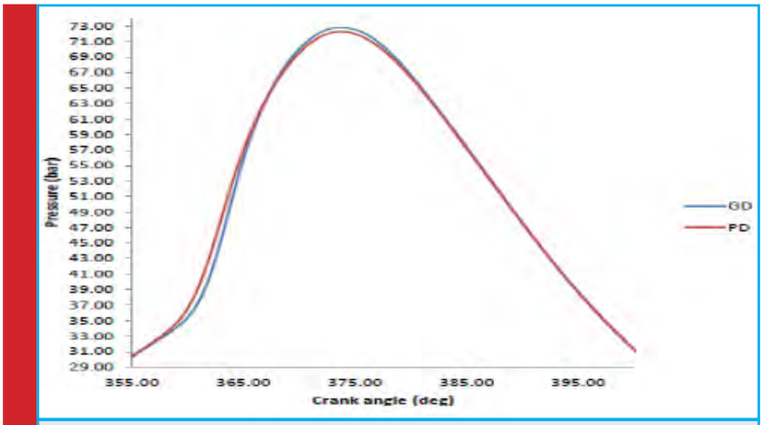

FIGURE 5. In cylinder pressure variation with crank angle (near the TDC position)

2011). The green diesel contains a narrow range (i.e. 11 to 17) which leads to high intensity combustion occurs with. The high pressure values of green diesel fuel may be a result of better combustion near top dead centre (TDC). Green diesel has the high cylinder pressure peak as compared to petro-diesel. The peak pressure in case of green diesel was 72.8 bar at 376° crank angle and for petro-diesel was 72.2 bar at 376° crank angle. Figure 4 shows the plots of the pressure rise with crank angle for both fuels for complete cycle where as figure 5 show this variation at top dead centre

The rate of pressure rise for the green diesel fuels is also noticeably large as compared to petro-diesel fuel. This mainly depends on the fuel's chemical and physical properties, which affect the mixing and ignition process. Higher increasing rate of in-cylinder pressure generally leads to more combustion noise. Figure 6 show the rate of pressure with crank angle

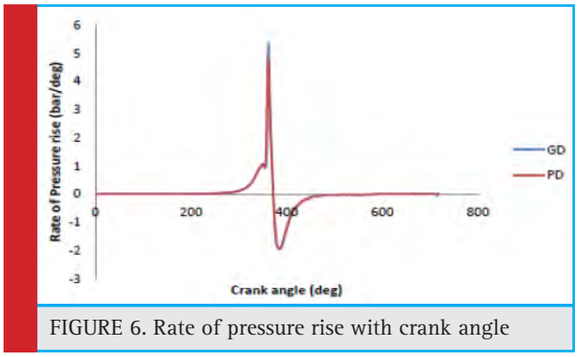

FIGURE 6. Rate of pressure rise with crank angle

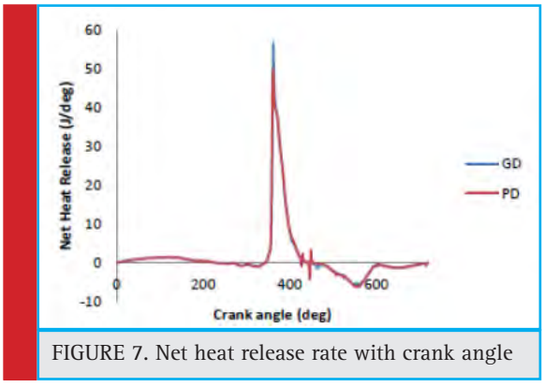

FIGURE 7. Net heat release rate with crank angle

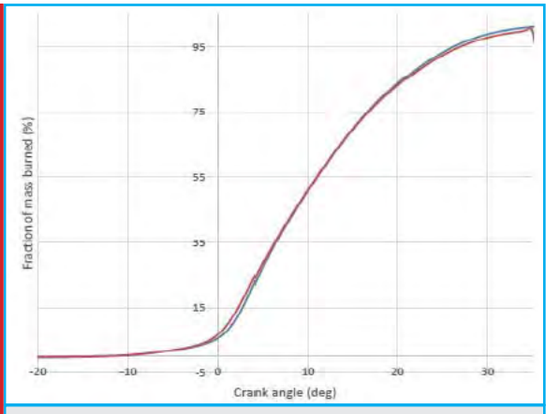

FIGURE 8. Fraction of fuel mass burned with crank angle

study the combustion results show that the green diesel might not contain smaller hydrocarbons and gaseous compounds. The combustion results of green diesel are somehow different from the previous publications. This might be due to the specific conditions for the current green diesel production process and experimental setup. More experiments are required by altering the production parameters.

Figure 8 shows the fraction of mass burned with crank angle for both green diesel and petro diesel. From the graph it is observed that at initial stage when the fuel is injected, the fraction of petrol diesel consumed is more than green diesel, at the middle stage it is equal and in later stage fraction consumed of green diesel is more as compared to petro diesel. This is due to high cetane number of green diesel. It will burn at low temperature as compared to petro diesel which have low cetnane number. If a tinny drop of green diesel is injected inside the cylinder it starts burning immediately but when tiny drop of petro diesel is injected in cylinder it will not burn at this temperature and pressure. Meanwhile another drop injected at same time and piston moves more upward which create more compression. Due to this, temperature will increases which in turn increases the rate of fuel burn.

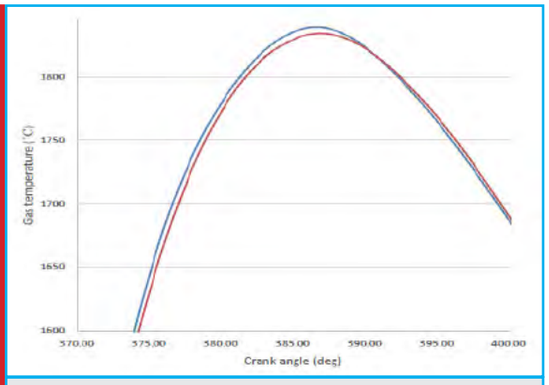

FIGURE 9. Variation of gas temperature inside the cylinder with crank angle

Figure 9 show variation of gas temperature inside the cylinder with crank angle near the top dead centre. From the figure 9 it is concluded that the temperature rise in initial phase is more for green diesel than petro diesel. The reason behind this is the shorter ignition delay of green diesel due to which fuel burn rate is high in this phase as compared to petro diesel which lead to high temperature. In later stage temperature generated is slightly more for petro diesel. Petro diesel have higher ignition delay which causes long lasting of burning fuel as compared to green diesel

CONCLUSION AND RECOMMENDATIONS

Green diesel has quality attributes comparable to those of syndiesel, including complete compatibility with petroleum diesel, high energy density, low specific gravity (0.78), excellent storage stability. Results from combustion show shorter ignition delays for green diesel which can overcome the problem of knocking in CI engine. Green diesel has the high cylinder pressure peak as compared to petro-diesel. The peak pressure in case of green diesel was 72.8 bar at 376° crank angle and for petro-diesel was 72.2 bar at 376° crank angle. In India due the poor economic condition and lack of awareness, used oil is not dumped, and is reused for cooking purpose. If the government introduces some program related to awareness of the harmful effect of reusing the used oil and also provides proper waste oil collection system, then there is huge scope to produce bio fuel from waste oil in India also.

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Engineered Nanomaterials and Their Properties: A Review

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ABSTRACT

Electronic state of a material is an arrangement allowed by the laws of quantum mechanics of electrons within an atom, molecule or system of molecules. Low-cost nano materials single wall carbon nano tubes and multi walled carbon nano tubes based on semiconductor devises. In case of a metal, the quasi-continuous density of states in the valence and the conduction bands splits into discrete electronic levels, the spacing between these levels and the band gap increases with decreasing particle size. Discussion of electronic and optical properties of carbon nano tubes depends upon size, morphology and synthesis methods. They are smaller than the wavelength of visible light and a hundred-thousandth times the width of a human hair. At this range, unusual properties of materials emerge out that can be applied to yield technologies and products with undividedly new abilities and applications.

KEY WORDS: NANO ENGINEERING, QUANTUM DOTS, MORPHOLOGY, CNT

INTRODUCTION

Engineered Electronic State of Nanomaterials:- Nanomaterials are the soul of nano engineering and nanotechnology. These materials possess at least one dimension in the 10–9 m, nanometer, range which is one billionth of a meter. In nanomaterials surface-to-volume ratio increases (Siegel et al, 1993; Collins et al, 2000, Alagarasi 2011). The number of atoms on the surface may be similar to or higher than those located in the crystalline lattice core and the surface properties are no longer negligible. When no other molecules are adsorbed onto the nano crystallites, the surface atoms are highly unsaturated and their electronic contribution to the behavior of the constituent particles is absolutely diverse from that of the inner laying atoms. Electronic State of a material is an arrangement allowed by the laws of quantum mechanics of electrons within an atom, molecule (or system of molecules) (Bate et al, 1975; Frolov et al, 2001).

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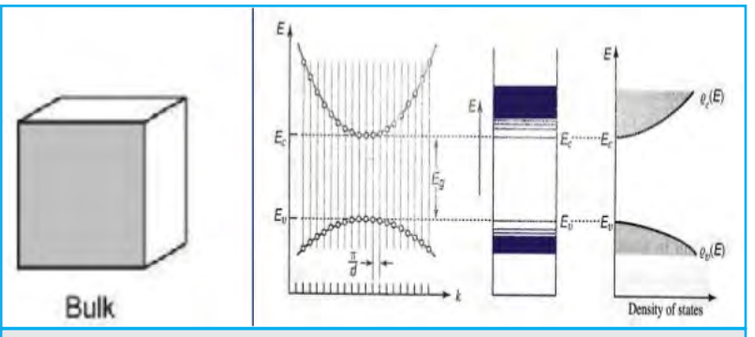

FIGURE 1. Electronic structure of bulk(Bate et al, 1975).

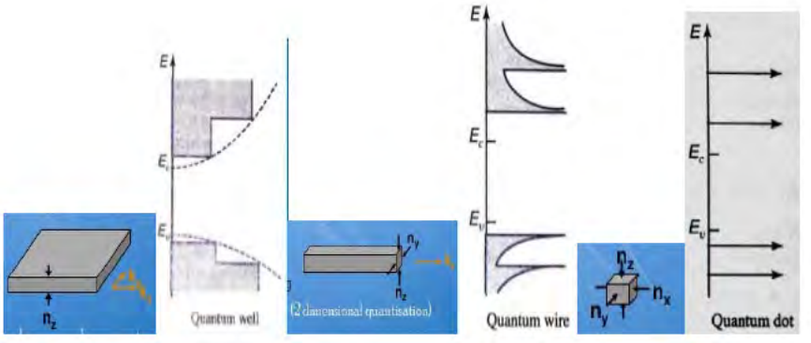

FIGURE 2. Quantization in one dimension i.e. 2D strucutres (Quantum well), two dimensions i.e.1D, three dimensions i.e. 0D (Frolov et al, 2001).

Fig. 1.1 and 1.2 give the electronic structure of bulk semiconductors and low dimensions respectively. In bulk, the electrons in conduction band and holes in valence bands have degree of freedom in all 3 dimensions.

As the size of the nanoparticle increases, the band structure evolves gradually i. e., molecular orbital change to delocalized band states. Figure 1.3, shows the size quantization effect which leads to conversion between a bulk metal or semiconductor, and cluster species. In case of a metal, the quasi-continuous density of states in the valence and the conduction bands splits into discrete electronic levels, the spacing between these levels and the band gap increases with decreasing particle size (Mekala et al, 2000; Bogue at el, 2010; Medintz et al, 2005). In the case of semiconductors, a band gap already exists in the bulk state. This band gap increases as the size of nanoparticle decreases and the energy bands gradually convert into discrete molecular electronic levels (Kim et al, 2011; Patil et al, 2012; Sharma et al, 2013 Tripathi et al, 2016).

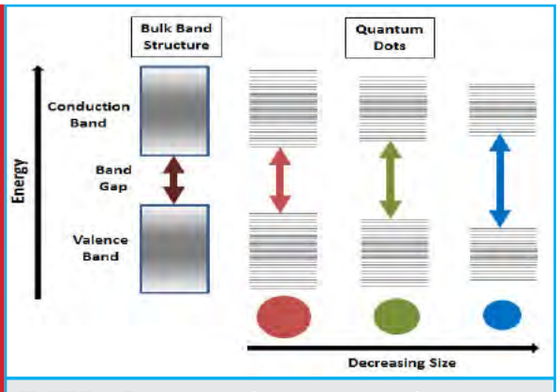

FIGURE 3. Engineering electronic states of nanomaterials using size quantization effect (Kim et al, 2011)

Engineered Nanomaterials are the manipulated matter on the nanoscale. Electronic states of nanomaterials can be engineered by tuning the following parameters: Size, aggregation or dispersion state and the extent of dissolution.

Size, morphology, and aggregation state

Some Engineered nanoparticles dispersions may have narrower size distributions than their naturally occurring counterparts as nanomaterials produced anthropogenically are tailored for specific, size-dependent properties; whereas, naturally occurring nanomaterials generally will not have such restrictions. One other technique

capable of sizing nanoparticles is single particle inductively coupled plasma mass spectrometry (SP-ICP-MS), which can provide element-specific information about individual particles that are ablated in the plasma (Pace et al, 2012; Mitrano et al, 2012 Tripathi et al, 2016).

SP-ICP-MS has been employed to size a diversity of NMs such as silver, gold, and metal oxide particles in various complex media (e.g., wastewater, bovine serum albumin). Although this technique requires information regarding particle shape and is currently hemmed in by the nano particle's size (Pace et al, 2011; Reed et al, 2012).

Dissolved ions vs. nano particulate

In spite of incidental and naturally occurring nanomaterials, various samples in environment comprise a high concentration of dissolved ions that, when using chemical analysis-based methods, may overestimate the amount of material present in nanoparticle form. One way of distinguishing between nanoparticles and dissolved forms of the material is through filtration methods, whereby particles can be size-fractionated, with the remaining fraction composed of dissolved ions. Similarly, centrifugation can put to use where particles may settle out under the centrifugal force, and the supernatant that is decanted, should contain the dissolved forms of the material. As the dimensions of the material is reduced the electronic properties change radically as the density of states and the spatial length scale of the electronic motion are reduced with decreasing size (Liu et al, 2008; Chen et al, 2012; Tripathi et al, 2016).

Electrical properties

Electrical Properties of nanomaterials hold forth the essentials of electrical conductivity in nanorods, nanotubes, nanocomposites and CNT and optical properties of nanorods as well. The low dimensions of a nanowire and measurement of the electrical current at a constant applied voltage is one of the methods to validate the quantum conductance. Here with decreasing diameter of the wire, the number of electron wave modes contributing to the electrical conductivity becomes increasingly smaller by well-defined quantized steps (Liang et al, 2011; Benn et al, 2010; Danniel et al, 2004). In CNT's, single electron wave mode is observed that conducts the electrical current. Different lengths and orientations of the carbon nanotubes provide two information: (i) the influence of carbon nanotube length on the resistance; and (ii) the resistances of the different nanotubes.

Selected Application of Engineered Nanomaterials

Nanomaterials have extensive application in the field of electronics, fuel cells, batteries, agriculture, food industry, and medicines, etc. Nanomaterials holds supercilious chemical, physical, and mechanical properties and of their exceptional formability.

Fuel cells

A fuel cell is an electrochemical energy conversion device that converts the chemical energy from fuel (on the anode side) and oxidant (on the cathode side) unswervingly into electricity. The soul of fuel cell is the electrodes. The structure of electrode must provide plenty surface area, maximum contact of catalyst, reactant gas and electrolyte, facilitate gas transport and provide good electronic conductance (Smijs et al, 2011).

Carbon nanotubes-Microbial fuel cell

In Microbial fuel cell bacteria devour water-soluble waste such as sugar, starch and alcohols and produces electricity in addition to clean water. This technology generates electricity using domestic or industrial wastewater. Microbial fuel cell metamorphoses various polysaccharides substrates present in wastewaters into a source of electricity. The performance of the fuel cell is dominantly affected by the efficient electron transfer between the microorganism and the anode of the microbial fuel cell.

Carbon nanotubes (CNTs), due to their special properties of high chemical stability and mechanical strength and large surface area, are ideal for the design of sensors. Since carbon nanotubes promote cell growth, they can be used to build electrodes of microbial fuel cells (Sajith et al, 2010; Sharma et al, 2015).

Table 1. Some synthesis methods and characteristics of engineered nanoparticles

Process	Elemental Composition Examples	Characteristics
Chemical reduction	Zerovalent metals Au, Ag, Fe	Mono-elemental in composition, (Au, Ag) monodisperse and often as spheres or wires
Sol-gel	Metal oxides SiO_2, TiO_2, ZnO, CeO_2	Single metal, moderate polydispersity, 2¬D and 3-D nanoscale dimension
Solvosynthesis	Semiconductors CdSe/ZnS, CdTe	Multi-metal composition, low polydispersity
Vapor-phase	Carbonaceous materials, metal oxides Carbon nanotubes, fullerenes	Polydisperse, 2-D nanoscale dimension
Organic synthesis	Multi-functional polymers	Carbon-based, monodisperse, 3-D nanoscale dimensions

Phosphors for High-Definition TV

The resolution of a television screen, or a computer monitor, greatly depends on the dimensions or size of the pixel. These pixels are essentially made up of materials called "phosphors," which radiate when a stream of electrons from the cathode ray tube (CRT) is made to impinge upon it. The resolution enhances with a reduction in the magnitude or size of the pixels, or the phosphors. Nanocrystals of zinc selenide, zinc sulfide, cadmium sulfide, and lead telluride which are synthesized by the sol-gel techniques turns up to be the useful for improving the resolution of computer monitors. The use of nanophosphors is intended to reduce the cost of these displays so as to render high definition televisions (HDTVs) and make personal computers to be affordable to be purchased (Baughman et al, 2002; Niemann et al, 2008).

Next-Generation Computer Chips

The appetence for miniaturization has emphasized the microelectronics industry, to reduce in size the circuits, such as transistors, resistors, and capacitors. By attaining a noteworthy decrease in their size, the microprocessors, which enclose these components, can run much faster, thereby enabling computations at a far greater speed (Kimling et al, 2006; Tripathi et al, 2015). Though, there are various technical impairments to these developments, which includes the absence of the ultrafine precursors to produce these components; poor dissipation of large amount of heat released by these microprocessors due to faster speeds; short mean time to failures (poor reliability) etc. Nanomaterials help the industry breach these barriers down by furnishing the manufacturers with nanocrystalline starting materials, ultra-high purity materials, materials having improvised thermal conduction, and long lasting, durable interconnections in the microprocessors (Lakshmi et al, 1997; Srivastava et al, 2014).

Nanowires for junction less transistors

Transistors are miniaturized to the dimension of sub assemblies of electronic systems and deliver smaller and smaller devices, but it is challenging to create high-quality junctions. In particular, it is very tough to alter the doping concentration of a nanomaterial over distances shorter than about 10 nm. Researchers have gained the ground in making the junction less transistor having closely ideal electrical properties. It could possibly function faster and use lesser power than any conventional transistor in the market today (Dabbous et al, 1997; Swihart et al, 2003).

The device consists of a silicon nanowire with perfectly controlled current flow by a silicon gate, separated using a thin insulating layer. The whole silicon nanowire is heavily n-doped, proving it to be an exceptional conductor. However, the gate is p-doped and its presence has the effect of depleting the number of electrons in the region of the nanowire under the gate. Also, the device has near-ideal electrical properties and behaves like the most perfect of transistors without suffering from current leakage like that in conventional devices and operates faster and consumes lesser energy (Gopidas et al, 2003; Kimling et al, 2006).

Elimination of Pollutants

Nanomaterials possess tremendously big grain boundaries as compared to their grain size. Hence, they are very dynamic in terms of their chemical, physical, and mechanical properties. Owing to their boosted chemical activity, nanomaterials can be used to serve as catalysts to react with such harmful and lethal gases as carbon monoxide and nitrogen oxide in automobile catalytic converters and power generation equipment to prevent environmental pollution arising from burning gasoline and coal (Drexle et al, 2001; Tripathi et al, 2015).

Sun-screen lotion

Prolonged UV exposure causes skin-burns and cancer. Nano skin blocks (ZnO, TiO_2 and BiOCl) have added advantage as they protect the skin by resting onto it rather than penetrating into the skin. Thus they inhibit UV radiation effectively for prolonged duration. Additionally, they are transparent, thus retain natural skin color while working better than conventional skin-lotions (Tripathi et al, 2016; Tripathi et al, 2017).

Sensors

Sensors depend upon the highly active surface to initiate a response with minute variation in the concentration of the species to be detected. Engineered monolayers (few Angstroms thick) on the sensor surface are exposed to the environment and the peculiar functionality (such as change in potential as the CO/anthrax level is detected) is utilized in sensing (Shrivastva et al, 2014).

Nanowires based Device: The Hub for 21st Century Nanoelectronics

The objectives of study of this topic are to gain an understanding of nanotechnology, the fascinating technological revolution of the 21st century, to review the evolution of nanotechnology and nanomaterials and, to study carbon nanotubes with a view to establishing it as nanoelectronics hub. Nanotechnology is a global phenomenon and being one of the most interesting and wide areas of research of the present century, it is changing the lifestyle, creating scientific developments

and new products that are smaller, faster, stronger, safer, and more creditable. This embellishing field has penetrated virtually into all the areas of science and technology. Furthermore, it is one big-league application of miniaturization. In point of fact, miniaturization results in the conception of mechanical, optical, and electronic products and devices have usage in smaller scales materials and devices. This is done with the understanding that items which take up less space are more desired than items which are bulkier since they are easier to carry, easier to store, and quite handy to use.

Nanotechnology, "the engineering technology of 21st century", is defined as the manipulating matter at dimensions of roughly 1 to 100 nanometers. Also, it can be termed as the application of science, engineering and technology to unfold novel materials and devices in different fields in the nano-range. A nanometer (nm) is a measurement system used to measure small particles like atoms and molecules and is equal to one billionth (10^{-9}) of a meter. It is smaller than the wavelength of visible light and a hundred-thousandth times the width of a human hair. At this range, unusual properties of materials emerge out that can be applied to yield technologies and products with undividedly new abilities and applications (Drexle et al, 2004; Onyeje et al, 2013).

In fact, at the nanoscale, physical, chemical, optical and electrical properties of materials bear no resemblance from the properties of matter at either smaller scale, such as atoms, or at the larger scales. It is anticipated that Nanotechnology will have an impact on nearly every industry. Hence, the U.S. NSF has hazarded a guess to attain $1 trillion or more within 20 years in the global market for nanotechnologies. According to its inventor, Raymond Kurzweil, nanotechnology is the next technological insurgency. Consequently, the Nanotech Age is expected to begin between 2025 and 2050, terminating the current Information Age which began in 1990. The Nano Revolution is expected to be at the most as transformative as the Industrial Revolution, but limited to just a few years. Materials that are used in nanotechnology are called nanomaterials; they are not merely an additional leap in the miniaturization of materials, they usually need quite different synthesizing approaches. Some nanomaterials are currently at the laboratory stage of manufacture, while some of them are being commercialized. Although, several nanomaterials exist till now but carbon nanotubes are widely used in nanoelectronics and nanodevices as they exhibit exquisite properties (Frolov et al, 2001; Medintz et al, 2005, Kimling et al, 2006; Moore et al, 2010).

Nanodevices

In the direction of producing nanodevices, there is the need to understand the fundamental phenomena, the synthesis of appropriate materials, the use of those materials to fabricate functioning devices and the integration of these devices into working systems. Nanofabrication interpolates the building of machines that operate on an atomic or molecular scale. These are minor, faster and devour less power than conventional electronics and because you can pack so much on to one computer chip, you can have many more functions. Such technology has huge potential in the field of communications, data storage, solar cells and medical applications. Nanofabrication is of much interest to computer engineers since it takes one to super-high-density microprocessors and memory chips. The usage of nanotechnology in electronic apparatuses, especially transistors is referred as nanoelectronics. The eventual aim of nanoelectronics is the sustained recognition of Moore's law by deploying novel methodologies and nanomaterials to produce electronic devices with particular sizes on the nanoscale. Nanoelectronics often attribute to transistor devices which are so small that inter-atomic interactions and quantum mechanics properties need to be deliberated extensively. In addition to being small and implanting more transistors to be packed into a single chip, the even and regular structure of nanotube allows higher electron mobility and a higher dielectric constant (Liu et al, 2008; Moore et al, 2010; Kimling et al, 2006; Frolov et al, 2001; Medintz et al, 2005 Chen et al, 2012; Tripathi et al, 2016).

Applications of Carbon Nanotubes in Nanoelectronics

Substantial interest generated by carbon nanotubes has been in applications to electronic materials and some of them are:

Better Solar Cells

Usually solar cells use silicon semiconductors. However, a change occurs when carbon nanotubes are incorporated into the semiconductor. These days billions of CNTs could be tightly packed onto solar cells and release far more electricity per square inch than silicon as they're so tiny.

Better Thinner TVs

In field emission, a systematic arrangement of CNTs is used as they are excellent electron emitters. There is now new process of displaying pictures called field emission display. In this process, the miniaturization occurs by using tiny electron emitters positioned behind individual phosphorus dots displaying the excitation of the phosphorus dots, creating bright, high resolution displays. With the help of CNTs we can produce TVs that are only millimeters thick and dissipate lesser power than plasma and liquid crystal displays.

Better Capacitors that Replace Batteries

In a capacitor, the capacitance is a function of the surface area. Due to the extraordinary high surface areas of CNTs, use of them as the dielectrics increases the storing capability of capacitors to compete with modern batteries.

Electrical Wires and Cables

Electric wires and cables can now be made-up of pure nanotubes and nanotube-polymer composites. It is exciting to observe that latterly the wires have been fabricated using the highest conductivity carbon nanotube with specific conductivity exceeding copper and aluminum.

Paper Batteries

Batteries are essential electronic components and now there are paper batteries. It is a type of battery that is designed to use a paper-thin sheet of cellulose pervaded with aligned carbon nanotubes. The nanotubes serve as electrodes and thus conducts electricity in the storage devices. This type of battery can deliver a longer, steady power output comparable to a conventional battery. The paper battery is the integration of all the battery components in a single structure, making it more energy efficient.

Faster Computers

Carbon nanotubes can now be put together into chips in order to get improvement in its speed. With CNTs in computer chips, several billions of CNT transistors could be crammed against a single processing chip, giving miniaturized, faster computers and electronic devices.

SUMMARY AND CONCLUSION

Nanotechnology being an innovative and powerful technology is seen as the latest mega trend in science and engineering which will bring a wave of radical innovation, thereby sparking new industrial revolution in various application areas. For nanoelectronics applications, carbon nanotubes are fascinating due to their excellent electrical properties. A far-reaching research area is molecular electronics, for which molecules that are quantum electronic devices are designed and synthesized with the aim to use individual molecules as switches and carbon nanotubes as the wires in circuits which are expected to fruition in nonvolatile memories.

Another trend is in the appliance of nanowires in opto-electronics and nano electro mechanical devices. There is also nanomotor which is a molecular electronic device competent enough to convert energy into movement and nano electro mechanical systems. Nano electro mechanical systems typically bears the part of integrating the nanoelectronics with mechanical actuators, pumps and motors, and may thereby form different types of physical, biological, and chemical sensors. Metallic CNT's have also been proposed since they can conduct current with high value. Carbon nanotubes have acquired so much of fame that they are used in nano electro mechanical systems and the approaches have developed to link suspended carbon nanotubes to other nanostructures. This allows carbon nanotubes to be structurally set up to produce complicated Nano electric systems. Also, since some carbon nanotubes are semiconducting, they can be utilized in transistors.

Replacing silicon in the channel by CNT, allows the transistors to be made comparatively smaller and faster than today's transistors. Nowadays, there is an evolving trend of Pico technology that includes the modification of the assembly and chemical properties of individual atoms through the manipulation of energy states of electrons within an atom to produce metastable states with unusual properties, producing some form of exotic atom. The term picotechnology is a coinage intended to cognate the term nanotechnology. This can be termed as a hypothetical future level of technological manipulation of matter, on the scale of trillionths of a meter. Furthermore, coinage of the term femto technology is also a proposed term used in reference to structuring of matter which is 10^{-15} m.

This is a smaller scale as compared to nanotechnology and Pico technology. Carbon nanotubes have phosphorescent/scintillating future as they are extremely versatile. No other material is as strong, conducting, inert and so forth at the same time. Carbon nano tubes unexampled combination of properties paves its way for the rapid progress at the present scenario for example it holds forth a very special property i.e. high surface area which increases the amount of charge that can be stored. These are another possible material for worth use in an ultra capacitor, as they have high density interior, compacted size, steadfastness, and high capacitance.

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Investigating Corporate Social Responsibility Perceptions for Sustainable Development

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ABSTRACT

The Sustainable Development Goals (SDGs) 2030 set by the United Nations have led governments across the globe to restructure their developmental policies and align their national agendas inline with the SDGs. Sustainable development can be achieved through collaborative efforts of governments and the Corporate and Corporate Social Responsibility (CSR) can play a pivotal role in attaining the SDGs. The recent legislative provision of CSR mandate as per New Companies Act 2013 is expected to generate huge financial corpus for socio-economic and developmental activities if implemented in true letter and spirit. Thus it is imperative to create awareness in present and future generations regarding CSR for its effective implementation and to ensure that CSR funds are utilised judiciously and appropriately. The study examined the level of perception of Business students towards CSR. A sample of 600 MBA 2nd year students were selected through random stratified sampling for gender and stream and their levels of perception towards CSR were studied by using PRESOR scale. The findings showed that majority of marketing male students have average or high level of perception towards CSR, while majority of marketing females have low levels. Whereas majority of finance males show average level of perception towards CSR, majority of finance females have average or high level of perception towards CSR. The findings also indicate that there is a substantial percentage of management students with low levels of perception that highlights the need to create awareness among management students so that they can become instrumental in the implementation of CSR mandate for development of the society at large.

KEY WORDS: CORPORATE SOCIAL RESPONSIBILITY, PERCEPTION, SUSTAINABLE DEVELOPMENT

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INTRODUCTION

The coming years will be witnessing complexity, uncertainty and rapid change manifested in the form of a bewildering range of global issues like inequity, economic instability, climate change, loss of biodiversity and migration. A lot of efforts are being made across the globe to counter these challenges and the efforts can be fruitful only by targeting the achievement of sustainable development. Sustainable development is the development that aims at meeting the needs of the present situations without compromising on the ability of the future generations to meet their needs (WCED, 1987). It includes two key concepts, the concept of 'needs,' in particular that include the essential needs of the poor, that need to be prioritised and the idea of limitations that are imposed by the state of technology and by the social organization on the ability of the environment to meet present and future needs (Brundtland Report ,United Nations World Commission on Environment and Development). Sustainable development is a development that protects the environment, because a sustainable environment enables sustainable development (Duran et al., 2015).

The 2030 Agenda for Sustainable Development, adopted by all United Nations Member States in 2015, seek to build on the Millennium Development Goals and provide a shared blueprint for bringing in peace and prosperity for people and the planet, now and in the future. The 17 Sustainable Development Goals (SDGs) aim at addressing the global challenges related to poverty, inequality, prosperity, peace, climate, environmental degradation and justice, are integrated and indivisible; and balance the three dimensions of sustainable development: the economic, social and environmental (Knowledge Platform, Sustainable Development Goals, U.N). Several strategies and plans have been adopted for sustainable development & management and many regulatory and incentive policies, standards and indicators for assessment and measurement have been applied (Klarin, 2018), the implementation of the concept significantly depends on the level of socio-economic development, availability of financial resources, technology, and largely on the diverse range of global socio-economic and political goals and interests (Drexhage & Murphy, 2010). The three key elements of the concept identified include the concept of development i.e socio-economic development keeping in view ecological constraints, the concept of needs i.e fair and equal distribution of resources to ensure quality of life for everyone and the concept of future generations i.e exploring ways of long-term usage of resources for required quality of life for coming generations (Klarin, 2018).

Though governments are making various efforts towards attainment of the SDGs, these efforts need to be supported by the community at large, specifically by the Corporate Business houses who can accelerate the process of development, develop and propagate processes to ensure sustainability of development. It has been recognized that private entities, apart from their profit making character, have a crucial role to play in preserving and protecting earth's natural resources and well being of communities (McElhaney 2009) Also, Business processes have both positive and negative impacts on society and the environment and it is the Social Responsibility of organizations to show transparent and ethical behaviour regarding the impacts of its actions on society and the environment, called as Corporate Social Responsibility (CSR). In a modern perspective, CSR is considered as a business model and approach to deal with developments in sustainability and related regulatory mandates in an integrated manner (Alhaddi 2015). Managing business segments alongside value chains in sustainability-oriented contexts demands strategy-driven and implementing momentum. Such sustainability strategies ensure long-term performance required to establish sustainability as determinant driver of innovation and business success, ultimately (Gerner, 2019).

CSR is the strategies adopted by a firm to conduct business in an ethical and society friendly way that is beneficial to society in terms of development. The term CSR has been in debate since 1950's and the concept has gradually evolved in meaning and in practice. The term first gained importance Bowen talked about the social responsibility of business in the 1950's. In the 1970's Friedman propagated the Stockholder Theory (only goal being profit maximization) that the only responsibility of Business is to be answerable to its shareholders and comply with the law. In 1980's Freeman argued that business is responsible towards all stakeholders and that interests of all stakeholders - Financers, customers, employees, suppliers and communities should be kept in harmony (Freeman, 1980).

Carroll's (1979, 1991) model defined CSR as the ways in which organizations can meet the economic, legal, ethical, and discretionary expectations of their stakeholders. Carroll (1998) described these as the four components of a corporate citizen, thus suggesting that socially responsible organizations need to meet society's needs as reflected by these four components.

In the 1990's the emerging theme of sustainability rose and in 1997, Elkington's Triple Bottom line model developed from the concept of sustainable development became popular and gained acceptance in academic and the business world. The model asserted companies to put people, planet and profit i.e social, environmental and economic aspects in a proper balance.

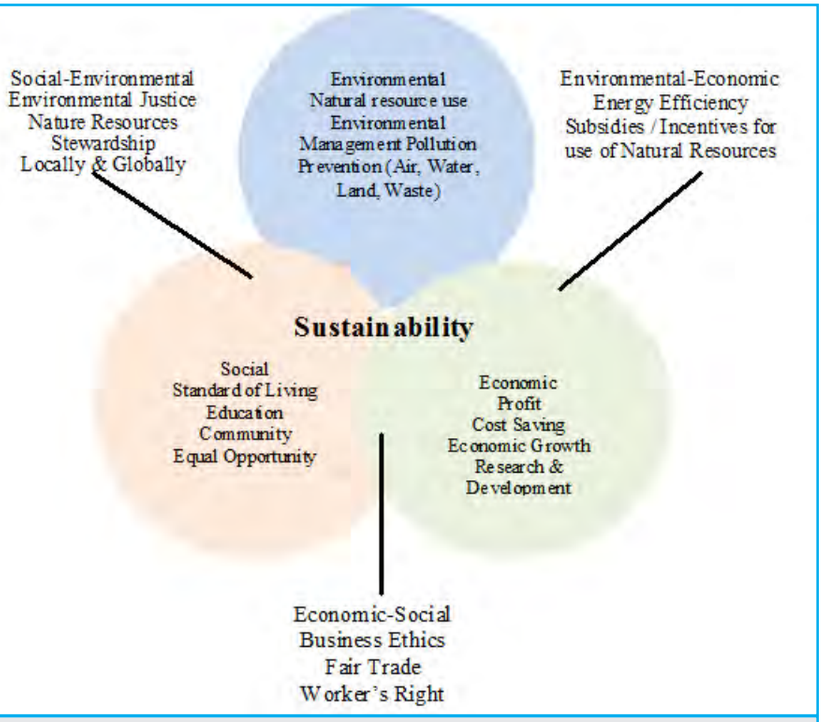

FIGURE 1. Elkington's Triple Bottom Line Model (Source: Elkington, J. (1997). Cannibals with forks: The triple bottom line of 21st century business.)

In the 2000's, CSR began to be viewed as a critical link between business strategies and sustainable development (Steurer et al. 2005). The triple bottom line captures the essence of sustainability for measuring impact of business activities on the environment, including both profitability and shareholder values with human, social and environmental capital (Savitz 2006). The Commission of the European Communities defined CSR as the responsibility of an enterprise for its impacts on society (2011).

There have been numerous changes in the area of CSR in the last few decades and governments and international bodies all over the world have taken various initiatives regarding its acceptance and implementation. A milestone step of the Indian Govt. in this direction was to make CSR spend mandatory as per in section 135 in The New Companies Act and India is perhaps among the leading nations to have made CSR legally mandatory. The Act directs companies (for a specified turnover /net worth / profit) to spend 2 % of their average net profits of last 3 financial years on CSR activities and report reasons in case of non compliance. The legislation has attached more importance to CSR and it is important to create awareness about CSR and its implementation (The Companies Act, 2013). The new provisions regarding Corporate Social Responsibility encompass around 20000 companies that will have to spend a particular amount on social and developmental activities.

Corporate Social Responsibility (CSR) is used as a synonym for referring to sustainability, responsible business conduct and human rights. Though different, they all address the responsibility of organisations for their impacts on society as defined by the European Commission in its 2011-2014 strategy on CSR. (EU Multi Stakeholder Forum on Corporate Social Responsibility, 2015). The global megatrends, such as population growth, urbanization and demographic change; resource scarcity and role of renewables; digitalization; climate change, and responsibility and compliance, represent many long-term opportunities and challenges for companies in the future. They also drive demands for sustainable solutions, new technologies and responsible business practices (UPM, 2017).

CSR and sustainability have gradually evolved over the past years and approaches such as shared value, the circular economy, caring economy, conscious capitalism and purpose economy have emerged and new initiatives and frameworks such as GRI, ISO, and UN-SDGs have been developed (Zu, 2019). The consolidation of the concept of Corporate Social Responsibility (CSR) in the business world has increased the demand and need for professionals who are qualified in competences for

responsible management. Sustainable development (at the three economic, social and environmental levels) largely depends on the responsible behaviour of organizations and educational institutions. The need of the hour is to study the perceptions of present generations towards CSR.

Perception towards Corporate Social Responsibility

There may be numerous factors determining socially responsible behaviour of individuals and the factor that is likely to have a significant impact on the inclination of business managers to be engaged in ethical and socially responsible behaviour is the extent to which they agree such behavior to be critical for success and survival of business. Singhapakdi et al. (1996) emphasised that the perceived importance of ethics and social responsibility to organizational effectiveness may be a key determinant of whether an ethical problem will even be perceived in a particular situation and whether it will be considered as a determinant of factors such as importance of stakeholders and deontological norms .Singhapakdi et al. (2001) assert that managers need to first perceive ethics and social responsibility as vital to organizational effectiveness before they behave in a way that will be more ethical and will reflect greater social responsibility. Thus, the perceived importance of ethics and social responsibility to organizational success is considered to be an important determinant of real business behaviour (Shafer,Fukukawa & Lee, 2007). Many scholars and practitioners acknowledge that decision-makers must incorporate ethical and social responsibility considerations in strategic decision-making processes (Velasquez,1996; Shafer et al., 2007; Vitell et al., 2010.

Singhapakdi et al. (1996) developed a scale to measure perceptions of the importance i.e perceived role of ethics and social responsibility (PRESOR) to organizational success. The original work represented PRESOR through three factors - social responsibility and profitability, long-term gains, and short-term gains , additional research identified only two factors - importance of ethics and social responsibility and subordination of ethics and social responsibility and the results also merged along two themes—stakeholder and shareholder views. The shareholder view is a narrow view of obligations of business emphasizing importance of organizational profitability and obligations only towards stockholders whereas stockholder view argues that an organization owes responsibility to all stakeholder groups and must act in an ethical and a socially responsible manner. Attitudes of managers towards the importance of corporate ethics and social responsibility influence his business decisions and behaviour in an ethical issue related situations will be based on perception of the possibility of the actions possibly creating a desired outcome. Thus, perception holds a significant place in regard to practicing and implementing CSR and it is the need of the hour that the role of the management students in promoting CSR is highlighted.

The review of literature reflected that researches on the topic studied PRESOR attitudes of various populations like business students (Simmons et al., 2009) and managers and employees (Kujala, 2010). Studies have also made comparisons between perceptions of students and managers (Cole and Smith, 1996); religiosity and ethical perspectives were also studied as determinants of perception (Shaffer and Simmons, 2008). Researchers found gender to be a significant determinant of perception towards CSR (Gill, 2010, Bhullar, 2019) whereas other studies found that there was no significant impact of gender on perception (Gholipur et al., 2012). Researches also examined differences based on academic backgrounds and streams, while few found no differences (Sharma & Singh, 2018) other studies found significant differences (Sharma & Gupta, 2019). The current quantum of research on CSR does not have many researches that study the levels of perception of business students and the previous work in the field does not indicate any clarity regarding the topic, and thus suggests scope for further research on perception towards CSR.

The growing importance of CSR emphasized by the new legislative provisions regarding CSR mandate highlights the emerging need of understanding the role of business students who are future managers in planning and conducting sustainable business processes. Thus, the investigator felt a need of research for studying perception of business students towards CSR, in the Indian context.

Research Question

What is the level of perception of business students towards CSR?

MATERIALS & METHODS

A sample of 600 MBA 2nd year students was taken for the present study and was selected by random stratification method at two levels for for gender (male and female) and stream (marketing and finance). First stratification was done according to gender and 2 groups of 300 male students and 300 female students were selected. In the second stratification, 300 male students were divided according to streams into 2 groups of 150 marketing males & 150 finance males and similarly 300 female students were divided into 2 groups of 150 marketing females and 150 finance females for the present study. PRESOR Scale by Singhapakdi , Vitell , Rallapalli and Kraft (1996) was used to measure Perceived role of Ethics and Social Responsibility.

Table 1. Table showing percentage of Low, Average and High levels of Perception of Marketing Male group, Marketing Female group, Finance Male group and Finance Female group of Management students towards CSR

			Group				Total
			Marketing Males	Marketing Females	Finance Males	Finance Females	
Perception	Low	Count	40	69	25	28	162
		% within Perception	24.7%	42.6%	15.4%	17.3%	100.0%
		% within Group	26.7%	46.0%	16.7%	18.7%	27.0%
	Avg	Count	55	57	102	62	276
		% within Perception	19.9%	20.7%	37.0%	22.5%	100.0%
		% within Group	36.7%	38.0%	68.0%	41.3%	46.0%
	High	Count	55	24	23	60	162
		% within Perception	34.0%	14.8%	14.2%	37.0%	100.0%
		% within Group	36.7%	16.0%	15.3%	40.0%	27.0%
Total		Count	150	150	150	150	600
		% within Perception	25.0%	25.0%	25.0%	25.0%	100.0%
		% within Group	100.0%	100.0%	100.0%	100.0%	100.0%

Table 1 shows that out of total 150 Marketing Male group, 40 students i.e. 26.7% of the marketing male students had low perception towards CSR, 55 students i.e. 36.7 % of the marketing male students had average perception towards CSR, 55 students i.e. 36.7% of the marketing male students had high perception towards CSR. The results show that majority of marketing male students have high and average level of perception towards CSR and substantial percentage of marketing male students have low level perception towards CSR.

Out of total 150 marketing female group, 69 students i.e. 46 % of the marketing female students had low perception towards CSR, 57 students i.e. 38 % of the marketing female students had average perception towards CSR, 24 students i.e. 16 % of the marketing female students had high perception towards CSR. The results show that majority of marketing female students have low level of perception towards CSR, a substantial percentage of marketing female students have average level perception towards CSR and a relatively small percentage of marketing females have high level of perception towards CSR.

Out of total 150 finance male group, 25 students i.e. 16.7% of the finance male students had low perception towards CSR, 102 students i.e. 68% of the finance male students had average perception towards CSR, 23 students i.e. 14.2% of the finance male students had high perception towards CSR. The results show that majority of finance male students have average level of perception towards CSR, an almost equivalent percentage of finance female students have low and high level perception towards CSR.

Out of total 150 finance female group, 28 students i.e. 18.7% of the finance female students had low perception towards CSR, 62 students i.e. 41.3% of the finance female students had average perception towards CSR, 60 students i.e. 40% of the finance female students had high perception towards CSR. The results show that majority of finance female students have average and high level of perception towards CSR, and a relatively lesser percentage of finance female students have a low level of perception towards CSR.

RESULTS AND DISCUSSION

It may be concluded from the findings of this study that majority of marketing male students have average or high level of perception towards CSR, while majority of marketing females have low levels. Whereas majority of finance males show average level of perception towards CSR, majority of finance females have average or high level of perception towards CSR. This may be attributed to varied social roles executed by women and men execute and different exposures and trainings given in marketing stream. The findings reflect that a significant number of management students have low level of perception and indicate a need for developing positive attitudes and awareness towards the concept of CSR for building stronger perceptions towards CSR and effective implementation. It is argued that managers who have high CSR perceptions will not just be better in implementing explicit CSR policies but will also ensure making company's day today activities socially responsible. (Dash & Sahoo, 2018). Sustainable development is the strategic base for social responsibility in modern business and satisfying social needs & economic needs by providing degrading natural and social capital is a

necessary condition for survival of a company in long perspective. Corporate commitment to and for sustainability is essential in the challenging times of economic turbulence and rapid changes and is representative of the window of opportunity for revisiting assumptions and value propositions for long-term business models (Gerner, 2019). The findings present important implications for policy makers and academicians and highlights that it is imperative to create awareness in the future generations regarding CSR. Business students are future managers and they need to be made aware of their role in implementing CSR for a sustainable future.

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On the reduction of health hazards caused by modified genes in indigenous rice plant varieties

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ABSTRACT

Genetically changed or designed foods are made from speedily increasing technologies that have sparked international debates and considerations regarding health and safety. These considerations target the potential dangers to human health, the risks of genetic pollution, and also the death of other farming techniques additionally as theft and economic exploitation by massive non-public firms. Transgenic or genetically changed plants possess novel genes that impart useful characteristics like weed killer resistance. One amongst the smallest amount understood areas within the environmental risk assessment of genetically changed crops is their impact on soil- and plant-associated microorganism communities. The popularity that these interactions might amendment microorganism multifariousness and have an effect on scheme functioning has initiated a restricted range of studies within the space. Moreover, novel proteins are shown to be free from transgenic plants into the soil scheme, and their presence will influence the multifariousness of the microorganism community by selection stimulating the expansion of organisms that may use them. Microorganism diversity is altered once related to transgenic plants; but these effects are each variable and transient. Minor alterations within the diversity of the microorganism community might have an effect on soil health and scheme functioning, and so, the impact that plant selection might wear the dynamics of the rhizosphere microorganism populations and successively plant growth and health and scheme property need additional study. Our aim is to identify the genes by the multiple sequence alignment (MSA) or the proteins related to the gene which are causing health hazard in human and to reduce the risk by homology modeling and spectroscopic analysis

KEY WORDS: GENETICALLY MODIFIED CROPS, HOMOLOGY MODELING, MULTIPLE SEQUENCE ALIGNMENT, SPECTROSCOPIC ANALYSIS, TRANS-GENES

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INTRODUCTION

Ranchers have with progress turned to hereditarily rising their yields through pondered plant rearing for a huge number of years, however, its logical premise wasn't set up till established botanist hereditary science were rediscovered inside the mid-twentieth century. For centuries, the typical plant reproducing practices of ranchers have the diode to fixing the cosmetics and advancement of harvests. amid this sense, ranchers are thought of to be the essential hereditary designers (Dunfield et al. 2004) desoxyribonucleic (DN) corrosive sequencing from qualities (Walters, 2004) exploitation DN corrosive markers (Acosta and Chaparro, 2008) for building hereditary maps, concocting PCR-based procedures for picking and portraying qualities, and DN corrosive exchange advancements between totally unique species have all set the establishments for the popular generation of the hereditarily designed plants and yields by and by available (Fischer and Emans,2000; Uzogara, 2000; Margulis, 2006; Singh et.al. 2006).

The apprehensions of the general population contradicting the innovation creating GM crops are related with a more extensive range of issues including the inclination that transnational organizations are more keen on expanding their benefits than in ensuring the earth or easing hunger, the likelihood that transgenic harvests may attack wild biological systems with hindering consequences for biodiversity, the unjustifiable challenge with other rural frameworks, for example, natural, agro-environmental and customary ones, the negative impacts that GM sustenance may deliver on human wellbeing, (ILSI, 2003 and 2004; Halpin, 2005; Brookes et al., 2006; ISAAA, 2006; Made et al., 2006; Taylor et al., 2006; Jeong et al., 2007;) the conceivable negative effect of GM crops on nourishment supply security, and an absence of trust in the offices in charge of directing GM crop bio-security. Commentators of GM crops contend that transgenic innovation has genuine ramifications for ranchers in creating nations as this remote global innovation may crush ranchers' abilities worked around indigenous agrarian frameworks, subsequently intensifying social rejection on account of subsistence ranchers (Chakraborty et al., 2001; WAL, 2001; Bucchin and Goldman, 2002; Newell-Mcgloughlin, 2006; Alexander et al., 2007; Oliver et al., 2007; Raney and Pingali, 2007).

As per some examination, numerous anti-microbial gatherings have been advancing neoliberal rationale as they have concentrated on the contemporary markets as the most ideal method for guaranteeing elective agribusiness. Their strategies stay concentrated on what should be possible at the ware level (Hodgson, 2001; Setamou et al. 2002; Tolstrup et al, 2003; Munoz et al, 2004; Poulsen et al., 2007; Schrøder et al. 2007). The original GM crops designed with info qualities to give improved agronomic execution and monetary and natural advantages have been related to ranchers' interests. Be that as it may, there is an expanding pattern towards creating the second era GM crops by exchanging esteem included yield qualities, fundamentally profiting customers and processors (OCTM, 2001; Azevedo and Welington 2003; ISO 2005 and 2006). The entry of sustenance DNA pieces over the intestinal divider is a characteristic and physiological marvel, chiefly when DNA is at high focuses in the nourishment. Given that domesticated animals expend significant measures of GM crop-inferred plant feed, open worry about the utilization of creature items containing transgenic DNA and protein have prompted examinations identified with their destiny inside the gastrointestinal tract of animals and the conceivable gathering of trans-qualities and their encoded proteins inside tissues, (Tylecote 2019, Kunling et.al 2019).

Endogenous and transgenic DNA pieces from low-duplicate qualities have been recognized in creature tissues, however in lesser sums than that distinguished on account of high-duplicate qualities. Section of plant DNA parts, endogenous or not, over the intestinal hindrance does not seem to have effectively affected animals (McClements, 2019). Proteins communicated by GM crops have raised some worry as they might be associated with nourishment sensitivities. Surveying allergenicity to GM crops has additionally included physicochemical and biochemical measures relating protein strength to warmth, corrosive and stomach related chemicals. In addition, transgenic innovation has been helpful in creating hypoallergenic crops by meddling with the outflow of qualities encoding significant allergens. There is scientific confidence that GM crops do not represent greater risks than those already present in conventional agriculture and that any new risk posed by GM crops could be identified, managed and prevented (Zhiguang et al., 2019, Wen-Ching et al. 2019).

However, conventionally modified genes are numerous and their functions remain essentially unknown, whereas trans-genes are controlled by their nature, making them more reliable in terms of obtaining the desired outcome. Our aim is to detect the abnormalities in the GM rice varieties and the effect of it in the human being and also to modify the changes in a way so that the yield does not vary and also the effect on human being is not harmful. This work was designed to modify the crops without harming the body.

MATERIALS AND METHODS

Materials: Every traditional variety has some nutritive or medicinal property. Today unfortunately, we have

narrowed our choices to a handful of varieties and consume them polished devoid of fiber and minerals. We, instead, eat a range of processed foods made with the same grains and mistake variety for diversity. However, it is diversity that will give us a range of nutritional elements – apart from fulfilling our need for varied taste. Each of the different rice varieties have their particular taste profile, and often, most people develop their personal favorites. Two GM rice samples, FR0502519 and FR0502520, were kindly provided agriculture extension department of Centurion University. The samples were labelled as "Anti-pest Shanyou 63" and as "Anti-pest Jinyou 63".

Sample material and DNA extraction: DNA was separated from ground material utilizing a changed CTAB nucleic corrosive extraction technique (ISO, 2006). 1.5 mL CTAB extraction cradle (20 g/L cetyl-trimethyl-ammonium bromide, 1.4 mol/L NaCl, 0.1 mol/L Tris–HCl, 0.02 mol/L Na2EDTA, pH 8.0) and 10 µL Proteinase K arrangement (20 mg/mL) were added to 200 mg of the processed rice test. The examples were brooded at 60 °C under steady fomentation medium-term and afterward centrifuged for 10 min at 13,000×g. The supernatant was moved into another vial, 750 µL of chloroform was included, vivaciously shaken and after that centrifuged at 13,000×g for 5 min. The upper stage was moved into another vial, its volume was resolved and blended with two volumes of CTAB precipitation support (5 g/L cetyl-trimethyl-ammonium bromide, 40 mmol/L NaCl). After hatching for 60 min at room temperature without tumult, the examples were centrifuged for 15 min at 13,000×g, the supernatant was disposed of and the pellet was resuspended in 350 µL of a 350 mmol/L NaCl arrangement. Chloroform (350 µL) was included, the examples were blended on a Vortex and centrifuged for 10 min at 13,000×g. The upper stage was joined with 0.6 volumes of isopropanol for nucleic corrosive precipitation and after 20 min brooding at room temperature the examples were centrifuged 10 min at 13,000×g. The supernatant was disposed of, the pellet was washed with 500 µL ethanol arrangement (70% EtOH) and settled in 200 µL TE support (1 mmol/L Tris– HCl, 0.1 mmol/L Na2EDTA, pH 8.0).

The separated DNA was evaluated in a fluorometric examine utilizing the PicoGreen dsDNA restricting color (Invitrogen) as per the producer's guidelines. Estimations were led in an ABI PRISM 7900 (Applied Biosystems) at 525 nm. A 100 bp sub-atomic size DNA marker with a centralization of 0.1 µg/µL DNA (Fermentas) was utilized for adjustment. For explicitness tests, DNA was removed from two financially accessible regular rice brands purchased in Germany ('Wurzener Parboiled Reis', Lot L 60531E, Wurzen, Germany and 'Gut and Günstig Spitzen-Langkorn-Reis', Lot 07\02\2008, Hamburg, Germany), from GM rice line LLRICE62, GM soya line GTS40-3-2 and GM maize lines T25, Bt11, Bt10, Bt176, MON810, MON863, NK603, TC1507, GA21 and CBH351.

For affectability tests, a sequential weakening of DNA extricated from tests FR0502519 and FR0502520 was set up by stepwise fourfold weakening with 0.1× TE cushion. Another sequential weakening likewise used to test for the affectability was set up similarly with DNA extricated from test FR0502519 and a blend of genomic DNA from ordinary rice ('Wurzener Parboiled Reis', 10 µg/mL) and maize (10 µg/mL). The mass portion blends with various GMO substance were readied utilizing ground customary rice and GM rice test FR0502519. A blend of 9.5 g traditional rice and 0.5 g of rice test FR0502519 bringing about a 5% (w/w) test was utilized a beginning material. 2 g of this 5% (w/w) blend was added to 8 g of regular rice giving a 1% (w/w) test. A consequent 0.5% (w/w) blend was readied utilizing 5 g of the 1% (w/w) test and 5 g of customary rice, and a 0.1% (w/w) level was readied utilizing 8 g ordinary rice and 2 g of the 0.5% (w/w) test. For consequent quantitative continuous PCR investigations DNA was extricated from the 5, 0.5 and 0.1% mass part blends.

PCR and DNA sequencing: PCR was performed in a volume of 25 µL containing 2.5 µL 10× PCR support with 15 mmol/L MgCl2, 0.5 µmol/L of every preliminary, 0.625 U Taq polymerase (HotStar, Qiagen) and 1 µL of layout DNA relating to 25 ng DNA. For warm cycling, an underlying denaturation venture for 15 min at 95 °C was trailed by 45 cycles of 30 s at 94 °C, 30 s at 60 °C and 60 s at 72 °C with a last prolongation venture of 7 min at 72 °C. In traditional PCR and constant PCR focusing on the CaMV 35S advertiser and the nos eliminator groupings conventions portrayed somewhere else were utilized (Ehlers et al., 1997).

For arrangement assurance of the transgenic develop DNA separated from tests FR0502519 and FR0502520 was utilized in PCR explores different avenues regarding groundworks RiceActin1f (5'-ccc tct cct ctt tct ttc tcc g-3'; individual correspondence N. Hess) in blend with groundwork NOS180R (5'-TTg TTT TCT ATC gCg TAT TAA ATg T-3'; individual correspondence R. Reiting) at response conditions as depicted. Extra PCR items were created with oligonucleotides NOS-1 (5'-gAA TCC TgT TgC Cgg TCT Tg-3'), NOS-3 (5'-TTA TCC TAg TTT gCg CgC TA-3') and CryIA(b)F (5'-ACC ATC AAC AgC CgC TAC AAC gAC C-3') utilizing response conditions depicted somewhere else (Ehlers et.al, 1997).

PCR items were decontaminated with a QIAquick PCR refinement unit (Qiagen) and legitimately sequenced with the BigDye Terminator V 1.1 cycle sequencing pack (Applied Biosystems) in an ABI PRISM 310 instrument (Applied Biosystems). Sequencing was finished

Table 1. Description of the different real-time PCR systems used in the study. The length of the generated PCR product is given in brackets

Method	Name	Oligonucleotide sequence (5'–3')	Final concentration in PCR (nmol/L)
Bt rice construct (83 bp)	T51F	gAC TgC Tgg AgT gAT TAT CgA CAg A	400
	T51R	AgC TCg gTA CCT CgA CTT ATT CAg	400
	T51p	FAM-TCg AgT TCA TTC CAg TTA CTg CAA CAC TCg Ag-TAMRA	200
gos9 rice reference gene (68 bp)	org1	TTA gCC TCC CgC TgC AgA	400
	org2	AgA gTC CAC AAg TgC TCC Cg	400
	orgp	FAM-Cgg CAg TgT ggT Tgg TTT CTT Cgg-Dabcyl	200
sps rice reference gene (81 bp)	SPSF	TTg CgC CTg AAC ggA TAT	650
	SPSR	Cgg TTg ATC TTT TCg ggA Tg	550
	SPSP	FAM-gAC gCA Cgg ACg gCT Cgg A-Dabcyl	200

by a groundwork strolling procedure to produce successions in covering areas of the diverse develop components. Nucleic corrosive succession information were first contrasted and the Sequence Navigator programming (Applied Biosystems) and afterward investigated via looks in the GenBank arrangement database utilizing the PC calculation BLAST 2.

Real-time PCR: Real Time PCR was performed in an ABI PRISM 7900 instrument (Applied Biosystems). All responses were kept running as copies in 96-well plates. The 25 µL response blends contained 12.5 µL all inclusive ace blend (Applied Biosystems), the showed convergences of groundworks and test (Table 1) and 5 µL of format DNA. The response conditions were as per the following: Initiation venture for 10 min at 95 °C pursued by 45 cycles of 20 s at 95 °C and 1 min at 60 °C. Preliminaries T51F and T51R1 and test T51p (Table 1) were structured with the Primer Express 2.0 programming (Applied Biosystems). Groundwork and test successions for location of taxon-explicit rice reference qualities were taken from distributed continuous PCR measures.

RESULTS AND DISCUSSION

Sequence analysis of GM rice samples: Two rice seed tests taken at neighborhood Chinese wholesalers by Greenpeace in the year 2005 were utilized for DNA arrangement examination. These examples, FR0502519 and FR0502520, were at first examined with GMO screening tests. The two examples demonstrated positive responses in a CryIA(c) immuno-based Bt-protein test and in a DNA-based test for the nopaline synthase (nos) translation eliminator arrangement, though just a single example (FR0502520) was certain for the CaMV 35S advertiser succession in a 35S DNA-test. Re-examination of these outcomes with a 35S advertiser explicit continuous PCR test demonstrated that just a powerless 35S response with a Ct-estimation of ~35 is discernible with DNA from FR0502520 when contrasted with the Ct-estimation of ~24 acquired in the nos-explicit constant PCR utilizing a similar measure of test DNA. We accept that hints of another GMO is available in test FR0502520, since the build in the suspected 'GM Shanyou 63' line ought not contain the CaMV 35S advertiser and the expulsion of the 35S advertiser driven hph marker quality by isolation has been accounted for the parental CMS restorer line 'Minghui 63'.

A few distinctive groundwork blends focusing on the assumed transgenic build embedded in 'GM Shanyou 63' were utilized to create PCR items reasonable for direct DNA sequencing (Fig. 1). The nucleotide (nt) successions of these items were broke down with the BLAST likeness internet searcher of the National Center for Biotechnology Information (NCBI). The two locales chose for arrangements of the transgenic groupings with indistinguishable GenBank successions are appeared. 1 and 2). The 5' succession piece of the enhanced part indicates total character to the rice Act1 intron contained in a plant change vector (pPLEX-5013) over a stretch of 404 nucleotides (Fig. 1). This grouping is trailed by a 38 nt long spacer with parts of a various cloning site until a potential ATG begin codon of the Bt poison quality encoding perusing outline is found. The following 347 nt significant lot indicates personality to an engineered CryIA(c) quality (increase number Y09787). The succession of the PCR items produced with DNA from FR0502519 and FR0502520 demonstrated no distinctions in this area (information not appeared), showing that either the two examples speak to a similar GM rice occasion, or that the two examples get from change occasions with a similar build. The distinguished transgenic develop apparently compares to plasmid pFHBT1 which was utilized for creation of the 'GM Shanyou 63'

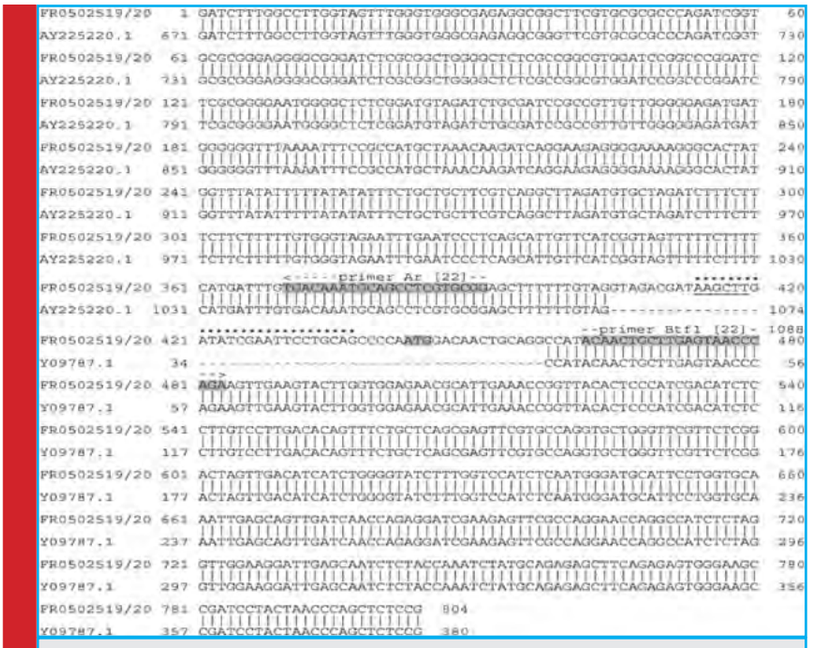

FIGURE 1. Alignment of the nucleotide sequences of PCR products generated with sample DNAs FR0502519 and FR0502520 with identical sequences identified by BLAST GenBank database searches. The sequence of AY225220.1 encodes the first intron of the rice Act 1 gene. Entry Y09787.1 represents a synthetic cryIA(c) gene sequence. The asterisks indicate a potential spacer sequence with similarity to a fragment of a multiple cloning site, containing also the HindIII site as described by (underlined). The shaded sequences are identical to primers 'Ar' and 'Btf1' which were used in a study for characterization of the transgenic restorer line TT51 and of its derived-hybrid GM Shanyou 63. The potential ATG start codon of the Bt fusion gene is also shaded.

line. Especially a short amino-terminal peptide succession (P-N-I-N-E-C-I) erased in the half and half cryIA(c) quality of pFHBT1 is likewise not present in the reasoned protein arrangement of the distinguished perusing outline.

To additionally break down the transgenic groupings in the rice test materials under scrutiny, it was tried whether the nos eliminator is likewise situated behind the Bt cryIA(b) and cryIA(c) combination quality as portrayed for the pFHBT1 build. Utilizing DNA from FR0502519 and FR0502520 as layout, particular PCR items were enhanced with preliminaries spreading over the district of the 3' part of the cryIA(c) quality and the nos eliminator. Sequencing results demonstrates that in this district no distinctions are available in the two explored rice tests (Fig. 2). The initial 393 nucleotides of the succession totally match to a GenBank database passage coding for an engineered cryIA(c) quality. Toward the finish of the cryIA(c) grouping homology a 26 nt long spacer with no similitude to realized successions is found, trailed by a 169 nt long arrangement indistinguishable to the nos eliminator got from *Agrobacterium tumefaciens*.

Crop-to-crop gene flow: All in all, developed rice is portrayed by high rates of self-fertilization and almost no cross-fertilization between nearby plants or fields (ordinarily under 1 percent). Tests in Italy demonstrated that dust intervened quality stream from a transgenic, herbicide-safe rice assortment to adjoining plants of a nontransgenic partner was 0.05 to 0.53 percent. Present day rice cultivars are regularly become close more established landraces (privately adjusted assortments that were trained and improved by customary ranchers) in Asia, and hybridization rates between these two gatherings additionally seem, by all accounts, to be extremely low . These discoveries are steady with the little separations that are suggested for detaching and keeping up the virtue of developed rice developed in seed nurseries. In the United States, for example, rice plants that are developed for ensured seed to be sold to ranchers must be segregated from other rice assortments by just 6 meters (m) or less.

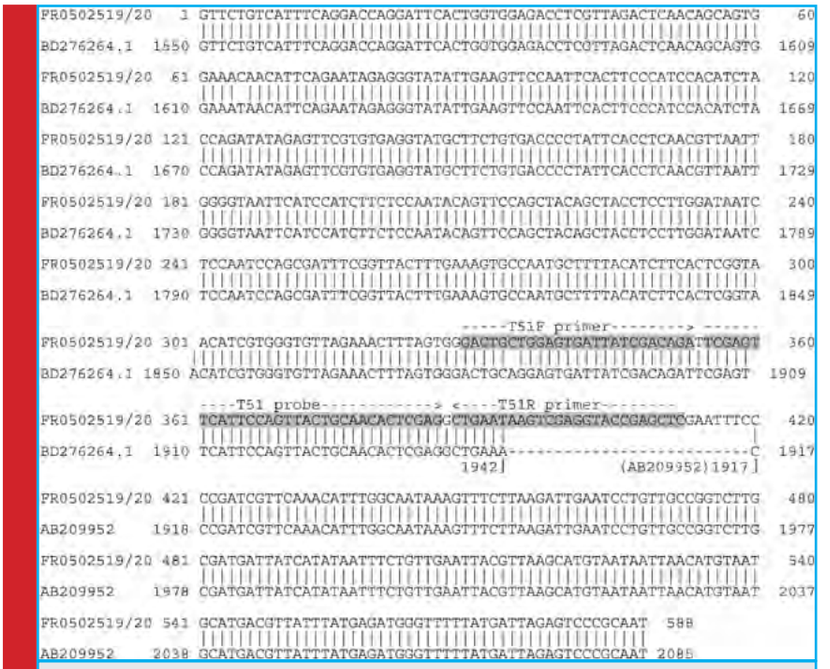

FIGURE 2. Alignment of sequences of PCR products amplified with DNAs extracted from samples FR0502519 and FR0502520. The sequence of BD276264.1 represents a patented GeneBank data base entry encoding the CryIA(c) gene. The sequence of AB209952 is taken from a GenBank entry containing the nos terminator sequence present in glyphosate tolerant GM soybeans. The positions of the primers and the probe used by the construct-specific Bt rice detection method described in this study are shaded.

Crop-to-weed gene flow: Another vital yet little-examined part of quality stream is the ingenuity of yield qualities following crop– weed hybridization. Similarly as half and half life is seen when ingrained, developed lines are crossed to deliver "mixture" rice, so may weedy rice profit by hybridizing with the harvest, if this outcomes in more prominent heterozygosity. I a work it has found that more prominent power in crop– weed cross breeds than in their weedy guardians, and frequencies of yield alleles in weedy rice were as high as 52 percent after just two years of contact with the harvest. In any case, in Arkansas, original cross breeds among developed and weedy rice blossomed so late that they had much lower wellness than their weedy guardians. Along these lines, the developmental significance of half breed energy in weedy rice populaces gives off an impression of being variable and ought to be examined all the more extensively.

Through the span of a few ages, crop qualities that are firmly pernicious to weedy rice, just as different qualities that are connected to malicious harvest qualities, are probably going to be cleansed from weedy populaces by normal choice and choice weights from ranchers. Then again, connected qualities that are related with more prominent survival and propagation are required to increment in recurrence following scenes of hybridization.

Spread of transgenic herbicide resistance: Transgenic herbicide opposition is a quality that could without much of a stretch be procured by weedy rice. Weed control in rice fields is progressively subject to herbicides in both created and creating nations, halfway in light of the fact that more established strategies for hand transplanting youthful rice plants into overflowed fields are being supplanted by direct seeding methods. This progress has brought about more awful issues with weeds, since weed seedlings can smother the development of rice seedlings. Rice handle that become intensely pervaded with weedy rice can end up unusable, in light of the fact that the weed is a successful copy of the harvest and its seemingly perpetual seed bank makes it exceptionally hard to destroy. Consequently, rice producers who can manage the cost of the expense of herbicides are anxious to embrace herbicide-safe rice assortments, despite the fact that the advantages of this methodology could be brief.

Effects of other fitness-related transgenes: As a rule, different transgenes are not expected to continue and

spread in weedy or wild populaces. On the off chance that the transgenes encode qualities that don't upgrade the plants' survival or multiplication, the departure of these qualities is probably not going to result in natural issues, in light of the fact that the new qualities will be uncommon. In like manner, if the transgenes give a wellness cost, as diminished survival or fertility, people bearing these qualities will be more averse to pass them on to their descendants. Numerous transgenic characteristics identified with wholesome quality and grain arrangement are probably going to have nonpartisan or negative consequences for the wellness of weedy and wild relatives of the yield. As opposed to these precedents, the wellness of wild and weedy rice populaces may be improved by transgenes that give better vermin opposition, more noteworthy resistance of abiotic stresses, for example, dry spell and saltiness, and upgraded yields. Contingent upon nearby conditions, these transgenes may discharge weedy or wild populaces from natural weights that confine their neighborhood plenitude or breaking point their territory prerequisites.

A few interrelated inquiries emerge in regards to the natural impacts of wellness upgrading transgenes. To start with, given these qualities' capability to spread and endure, their conceivable negative impacts on non target species ought to be considered. Would the transgene or its items lead to hurtful impacts on gainful bugs, natural life, or different species, and how might these impacts contrast and conceivable mischief coming about because of traditional farming practices. This lead us to the different practices which are causing hazards to the body. This MSA provide us the data of the genes affected in GM crop and also the data of the genes affected by the GM crops in human from which we can identify the genetic rectifications and the cause of it and through which we can stop the genetical modification in human which actually increasing day by day.

CONCLUSION

Taking everything into account, it merits stressing that quality stream is a characteristic procedure that happens unavoidably under normal conditions. Based on current natural and transformative learning, we trust that the dispersal of numerous sorts of transgenes from rice won't be unsafe to the earth. Transgenes that don't have critical common specific focal points should cause constrained ecological effect, assuming any. Then again, transgenes that lead to more prominent bounties of weedy or wild rice could present natural issues, as depicted previously. Before allowing the business arrival of new sorts of transgenic rice, policymakers should most likely gauge the net natural advantages and dangers of such transgenes against different elements, for example, the foreseen impacts of transgenic assortments on human wellbeing, nearby flourishing, and exchange. Natural evaluations that are deductively thorough and freely available are fundamental for the long haul accomplishment of this imperative innovation.

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Antimicrobial activity of web of spider, *Stegodyphus sarasenorum* on *E. coli* and *S. aureus.*

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ABSTRACT

Antimicrobial activity of web of spider *Stegodyphus sarasenorum* was studied on two different bacteria. Web extract prepared in three different solvents Ethanol, Methanol and Acetone. Zone of inhibition was observed for two bacteria *E. coli* and *S. aureus* after 24 hours, for all solvents. Web extractin Ethanol has shown inhibition diameter 6 mm for *E. coli* and no antimicrobial activity for *S. aureus*. Methanol extract shown zone of inhibition 12 mm for *E. coli* and 14 mm for *S. aureus*. For Acetone it was 14 mm for *E. coli* and 18 mm for *S. aureus*.

KEY WORDS: ANTIMICROBIAL ACTIVITY, WEB, *STEGODYPHUS SARASENORUM*, *E. COLI* AND *S. AUREUS*, SOLVENTS

INTRODUCTION

An antimicrobial is an agent that kills microorganisms or stops their growth. Nowadays in this ail-full life nature is being a great source as an answer for many therapeutical problems and also to treat the infection with alternative means. Many Bacterial diseases are contagious and can result in many serious and life threatening complications. *Escherichia coli* and *Salmonella* cause food poisoning. *Helicobacter pylori* causes gastritis and ulcers. *Neisseria gonorrhoea* causes sexually transmitted disease gonorrhoea. *Neisseria meningitides* causes meningitis. *Staphylococcus aureus* causes variety of infections in the body including boils, cellulitis, abscesses, wound infections, toxic shock syndrome, pneumonia and food poisoning. *Streptococcal* bacteria causes ear infections, strep throat, meningitis and many other infections.The natural world is a good source of therapeutic products that are able to inhibit the growth of bacteria. The peptides with antibacterial activity have been found in plants and the whole animal kingdom, from bacteria and different insect orders to amphibians, mammals and humans (Haeberli *et al*., 2000). Phospholipids hydrate and potassium nitrate available at spider

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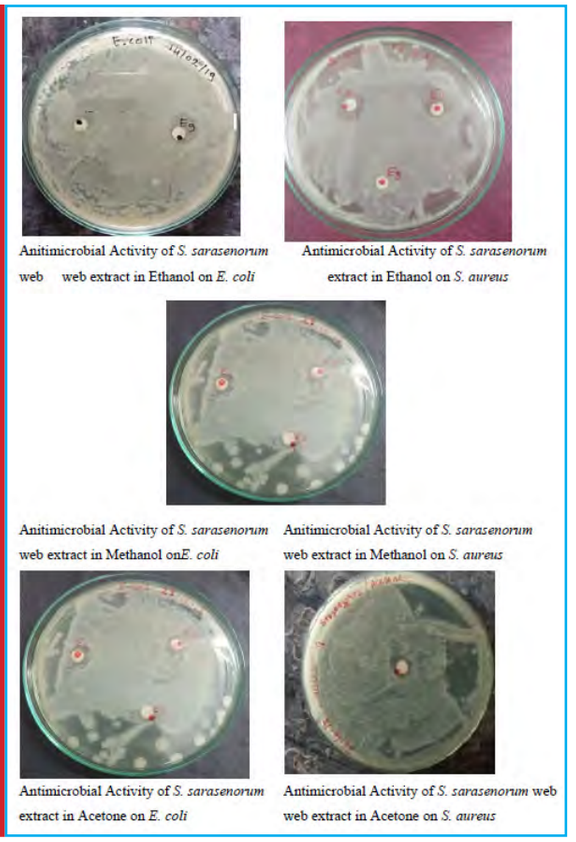

Anitimicrobial Activity of *S. sarasenorum* web web extract in Ethanol on *E. coli*

Antimicrobial Activity of *S. sarasenorum* extract in Ethanol on *S. aureus*

Anitimicrobial Activity of *S. sarasenorum* web extract in Methanol on*E. coli*

Anitimicrobial Activity of *S. sarasenorum* web extract in Methanol on *S. aureus*

Antimicrobial Activity of *S. sarasenorum* extract in Acetone on *E. coli*

Antimicrobial Activity of *S. sarasenorum* web web extract in Acetone on *S. aureus*

silk can prevent from the growth of fungi and bacteria on the silk (Chakraborthy*et al.* 2009; Gomes *et al.* 2010, WSC 2016, Deshmukh 2017).

It has been observed that there is no any microbial growth on spider's web even after it is rich source of proteins. The indication of its resistance to microorganism is its longitivity. Studies by Vollrath *et. al.* (2006) have investigated the compounds present in spider silk and have found that spider silk contains molecules that are known to have antimicrobial properties. There are many ancient examples of applications of spider silk in medicines. According to Heimer (1988) one traditional use of spider's web seen in ancient times that it was used by the peasants in Carpathian Mountains for healing wounds; they used the web of *Atypus* spider. All spiders do not spin the web, some only secrete silk to protect egg sacs or form simply drag line. Where as many spiders construct the large sized webs, there are different types of web varies according to size and shape. Orb-weaving spiders are the members of family Araneidae which spin orb webs. They have spoke like wheels with a spiral design. Cob web are the messy webs in corners especially along top of the walls. Spider belongs to family Theridiidae spin cob webs. Sheet webs are made from dense layer of silk to trap the prey. *Stegodyphus* have unkempt, irregular and large webs.

Table 1. Zone of inhibition observed for web of *Stegodyphus sarasenorum*

Solvents	Length in mm	
	E. coli	S. aureus
Ethanol	6 mm	-
Methanol	12 mm	14 mm
Acetone	14 mm	18mm

S. sarasenorum is also known as a social spider. It is native to India, Nepal, Sri Lanka and Myanmar (Karsch 1892, WSC 2016). This spider exhibits communal predation and feeding (Willey *et. al* 1993) where individual live in large cooperatively build nest or retreat constructed of silk woven using leaves, twigs and sheet webs for capturing prey (Chakraborthy *et. al.* 2009). It has been observed that in life cycle of this spider at final instar stage female devotes her life for the spiderlings to use her body fluids and then dies (Deshmukh 2017).

Spider web used in this study was of *S. sarasenorum* from family Eresidae. Webs were collected from different locations, from the places of their abundance. Spider's fresh web was collected with the help of brush and forceps. Web is kept in polythene bags of 50 micron, maintaining aseptic conditions. Bacterial cultures used were *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive). Bacterial cultures were collected from Department of Microbiology Bhartiya Mahavidyalaya, Amravati. Cultures were in the form of nutrient broth.

Desired bacteria *E. coli* and *S. aureus* were suspended separately in a liquid nutrient medium called Luria broth in an upright flask from which large amount of bacteria were cultured.

Spider's web was washed using distilled water and oven dried. Followed by drying web was weighed. Extract was prepared using three different solvents i.e. Ethanol, Methanol, Acetone. 1 gm. of web is dissolved in 10 ml of Ethanol, Methanol and Acetone separately for a week. Extract made was centrifuged at 4000 rpm for 30 minutes. Extracts of web in different solvents showed different colour appearance of supernatant.Agar gel was prepared. Inoculation was done in laminar airflow. Agar liquid was poured in petri plates and kept undisturbed for a while until it gets solidified into gel. Then using micropipette 5µl desired bacteria was inoculated. Spreading was done using 'L' shaped loop. A place was marked on the petri plate; a punched filter paper was deep into the extract in a cavity block and kept on the mark place.Incubation was done in incubator at 37^0 c, for 24 hours. After 24 hours petri plates were observed.

Antimicrobial activity of *S. sarasenorum* of family Eresidae was observed. Two bacterial strains were used i.e. *E. coli* and *S. aureus*. Three extracts of spider web

was prepared using three solvents Acetone, Methanol and Ethanol. Firstly these three solvents (Ethanol, Methanol, and Acetone) were tested against bacterial strains and it has been observed that there was no inhibition for growth of bacteria. Extract of web in distilled water does not show any zone of inhibition. The extracts of spider web in three solvents Acetone, Methanol and Ethanol used to study antimicrobial activity. Length of zone of inhibition is measured in mm, measured as diameter of zone with the help of paper scale.Extract of web of *S. sarasenorum* was prepared by using three solvents, Ethanol, acetone, methanol, by taking concentration 1:10. After inoculation the extracts of web of *S. sarasenorum* was placed on the inoculated plate of *E. coli* and *S. aureus* and then incubated for 24 hour.After 24 hour following results were observed: For three different solvents Ethanol, Methanol and Acetone zone of inhibition was observed for two bacteria *E. coli* and *S. aureus* gram negative and gram positive bacteria respectively. For *S. sarasenorum* Ethanol extract has shown the diameter 6 mm for *E. coli* and no inhibition for *S. aureus*. Methanol extract shown zone of inhibition was 12 mm for *E. coli* and 14 mm for *S. aureus*. For Acetone it was 14 mm for *E. coli* and 18 mm for *S. aureus*.

Findings of the work indicate that the web of *S. sarasenorum* possesses antimicrobial activity, when the extracts are prepared with Acetone, Ethanol and Methanol. Web of *Stegodyphus* resulted into maximum antimicrobial activity in Acetone for *S. aureus*. This study will be the base for further investigations on advance purification.

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Optimization of Cultivation Conditions for Microbial Lipid Production by *Rhodotorula glutinis*, an Oleaginous Yeast

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ABSTRACT

Microbial fuels are the 3rd generation biofuel. These fuels are made by the conversion of microbial lipid into fuel. *Rhodotorula glutinis*, a pink oleaginous microbe has the capacity to produce microbial lipids from cuture medium containing carbon and nitrogen sources. These microbial lipids can further be converted in to fuel. In this work, the growth conditions for R. glutinis were optimized with different C:N ratios by having various concentrations of Carbon and Nitrogen sources. Glucose, Fructose, Sucrose, Galactose and Xylose were evaluated as carbon sources (70 g/L). ($NH_4H_2PO_4$, NH_4NO_3, $(NH_4)_2SO_4$, Urea, Aspargine were evaluated as Nitrogen source (20g/L). Subsequently, the influence of surfactants (Tween 20 and Tween 80 (0.5 ml/L), pH (3, 4,5) and incubation temperature ^{0}C (25,30,35 and 40) were also analysed in intial media composition. *Rhodotorula glutinis* showed significant growth with maximum biomass and lipid production in the media containing sucrose as carbon source, NH_4NO_3 as nitrogenous source at pH 4 and temperature 30 °C. The surfactant has shown no effect in lipid production. Thus, the results of this study indicated that these optimized conditions could be used to produce maximum production of lipids as biodiesel feedstock.

KEY WORDS: *RHODOTORULA GLUTINIS*, MICROBIAL LIPIDS, MICROBIAL FUELS, OLEAGINOUS MICROBE

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INTRODUCTION

The price of fuel rises every year with a worrying concern of depletion and complete loss of the available natural resources. With the idea of sustainable development and cutting the economic values of the fuels other options are like usage of plants, animals, aqua life and microbes are acquitted for research and development. Biofuels, namely bioethanol, butanol produced from cellulose are therefore of considerable interest to various researchers as well as governments (Deman et al., 2005; Hill et al., 2006, Zhou et al., 2014; Choudhary et al., 2017).

Yeast is generally being employed on a large scale yielding vitamins, industrial enzymes and pharmaceutical polypeptides. The oleaginous attribute of yeast renders it advantage over its proponent viz. bacteria, molds, and alga, primarily due to higher rates of proliferation & also due to the propensity to greater lipid yields (Saenge et al., 2011). High degree of lipid accumulation is due to carbon surplus and a nitrogen or some other nutrient deficit in the growth medium. (Ratledge & Boulton, 1985; Ratledge, 1986). Hence fatty acid profile of the microbial oil like the oleaginous yeast makes it potential host for biodiesel industry due to the accumulation of polyunsaturated fatty acid triacylglycerol inside the cells, which is similar to vegetable oils (Dai et al., 2007; Kot et al., 2016).

Jiru et al., (2017) optimized the biotechnological production of lipid by *Rhodotorula kratochvilovae* (syn, *Rhodosporidium kratochvilovae*) SY89 for biodiesel preparation .It could serve as a replacement for conventional oil sources like crude oil replenishing the necessities of the energy sector & also industries like food, pharmaceutical or cosmetic. *Rhodotrula glutinis* is aerobic yeast characterized by pink, smooth colonies with a moist appearance, sexually reproduce via basidiospores arising from a teliospore developed primarily due to the existence of mycelial clamp connection. Multipolar budding serve as reproductive avenue. The significance of *Rhodotorula glutinis* yeast is being acknowledged & published in different journals for the production of numerous useful metabolites such as lipid (Li, et al., 2013), carotenoids having anticancerous and antioxidant properties (Gupta et al., 2012: Yen et al., 2015) and many industrially useful enzymes. *Rhodotorula glutinis* can produce brewery effluent (Yen et al., 2012), crude glycerol (Schneider et al., 2013), microbial lipids, carotene, cellulase and carotenoids by using cheap energy sources like carbon, nitrogen, Sulphur, (Karamerou et al., 2016; Pi et al., 2018; Elfeky et al., 2019). In this research Optimization of Microbial lipid production by *Rhodotrula glutinis* was done by using various carbon and nitrogen substrates at different pH, Temperature and time of cellular growth.

MATERIALS AND METHODS

Yeast *Rhodotorula glutinis* strain was obtained from IMTECH (Chandigarh, India). The lyophilized culture was hydrated in medium containing Glucose (70g/l), $(NH4)_2SO_4$ (20g/l), a slightly modified basal medium recommended by Bhosale and Gadre (2001). Then a culture was transferred to slant tubes containing medium with the same composition, including 15 g/l agar and incubated at 30° C for 60 hr. After growth, the slants were kept at 4° C and were subcultured each 2 months. The inoculum was prepared in Erlenmeyer flasks of 250-ml with 100 ml of medium.

Optimization of Growth Conditions: The growth conditions for *R. glutinis* were optimized with different C:N ratios by having various concentrations of Carbon and Nitrogen sources. Glucose, Fructose, Sucrose, Galactose and Xylose were evaluated as carbon sources (70 g/l) with Ammonium Sulphate (20 g/l) as nitrogen source .$NH_4H_2PO_4$, NH_4NO_3, $(NH_4)_2SO_4$, Urea, Aspargine were evaluated as Nitrogen source (20g/l) with glucose (70g/l) as carbon source. Subsequently, the influence of surfactants (Tween 20 and Tween 80 (0.5 ml/l), pH (3, 4,5) and incubation temperature ^{0}C (25,30,35 and 40) were also analysed in initial media composition.

R. *glutinis* were inoculated and incubated at 30°C for 60 Hr. The dry weight of R. *glutinis* was measured after each 12Hr. Dry mass of the cell was taken by centrifugation of one ml culture medium and dried the pallet at 80 °C for 12 hr. then the weight of dried mass was taken and converted into g/l.

Isolation of lipid from R. *glutinis*: Extraction of lipids was done by Soxhlet method in which hexane was used as solvent for the extraction from R. *glutinis*. The dried yeast was used for the isolation of lipid. The moisture content of the yeast sample should not exceed 10%. Weighed exactly 1 g of yeast sample which was kept on Whatman filter. Covered the top of each thimble with glass wool to prevent floating. Weighed the pre-dried flat-bottom extraction flask with a few boiling chips or glass beads. Extract lipids with 150 to 200 ml of hexane at the boiling point for 7 to 12 h in a Soxhlet extractor using a heating mantle. The condensation rate for the solvent was set at about 2 to 6 drops per second, depending on the extraction period envisaged. For longer extraction periods, a lower condensation rate was selected and vice versa. Usually,an extraction period of 8 hr at a rate of 150 drops per min was considered adequate. The sample was made to cool. Finally solvent was removed from

the sample extract using rotary evaporator at 40°C under reduced pressure. Further calculations were performed for the amount of lipid recovered and its percentage in the original sample using following equation: –

Mass of lipid = (weight of the flask + boiling chips + extracted oil) – (weight of the Flask + boiling chips)

RESULTS AND DISCUSSION

The *Rhodotorula glutinis* was grown on different carbon source (Glucose, Fructose, Sucrose and Xylose) for different incubation time (12, 24, 36, 48, 60 hr.) As we can see from the **Table 1 & Figure 1**, both carbon source and incubation time play an important role in the production of lipid. The maximum biomass (17.14 g/l), lipid production (8.82 g/l) and % lipid yield (51.45%) were found with Sucrose in the incubation period of 60 hrs followed by glucose, xylose and fructose.

Table 1. Growth of *Rhodotorula glutinis* and production of lipid at different carbon sources.

Carbon Source	Time (Hr.)	Biomass (g/l)	Lipid (g/l)	% Lipid yield
Glucose	12	0.76	0.29	38.15
	24	3.71	1.41	38.00
	36	8.73	4.11	47.07
	48	14.13	6.91	48.90
	60	14.63	7.36	50.30
	72	13.95	6.92	49.60
Fructose	12	0.97	0.26	26.80
	24	4.65	1.94	41.72
	36	7.23	3.63	50.20
	48	10.84	5.21	48.06
	60	17.32	5.62	32.44
	72	16.82	5.34	31.74
Sucrose	12	1.64	0.47	28.65
	24	8.81	3.25	36.88
	36	13.21	6.63	50.18
	48	16.62	8.38	50.42
	60	17.14	8.82	51.45
	72	16.74	8.55	51.07
Xylose	12	0.83	0.31	37.34
	24	7.65	3.13	40.91
	36	10.93	5.32	48.67
	48	13.66	6.89	50.43
	60	13.79	6.91	50.10
	72	13.67	6.63	48.50

Among the various nitrogen sources **Table 2 & Figure 2**, NH_4NO_3 was the best nitrogen source for production of biomass (17.11 g/l), lipid production (8.62 g/l) and % lipid yield (50.37%). Aspargine showed the poorest result.

Various nitrogenous sources are taken for the optimization of cultivation conditions viz. ammonium dihydrogen phosphate, ammonium nitrate, ammonium sulphate, urea & aspargine in which the best possible nitrogenous substrate was found out to be ammonium nitrate (NH_4NO_3) at an optimal temperature of 30 ^{0}C at the 60th hour incubation as far the microbial proliferation with respect to lipid yield is concerned.

The cell dry weight increased gradually with an increase in the *p*H of the broth (at pH 3 & 4) later got decreased by increasing the pH (pH 5). The optimum pH were found to be 4 & 3 as per **Table 3 & Figure 3** for maximum yield of biomass, lipid production and % of lipid yield.

R. glutinis was able to grow at all the temperatures examined ^{0}C (25, 30, 35, 40). A considerably increased biomass (18.65 g/L) 30^{o}C; whereas enhancement of temperature 35^{0}C to 40^{o}C causes reduction in the biomass. The reduction in the biomass primarily is an indicator of microbial competency to withstand the temperature alterations **(Table 4 and Figure 4)**. Biomass produced efficiently at 30^{o}C suggests that the rate of replication has increased significantly during the or in other words the doubling time of oleaginous organism has decreased vis-à-vis to all the other incubation temperature. The sampling time of incubated organism in order to assess the biomass yield as well as the lipid content is strategically being conceived at a time interval of 12 hours depending upon the microbial growth curve of the organism.

The illustration in the graphs primarily suggests the data which is being tabulated. The rate of production of the biomass is directly correlated lipid yielding efficiency of the biomass. As one can easily decipher from the depiction maximal biomass yield is being acquired at optimal mesophilic temperature i.e., 30^{o}C, invariably also suggests the ratio of production of biomass vis-à-vis lipid content which is 1:2

The effect of surfactant (0.5ml/l) on the growth of *R. glutinis* was measured at different time intervals and observed that surfactants did not effected much. The production of Biomass and lipid were almost same for both the surfactants **(Table 5 & figure 5)**

The table illustrates the implication of two surfactants being used viz. tween 20 & tween 80 which had no negative or positive effect on the biomass yield with respect to the lipid content. Hence one can imply the microbial culture or lipid yield remain unaffected with the aforementioned concentration (0.5ml/l) of surfactants being used

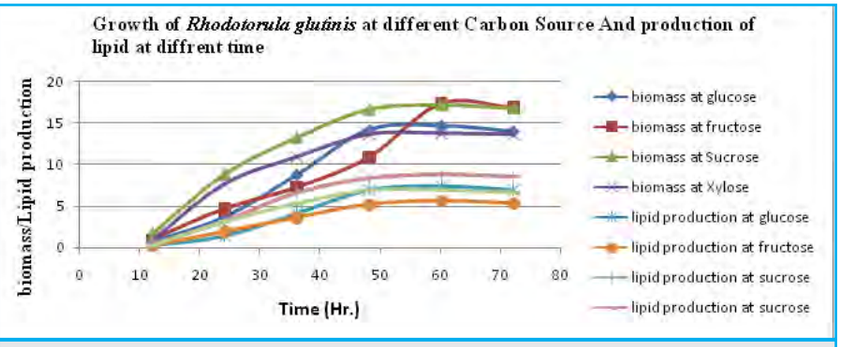

FIGURE 1. Growth of *Rhodotorulaglutinis* at different carbon source and production of lipid at different time

Table 2. Growth of *Rhodotorula glutinis* and production of lipid at different nitrogen sources.

Nitrogen Source	Time (Hr.)	Biomass (g/l)	Lipid Content (g/l)	Lipid Yield (%)
$NH_4H_2PO_4$	12	1.21	0.31	25.61
	24	5.62	2.19	38.96
	36	11.63	5.08	43.68
	48	14.25	7.11	49.89
	60	14.83	7.49	50.5
	72	14.52	7.29	50.2
NH_4NO_3	12	1.93	0.61	31.6
	24	8.65	4.09	47.28
	36	12.82	6.32	49.29
	48	16.89	8.53	50.5
	60	17.11	8.62	50.37
	72	16.79	8.41	50.08
$(NH_4)_2SO_4$	12	1.21	0.51	42.14
	24	3.18	1.21	38.05
	36	5.62	2.31	41.1
	48	8.93	4.31	48.26
	60	9.32	4.19	44.95
	72	9.12	3.96	43.42
Urea	12	0.919	0.27	29.37
	24	8.82	3.89	44.1
	36	9.12	4.19	45.94
	48	9.16	4.41	48.14
	60	10.14	4.62	45.56
	72	9.83	4.3	43.74
Aspargine	12	0.33	0.06	18.18
	24	0.81	0.31	38.27
	36	1.96	0.68	34.69
	48	3.92	1.56	39.79
	60	5.72	2.22	38.81
	72	5.65	2.15	38.05

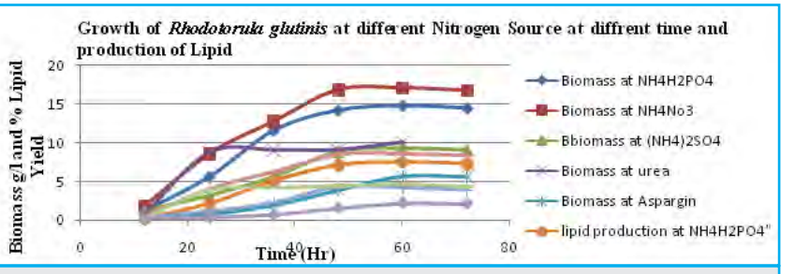

FIGURE 2. Growth of *Rhodotorulaglutinis* at different Nitrogen Source and production of lipid at a different time interval.

Table 3. Growth of *Rhodotorula glutinis* and production of Lipid at different pH and different time interval.

S. No.	pH of the media	Time Duration Hr.	Biomass g/l	Lipid production g/l	Lipid Yield %
1	3	12	0.61	0.23	37.70
		24	2.93	1.15	39.24
		36	7.63	3.43	44.95
		48	14.22	7	49.22
		60	15.61	7.97	51.05
		72	15.34	7.4	48.23
2	4	12	1.92	0.72	37.50
		24	4.32	2.17	50.23
		36	12.33	6.12	49.63
		48	17.61	8.92	50.65
		60	18.98	9.68	51.00
		72	18.56	9.32	50.21
3	5	12	1.62	0.41	25.30
		24	9.82	4.13	42.05
		36	11.31	5.67	50.13
		48	16.13	8.12	50.34
		60	16.92	8.42	49.76
		72	16.82	8.32	49.46

The graphical illustrations of tabulated data is indicative of the fact aforesaid i.e., surfactant remains inert to the microbial proliferation & hence lipid yield unaffected, hence the indictment for the biomass production & lipid yield remains stable as for as the exposure to surfactant is concerned.

When *R. glutinis* was incubated with the medium containing sucrose as a carbon source (70 g/l), NH_4NO_3 as

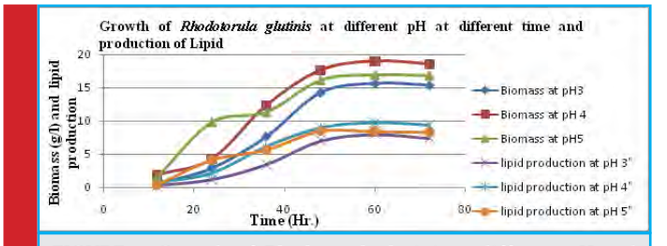

FIGURE 3. Growth of *Rhodotorulaglutinis* at different pH and production of lipid at a different time interval

Table 4. Growth of *Rhodotorula glutinis* and production of Lipid at different Temperature and different time interval.

S. No.	Temp. (C)	Time Duration (Hr.)	Biomass (g/l)	Lipid Content (g/l)	Lipid Yield %
1.	25	12	1.01	0.28	27.72
		24	13.13	3.72	28.33
		36	14.99	4.43	29.55
		48	16.43	5.43	33.04
		60	17.34	6.32	36.44
		72	16.98	6.12	36.04
2.	30	12	1.21	0.35	28.92
		24	15.08	7.18	47.61
		36	15.98	8.17	51.12
		48	18.32	9.24	50.43
		60	18.65	9.41	50.45
		72	18.43	9.21	49.97
3.	35	12	1.09	0.31	28.44
		24	2.93	1.29	44.02
		36	13.17	6.48	49.20
		48	14.68	7.32	49.86
		60	15.73	7.95	50.54
		72	15.21	7.12	46.81
4.	40	12	0.66	0.21	31.81
		24	4.32	2.22	51.38
		36	8.11	3.94	48.58
		48	12.13	5.93	48.88
		60	12.56	6.45	51.35
		72	12.09	6.38	52.77

a nitrogen source (20 g/l), yeast (15g/l) at 30^{0}C with pH 4. The yield of lipid was increased and reached 57.23% of total dry biomass with inoculums size 6%. **(Table 6 and Figure 6)**

Again the the table illustrates the significance of the optimized inoculum size with respect to optimal temperature & media components making growth maximal to its competency. The maximal biomass yield is achieved at pH 4 indicative of the operability of oleaginous organism to produce lipid under the influence of the acidic condition. Moving towards the alkaline scale the rate of microbial profillation decrese & withit the lipid content is invariably effected. The best possible microbial proliferation & lipid content is being percieved for the sucrose

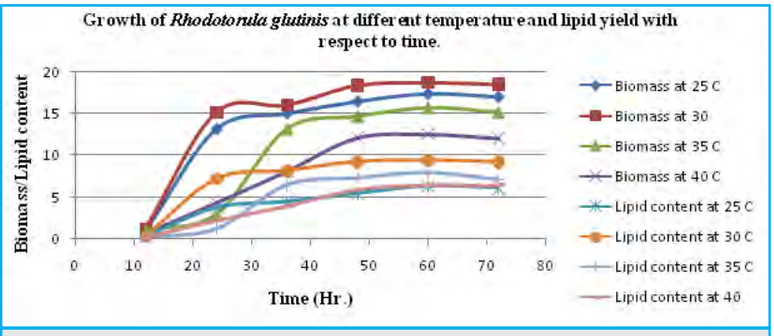

FIGURE 4. Growth of *Rhodotorulaglutinis* at different temperature and lipid yield with respect to time.

Table 5. Growth of *Rhodotorula glutinis* at different surfactant and production of lipid at different time interval

S. No	Surfactant	Time (hr.)	Biomass (g/l)	Lipid production (g/l)	Lipid content (%)
1	Tween 20	12	0.95	0.21	22.10
		24	6.52	2.91	44.63
		36	11.62	5.24	45.09
		48	13.73	6.36	46.32
		60	14.81	7.23	48.81
		72	14.65	7.09	48.39
2	Tween 80	12	0.81	0.17	20.98
		24	6.66	2.84	42.64
		36	11.38	4.15	36.46
		48	13.84	6.15	44.43
		60	14.38	6.9	46.52
		72	14.22	6.3	44.30

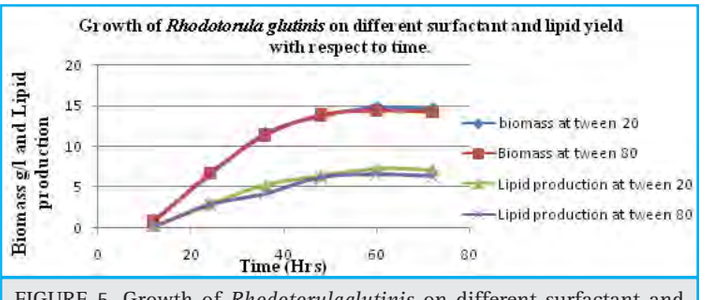

FIGURE 5. Growth of *Rhodotorulaglutinis* on different surfactant and lipid yield with respect to time.

as the carbon substrate followed by glucose & fructose. Xylose is least catabolized substrate as far as microbial replication & lipid production is concerned

CONCLUSION

In this present work, we optimize the growth conditions of *Rhodotorula glutinis* to get maximum production of Biomass and lipid content. Sucrose was found to be the best carbon source (70 g/l), NH_4NO_3 was found to be best nitrogen source (20g/l) with optimum pH 4, optimum temperature 30 ^{0}C and optimum time period 60 hrs. Surfactant does not have any effect on the growth and lipid production of Rhodotorula glutinis. This study reveals that cultivation conditions had an influence on lipid production. This study provides a valid and potential strategy for optimizing yeast cultivation conditions to improve the production of lipid with possibly broad biotechnological applications.

Table 6. The growth of *Rhodotorula glutinis* at the optimized condition, and effect of inoculums size and production of lipid at a different time interval

Media	pH	Temperature	Inoculum Size (%)	Biomass after 60 hrs of incubation (g/l)	Lipid production (g/l)	% Yield of lipid
70 g/l Sucrose	4	30 °C	5	19.5	10.3	52.82
20 g/l NH4NO3			6	22.2	12.6	57.27
15 g/l Yeast Extract						
7 g/l KH2PO4						
1 g/l MgSO4			7	20.1	10.7	53.23
15 g/l Agar						

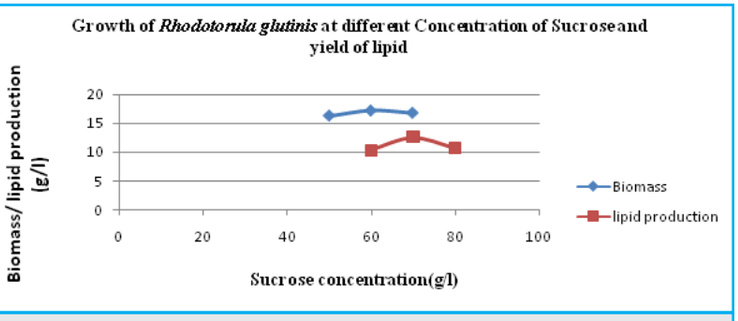

FIGURE 6. Growth of *Rhodotorulaglutinis* at different Concentration of Sucrose and yield of lipid

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BBRC Bioscience Biotechnology Research Communications

Comparative Study of the Zno and Zno Coated with Sio_2 As Potential Antimicrobial and Anticancer Drugs

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ABSTRACT

Zinc, as one of the major trace elements of the human body and co-factor of more than 300 mammalian enzymes, plays an important role in maintaining crucial cellular processes including oxidative stress, DNA replication, DNA repair, cell cycle progression and apoptosis. Thus, it is evident that an alteration in zinc levels in cancer cells can cause a deleterious effect. Research has shown that low zinc concentration in cells leads to the initiation and progression of cancer and high zinc concentration shows toxic effects. Zinc-mediated protein activity disequilibrium and oxidative stress through reactive oxygen species (ROS) may be the probable mechanism of this cytotoxic effect. ZnO has a neutral hydroxyl group attached to its surface, which plays an important role in its surface charge behaviour. Our aim is to show that the effect of Zinc Oxide and Silica coated Zinc Oxide on different microbes and cancer cells. Characterization of Zn nanoparticles have been done by using different analyzing techniques i.e. UV-Vis Spectroscopy, DLS (Dynamic Light Scattering) and SEM (Scanning Electron Microscope). The effect of the Zn nanoparticles on microbes has been measured by the cup disk method where as the effect on cancer cell line (HeLa) (Human Cervical Cancer Cell Line) has been measured by Fluorescence Anisotropy, MTT assay, Reactive Oxygen Species (ROS). The effect on different enzymatic action has also been measured. Regardless of antimicrobial medicinal consideration, dismalness and mortality identified with these microorganism contaminations remain high, somewhat because of the adaptability of those life forms to create protection from almost all anti-infection agents. Our aim is to develop new drugs spot and build up the resulting age of prescription or operators to manage microorganism contaminations.

KEY WORDS: ZINC OXIDE, REACTIVE OXYGEN SPECIES, FLUORESCENCE ANISOTROPY, MTT ASSAY

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INTRODUCTION

Bio nano molecules are those, whose size is comparable with nanoparticles, play an unavoidable important role in regulating various cellular cycles of the body and maintaining crucial cellular homoeostasis.With proper bio engineering, Nanoparticles can be sent in a localized condition in any system of the body and thus it can incorporate the activity of biological components, thus mimicking the biological system of the body according to the need for human benefit. Nanoparticles are highly soluble due to their small size and their solubility can be further increased by proper surface modification and the high surface area to volume ratio of those particle, make them having ample surface area to encapsulate drugs and other materials, thus providing higher therapeutic payload. Another property of these nano particles can be described as the selective targeting nature, thus Nanoparticles can specifically release a therapeutic payload onto the target, reducing the side effects on normal cells, (McNeil, et al, 2009, Wang, et al, 2013, Bisht and Rayamajhi, 2016; Hussain et al, 2019). Marco et.al, 2019).

Research and development in the field of nanotechnology are growing rapidly throughout the world (Vidya *et al.,* 2013). A major contribution of this field is the development of new materials in the nanometer scale (Sivakumar, *et al.,* 2011; Karthikeyan et.al, 2019). These are usually particulate materials with at least one dimension of less than 100 nanometers (nm), even the particles could be zero dimension in the case of quantum dots (Vidya*et al.,* 2013). Metal nanoparticles have been of great interest due to their distinctive features such as catalytic, optical, magnetic and electrical properties (Garima, *et al.,* 2011). Nanoparticles exhibit completely new or improved properties with larger particles of the bulk materials, and these novel properties are derived due to the variation in specific characteristics such as size, distribution, and morphology of the particles (Ravindra, *et al.,* 2011, Ravindran, et.al. 2016). Particularly, nanoparticles (NP) made from metal oxides with sizes less than 100 nm exhibit antimicrobial activities owing to their special characteristics (e.g. small particle size, large surface area), which micro- or macro-sized particles do not possess. Zinc oxide, with its unique physical and chemical properties, such as high chemical stability, high electrochemical coupling coefficient, broad range of radiation absorption and high photo stability, is a multifunctional material (Lou, 1991, Segets, et al. 2009). Recent studies have shown that some NP made of metal oxides, such as ZnO NP, have selective toxicity to bacteria but exhibit minimal effect on human cells (Brayner et al. 2006; Thill et al. 2006; Reddy et al. 2007; Zhang et al. 2007, Sadhukhan et al, 2019).

Compared with the organic materials, inorganic antibacterial reagents are more stable at high temperatures and pressures (Sawai 2003). Compare to the inorganic antibacterial materials, metal oxides such as zinc oxide (ZnO) have received increasing attention in recent years, not only because they are stable under harsh processing conditions, but also because they are generally regarded as safe materials to human beings and animals (Stoimenov et al. 2002; Fu et al. 2005; Kaushik et.al, 2019).

ZnO has a neutral hydroxyl group attached to its surface, which plays an important role in its surface charge behaviour. At high pH, ZnO exists as ZnO^- due to the transfer of adsorbed protons from its surface towards aqueous solution. At low pH (acidic condition), ZnO exists as $ZnOH_2^+$ due to the transfer of protons from the aqueous environment towards its surface. The isoelectric pH of ZnO nanoparticles is 9-10 (Orel, et.al, 2015, Vinardell et.al, 2015 Roy and Jong,2019).

Thus, ZnO nanoparticles exhibit positive charge under physiological conditions such as blood or tissue fluid (which has pH 7), etc. (Degen et.al,2000, Rasmussen et.al, 2010). On the other hand, cancerous cells usually have high concentration of (negatively charged) anionic phospholipids on their outer membrane (Abercrombie et.al, 1962). The re-emergence of infectious diseases and the continuous development of antibiotic resistance among a variety of disease-causing bacteria pose a serious threat to public health worldwide (Desselberger, 2000, Vandenesch et.al, 2003). Among these pathogenic microorganisms, *Enterococcus, Staphylococcus* and *Streptococcus* are common closely related species that cause a wide variety of infections and diseases (Boyce, 1997; Lowy, 1998; Hancook & Gilmore, 2000, Laura et.al,2019).

Despite antimicrobial therapy, morbidity and mortality associated with these bacterial infections remain high, partially as a result of the ability of these organisms to develop resistance to virtually all antibiotics. New strategies are therefore needed to identify and develop the next generation of drugs or agents to control bacterial infections. CuO nanoparticles are successful in murdering a spread of microorganism. Be that as it may, ZnO nanoparticles with high focus are expected to get the disinfectant effect. The disinfectant property of such nanoparticles relies upon their size, steadiness, and focus extra to the extension medium, that gives bigger maintenance time to microorganism NP association.

MATERIAL AND METHODS

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (Gibco™, Thermo Fisher Scientific), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Merck, India), TMA-

DPH (1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatriene *p*-Toluenesulfonate) (Thermo Fisher Scientific), Dry N,N,dimethylformamide (DMF; Merck, India), nickel chloride hexahydrate ($NiCl_2$, $6H_2O$, Mw = 237.69 g mol−1; Merck, India), sodium hydroxide (NaOH; Merck, India), ethyl alcohol (C_2H_6O; Merck, India), tetraethyl orthosilicate (TEOS; Merck, India), and ammonia solution 25% (NH_4OH; Merck, India) were used in this work. All the materials were used in the experiments without further purification.

Preparation Of Zno@Sio$_2$ Nanoparticle: Hydrothermal method has been used to synthesize the Zinc oxide nanoparticles (Dutta et al., 2017). Details of that hydrothermal procedure for synthesis of metal oxide nanomaterials has been referred from (Dutta et al., 2015). Modified Stóber (Stober et al., 1968) method, a widely used method for synthesis of silica nanoparticles has been used to synthesize the silica coated Zinc oxide. In this typical synthesis procedure, hydrothermally synthesized Zinc oxide nanoparticles (Dutta et al., 2017) were added to the solution of water and ethyl alcohol (in volume ration approximate 4:1). To achieve a well-dispersed mixture, the solution was sonicated for 10 min. After that, ammonia was added to the mixture (in volume ratio 1.4:50) drop by drop to catalyze the Zinc oxide nanoparticles in alcoholic media. The mixture was again sonicated for 40 min after the addition of ammonia, and finally, TEOS was added drop by drop to the mixture (in volume ratio approximately 0.4:50). The final mixture was kept under strong magnetic stirring (500 rpm) for 18 h. The well-mixed colloidal solution was centrifuged at 4000 rpm and washed by ethanol to remove the residuals from the product. The collected product was dried at 80 °C and employed for further characterization.

Anti Microbial Activity: Cup-Disc Method: The number of the zone of inhibition has been deduced from three parallel studies and those are taken as the mean value of those. These studies were compared with the known drugs available in the market. The ZnO and ZnO@SiO2 showed an average value of the zone of inhibition where the combination of these two nanoparticles showed a maximum zone of inhibition. The lower concentration of the mixed drug has an effect on the bacterial and the fungal growth which has been measured by calculating the zone of inhibition and the values are (+/-) SD of three parallel measurements.

Cell Culture: Human cervical epithelial malignant carcinoma cell lines (HeLa) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (Gibco™, Thermo Fisher Scientific), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere containing 5% CO2. HeLa cells at a concentration of 1.5×105 cells/mL were grown in a 25 cm^3 flask of complete culture medium. At 85 % confluency HeLa cells were and trypsinized, and seeded on a 96 well tissue culture plate for overnight according to the selection of experiments.

Mtt Assay: Approximately 1 × 10^5 mL^{-1} HeLa cells in their exponential growth phase were seeded in a flat-bottomed 96-well Tissue culture plate for 24h at 37°C in a 5% CO_2 incubator. Series of concentrations (5, 25, 50, 100, and 250 µg/mL) of ZnO and ZnO@SiO_2 nanoparticles in the medium were added to the plate in a triplicate manner. Cytotoxicity evaluation of NiO and NiO@SiO_2 nanoparticles was performed using MTT assay and MTT was added to each well and the plates incubated for 3 h in a dark chamber. 100 µl of DMSO was added to dissolve the formazan crystals and the absorbance read at 540 nm using ELISA reader (EPOCH, BIOTEK) (Zhu et al., 2001).) The % of survival was calculated using untreated cells as 100 %.

$$\% \text{ Cell Viability} = \frac{\text{(A) Control – (B) test}}{\text{(A) Control X 100}}$$

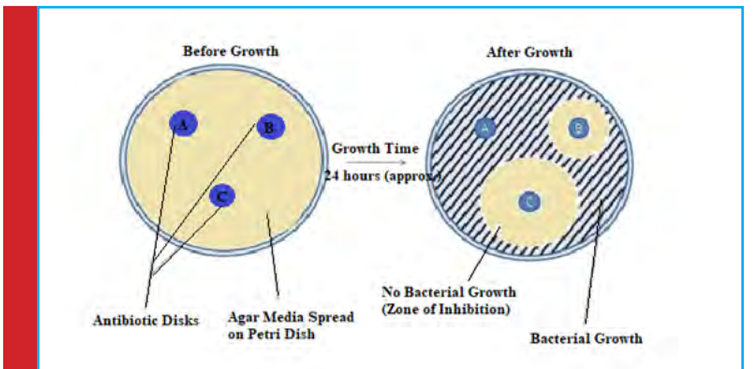

FIGURE 1. Schematic Diagram Of Cup Disk

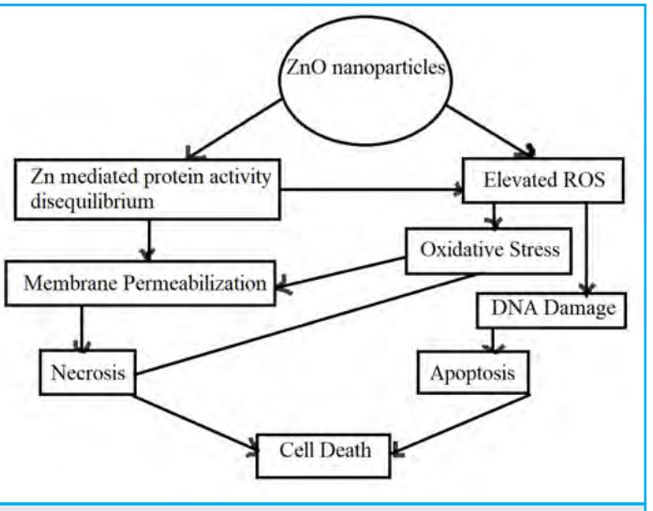

FIGURE 2. Schematic Diagram of the action of ZnO nanoparticles

Where B test is the absorbance of the test sample and A control is the absorbance of the control sample. Non-treated cells were used as the control, and the samples were imaged using an inverted photomicroscope. The Values of MTT assay correspond to mean and standard deviations of three independent experiments.

Fluorescence Anisotropy: The fluorescence anisotropy of HeLa was assessed by the determination of TMA-DPH steady-state fluorescence polarization after the cell membrane exterior phospholipid layer permeation of the probe (Dowell, 2002; Pearson,1996; Pearson, et.al,2001, Shrivastava, et.al. 2007; Katona,2004; Lakowicz,2004; Hollan, 1996).

For the measurement of the changes in the TMA-DPH fluorescent properties following the membrane permeation, we added 2.5µM TMA-DPH to a 2 ml of cell in the measuring cuvette. The cell suspension with the fluorescent probe was incubated for 30 min at 37°C. The measurement has been done between excitation and emission state, 360 nm and 430 nm respectively.

ROS Analysis: Membrane fluidity of cancer cells was shown to have a decisive role in the direct cell to cell contact and the modulation of the activity of membrane enzymes are to be affected by the increased release of reactive oxygen species (ROS) (Garden, 2001).

For the measurement of the intracellular ROS, DCF-DA was added to a 2 ml of HeLa suspensions. The cell suspension with DCF-DA was incubated for 60 min at 37^0 C in a dark condition. Cells without nanoparticles were used as control. Fluorescence intensity was measured in

Table 1. Zone of Inhibition of Bacteria: (Values are mean +/- SD of three parallel measurements = Number of zones of inhibition.) (Concentration in µg/ml and Zone of inhibition in mm)

Drug Name	Drug Concentration (µg/ml)	*E. coli*	*S. typhi*	*S. aureus*	*S.pyrogenes*
ZnO	5	-	-	-	-
	25	13	12	14	12
	50	15	14	16	13
	100	17	15	19	17
	250	20	17	20	20
$ZnO@SiO_2$	5	-	-	-	-
	25	8	9	7	6
	50	10	12	11	10
	100	13	13	14	12
	250	14	13	15	13

Table 2. Known drugs used for bacteria

Drug Name	Drug Conc. (µg/m)	*E.coli*	*S.typhi*	*S.aureus*	*S.pyrogens*
Ampicillin	5	13	14	11	10
	25	15	19	14	13
	50	17	15	15	16
	100	18	17	19	18
	250	20	21	22	20
Norfloxacin	5	23	19	18	20
	25	25	20	20	22
	50	26	22	23	26
	100	28	25	24	28
	250	30	26	24	32
Amoxiline	5	21	20	18	19
	25	23	23	20	22
	50	25	24	23	23
	100	27	26	24	25
	250	28	29	26	28
Cifroflloxacin	5	20	21	19	17
	25	22	23	21	20
	50	26	24	23	20
	100	28	27	25	22
	250	30	29	28	25

a fluorescence spectrophotometer (model Hitachi, USA) at excitation and emission wavelengths of 504 and 529 nm, respectively.

Antioxidant Enzymes Activities: Superoxide dismutase (SOD) and Catalase (CAT) activities were measured by commercially available kits. The cells were seeded into 12- well plates at a concentration of 7×10^5 cells/well and all measurements were performed according to supplier's recommendations.

RESULTS AND DISCUSSION

The Zinc oxide Nano Particles have shown better effect than the Silica coated nano Particles. In some cases the

Table 3. Anti Fungal Activity: Zone of Inhibition of Fungi: (Values are mean +/- SD of three parallel measurements = Number of zones of inhibition). (Concentration in µg/ml and Zone of inhibition in mm)

Drug Name	Drug Concentration (µg/ml)	*A. nigar*	*C. clavus*	*C. albicans*
ZnO	5	-	-	-
	25	14	17	15
	50	17	19	17
	100	19	20	18
	250	20	22	20
$ZnO@SiO_2$	5	-	-	-
	25	8	7	5
	50	10	9	8
	100	11	10	10
	250	12	11	12

Table 4. Known Drug Concentration

Drug Name	Drug Concentration (µg/ml)	*A. nigar*	*C. clavus*	*C. albicans*
Greseofluvin	5	17	18	19
	25	21	20	20
	50	22	23	21
	100	23	24	24
	250	27	28	26
Nystitin	5	19	17	18
	25	20	21	20
	50	20	22	21
	100	23	24	23
	250	28	27	25

Zinc oxide nanoparticles have shown good results in comparison to the known drugs which is a positive indication of using these nanoparticles as a potential antimicrobial drugs. We also checked their activity on the fungal growth and these particles have also shown a great effect on reducing the fungal growth. These nanoparticles can also be used to reduce the fungal growth and the contamination occurs from it. The results of the zone of inhibition in bacteria and the antibiotics have shown in table 1 and table 2 and the zone of inhibitions for fungi and available anti-fungal have shown in table 3 and table 4.

MTT assay was undertaken in order to evaluate the cell viability in cells stressed by Zinc oxide nanoparticles and Silica coated Zinc oxide nanoparticles. First we evaluated the effects of NiO Nanoparticles on HeLa cells viability. Incubation with 5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml, 250µg/ml for 3, 6, 12 and 24 h resulted in a concentration dependent decrease in cell viability, the LC50 was 79.83 ± 0.856 µg/ml. Based on these results Zinc oxide nanoparticles and Silica coated Zinc Oxide nano particles at submaximal concentrations after 12 h, 50 and 100 µg/ml were selected in this study. The main findings of this assay are the LC50 of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles was 79.83 µg/ml and submaximal concentrations of 80 and 100 µg/ml were selected in this study. Similar results were obtained in previous findings demonstrated a dose dependent reduction of MTT-value in HeLa cells treated with Zinc oxide nano particles and Silica coated Zinc oxide nanoparticles, though cells were different (Ahamed 2011 and Capasso, et al. 2014).

According to the National Cancer Institute (USA), vegetables crude extracts are cytotoxic considered when their IC50 values are less than 30 µg/ml (Da, et al. 2013). After a large screening, Zinc oxide nanoparticles and

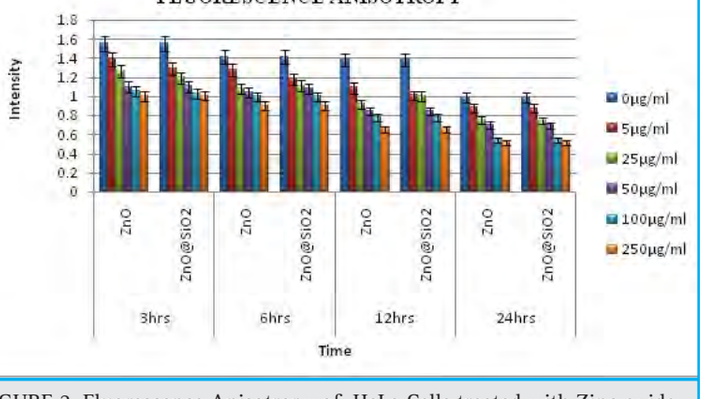

FIGURE 3. Fluorescence Anisotropy of HeLa Cells treated with Zinc oxide-nanoparticles and Silica coated Zinc oxidenanoparticles. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

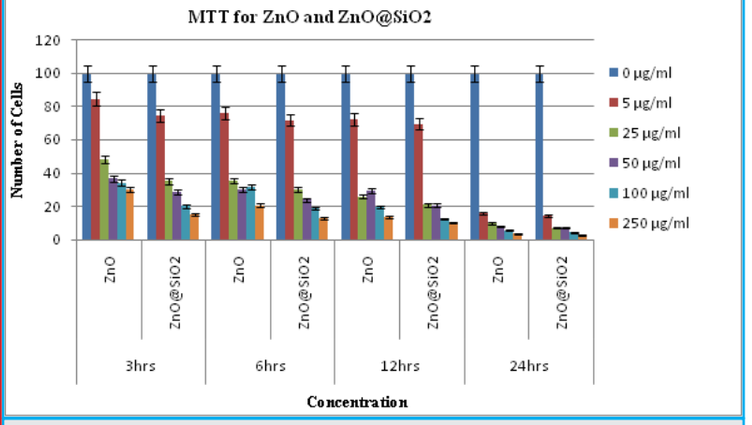

FIGURE 4. MTT Assay of HeLa Cells treated with Zinc oxidenanoparticles and Silica coated Zinc oxidenanoparticles. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

Silica coated Zinc Oxide nanoparticles (60 and 80 µg/ml) concentrations were selected due to their best actions. The present study agree with the results of Remila et al. (2015) who have demonstrated that pre-treatment of THP-1 cells with *P. lentiscus* extracts for 24 h strongly inhibited H_2O_2 damage, with maximum protection at 100 µg/ml (Remila, et al. 2015). The triplicate study of the cell culture has shown that the number of cells is decreasing by the increment of time and the concentration of the drugs respectively.

In order to investigate the effect of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles induced cytotoxicity mediated through ROS generation, HeLa cells were treated with the two selected concentrations of the Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles. We detected a significant decrease of ROS level in cells treated with Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles (Figs. 4 and 5). Oxidative stress, which is an imbalance between ROS production and the antioxidant systems favouring a ROS excess, has been identified as a common mechanism for cell damage. During oxidative stress, ROS are produced mainly from the mitochondrial electron transport chain. To minimize the damage induced by ROS, free radicals can be transformed to other less toxic molecules, for example, the superoxide anion is enzymatically converted into hydrogen peroxide by superoxide dismutase (SOD) and hydrogen peroxide may be enzymatically converted into water by catalase or glutathione peroxidase enzymes (Huerta-García et.al, 2014). Nanoparticles have been demonstrated to generate more free radicals and ROS than larger particles, likely due to their higher surface area (Sioutas, et al, 2005). NiO Nanoparticles have been reported to reduce

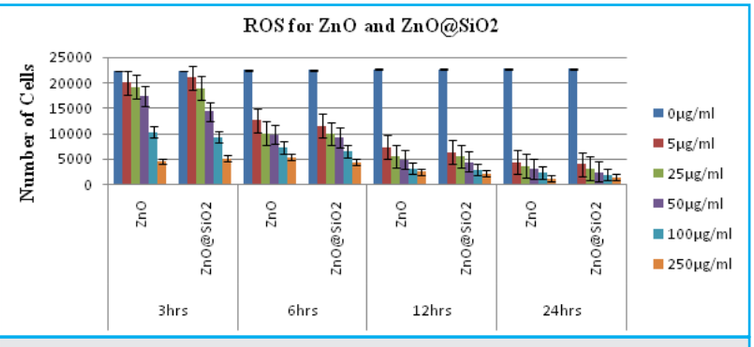

FIGURE 4. ROS of HeLa cells treated with Zinc oxidenanoparticles and Silica coated Zinc oxidenanoparticles. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

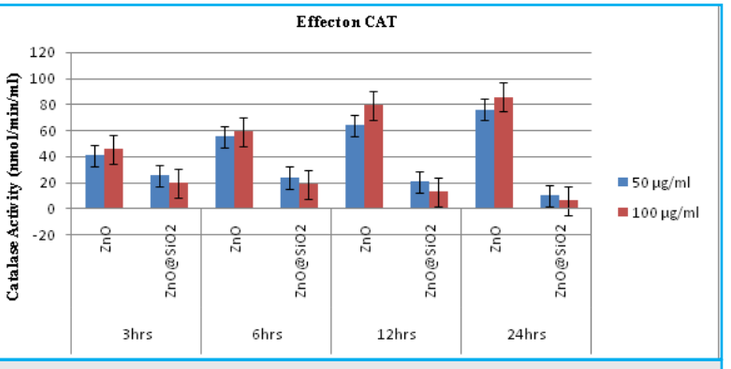

FIGURE 6. Effects of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles on CAT activity in HeLa cells. Cells were treated with different concentrations for 3, 6. 12 and 24 hours. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

cell viability and to induce oxidative stress by depletion of glutathione and induction of reactive oxygen species in HEp-2 and MCF-7 cells (Siddiqui, et al, 2013), cell death via apoptotic pathway and ROS generation in HepG2 cells in dose-dependent manner (Ahamed, et al, 2012), Zinc oxide nano particles also increased intracellular ROS, apoptosis and necrosis in BEAS-2B and A549 cells (Capasso, et al, 2014).

Our results confirmed that Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticlesare toxic to HeLa cells. In the Fig 6, ROS analysis has been shown in triplicate studies. These analyses showed that the requirement of the oxygen got low with the increase of time and concentration of the drug. These need of oxygen lead the cells to the apoptosis and thus the cell dies due to the treatment of the drugs. These have also coincided with the result of the MTT assay. With the increase of the concentration of the nanoparticles, the number of viable cells decreased. The nanoparticles showed the better result as the variation of the valance electron was increased as a result those reacted with the protein particles of the cells and dissociated it which leads the cells to destroy.

Pre-incubation of cells with both concentrations 50 and 100 µg/ml of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles led to enhance the antioxidant enzymes, SOD and CAT, activities shown in Figs. 5 and 6. Similarly, the Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticles also induce a significant depletion of antioxidants. The accumulation of ROS, e.g. superoxide radicals (O2%) and hydroxyl free radicals (%OH) decrease the defensive effects of cellular antioxidant enzymes, e.g. SOD, CAT (Li, et al. 2012). Exposure of HT22 hippocampal cells to CuO Nanoparticles resulted decrease in the activity of SOD and the other detoxification enzymes which has been founded in this work (Niska, et al. 2015).

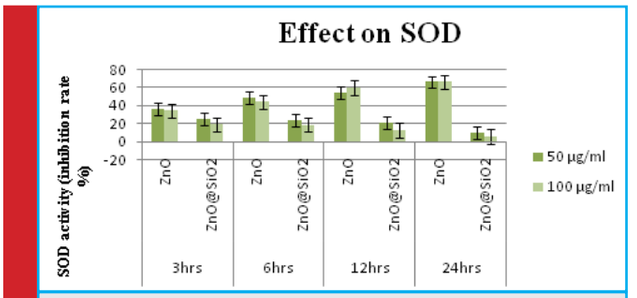

FIGURE 5. Effects of Zinc oxide nanoparticles and Silica coated Zinc Oxide nanoparticleson SOD activity in HeLa cells. Cells were treated with different concentrations for 3, 6. 12 and 24 hours. Data represented are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 treatments compared to control.

DISCUSSION

As per our research is concerned, we have found much more promising result on both anti microbial and anti cancer effect. We observed that the growth of both Gram-positive and Gram-negative bacteria was inhibited by increasing concentrations of ZnO NPs. We further explored the effect of the ZnO NPs on the cellular morphology (Hussain et al, 2019).

The recent data of Karthikeyan et al, (2019) have showed that the REM doped ZnO which is being costly but the procedure making of our doped particle is both cost effective and easy. Different review (Sadhukhan et al, 2019; Kaushik et al, 2019; Roy and Jong, 2019; Laura et al 2019; Marco et al, 2019; Xiuting et al, 2019) of the articles lead us to do the experiments with different gram positive and gram negative bacteria and our material has shown effect in much more lower concentration both in the bacteria and cancer cells. Our Spectroscopic data analysis also confirmed the lower concentration effects on the both. This work will completely open the new era of personalized drug for each.

CONCLUSION

Nanoparticles in medicine are a new and emerging topic of interest for researchers. With all their promising characteristics, the in vivo application of nanoparticles is still rare and there is currently a serious lack of in vivo research into nanoparticles. Hence, a much better collaboration between clinicians, biologists and material scientists is required for the in-depth understanding of cancer biology and intelligent design of NPs for their better clinical use. This is in fact an achievable aim, considering the highly promising characteristics of ZnO nanoparticles and their inherent nature of selectivity and toxicity towards cancer cells, making them unequivocally a key tool for next-generation cancer treatment. ZnO NP exhibited impressive antibacterial properties against different food borne pathogens as well as fungi and the inhibitory effects increased as the concentrations of ZnO nanoparticles increased. ZnO NP could distort bacterial cell membrane, leading to loss of intracellular components, and ultimately the death of cells. These results demonstrate that ZnO NP could be potentially considered as an effective antibacterial agent for protecting agricultural and food safety. Thus we have found that the ZnO can be a potential anti cancerous and antimicrobial drug for next generation of treatment.

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A report on the diversity of spider fauna from Charghad river basin of Morshi, Amravati India

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ABSTRACT

The present field study was carried to record variety and abundance of spiders in Charghad river basin of Morshi tehsil, Amravati district, Maharashtra, India. The field survey was carried from August 2017 to March 2018. The spiders were collected and photographed in quadrants covering all significant area with natural vegetation along 5 km of river patch. Ideally, all sites along the river basin were studied during this period. Diversity index and Evenness of spiders were calculated. This survey shows the occurrence of 48 species belonging to 12 families. Of which Salticidae was prominent (9 Genera with 14 species) followed by Araneidae (5 Genera with 13 species). Shannon Wiener diversity index (H) is 2 and Evenness of species found to be 0.80. The study suggests Charghad river basin has a rich diversified spider fauna.

KEY WORDS: SPIDER, DIVERSITY, ABUNDANCE, CHARGHAD, MORSHI TEHSIL

INTRODUCTION

Spiders make up a considerable proportion of the biodiversity of this vast and diversified nature. They are cosmopolitan and found in all types of ecosystems and habitat. Spiders are air-breathing predatory animals having two body segments, belonging to class Arachnida with about 45,776 species under 3974 genera distributed over 114 families (WSC, 2016). In India, they are represented by 1686 species to 438 genera of 61 families (Keswani *et al.,* 2012, WSC, 2015; WSC,2016). About 91 species from Mygalomorphae under 28 genera belonging to 8 families have been reported by Keswani *et al.,* (2012). Abundance of spider depends on the type of environment, vegetation and prey base. River basin provides the ideal hunting ground for spiders. Spider as an ecological indicator plays an important role in maintaining ecological equilibrium, (Halarnkar and Pai 2018).

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Charghad River is originated in Satpuda mountain ranges near village Velmandali of Amravati district and is tributary of Wardha River. Charghad River flows through Morshi tehsil and merges into Wardha River. Hippargi *et al.,* recorded an occurrence of spiders belonging to 19, 25 and 31 respective families from Lonar, Melghat and Southern Tropical thorn forest, Solapur. The diversity of spider in Satpuda ranges adjacent to this study area was studied by Deshmukh and Raut (2013) recorded 57 species belonging 35 genera under 14 families during 6-month survey in Salbardi forest (Satpura range). Again Deshmukh and Raut (2014) studied the seasonal diversity of Salbardi forest (Satpuda range) and recorded 104 species of 52 genera under 18 families during year 2014. Deshmukh and Chaudhari (2016) recorded 49 spider species belonging to 22 genera under 9 families from orange agroecosystem in the catchment area of upper Wardha dam, Amravati, Maharashtra. The diversity of spiders from Charghad river basin is not yet explored, so the attempt was made to study and make a checklist of spiders from this area. The ecosystem of Charghad river basin depends upon rainfall. The survey was made to study diversity and abundance of spider's fauna in Charghad River Valley of Morshi Tehsil, Amravati District, and Maharashtra, India. The study has been started in the month of August 2017 and extended to March 2018 along the river basin which is 45km from Amravati district of Maharashtra. The study area is located latitude 21.324196^0 N and longitude 78.013832^0 E at an elevation of 303 meters from sea level. Charghad River is originated in Satpuda mountain ranges near village Velmandali of Amravati district and passes through Morshi Tehsil being is tributary of Wardha River. Morshi Tehsil has a temperature range of 32 to 48^0 C in summer and 16 to 27^0 C in winters. The rainfall is with southwestern monsoon from June to September having annual an average rainfall of 758.40 mm.

The river basin is flourished with various flora with perennial plants like *Ficus bengalensis* (wad), *Acacia leucophela* (hiwar), *Bauhinia racemosa* (apta), *Zyzypheus vulgaris* (ber), *Acacia catechu* (khir), *Limonia acidissima* (kaut), Bamboo and dominant grasses like *Andropogon martini* (tikhari), *Sorghum halepense* (boru), *Cynodon dictylon* (hariali), *Ichamum sulcatum* (paonia), *Ichamum laxum* (sahada) and *Andropogon contorlus* (kusal) and *Lantana cammera*. Ideally all sites of the river basin were studied. The field work was designed in 10 quadrants covering all significant area with natural vegetation along 5 km of river patch. Where sampling sites of 10 sq. meters were selected and marked. Sampling was done from this 10 sq. meter quadrants in river basin every weekend; mostly during from early morning to late night. Visual search was carried out by walking through the habitat and visually searching for spiders, their webs or retreats (curled leaves, silk case). When walking in the grass, due to disturbances ground jumping of spiders was seen and by keeping their trail spiders were captured. Heavy insect net sweeps were used through soft vegetation or tall grassin a zigzag pattern in the marked area. After a few sweeps, dump the content on flat sheet and capture the spider. But it is less effective in wet condition. In this case, an inverted umbrella opened place was used under the bush or lowered branches. The branches were given vigorous shaking or striking them with sticks.

Spiders were dislodged and were collected in the umbrella. This is mostly used and successful technique which was also used in the present study. Any smooth plastic bottle of 10 cm diameter and 11 cm depth was buried within the ground surface with a funnel at top of the container. Spiders tumble into the container and captured in the collection bottle. Only mature spiders were collected for identification they were photographed and release back in natural habitat. The keys of Platnick (1981-1987); Barrion and Litsingerm (1995); Tikader (1987); Gajbe (2005) were used for species identification and to record classification. Statistical analysis was done using Shannon Wiener diversity index (H) and Evenness of species.

$$H = -\sum [(Pi) \times \ln (Pi)]$$

$$E = \frac{H}{H\,max}$$ Where,

$$\text{Summation of pi} = \frac{\text{Number of individual of species}}{\text{total number of species}}$$

S = Species richness
H max = maximum diversity possible.
E= Evenness

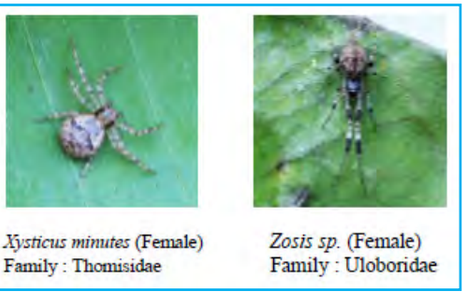
During a survey of 8 months in river basin, the individual belongsto 48 species of 31 Genera and 12 families (table no. 1) were recorded. This present study indicates

Table 1. Family wise distribution of Spider Species and Genus.

Sr. no	Name of Family	Number of Genus	Number of Species
1	Araneidae	5	13
2	Clubionidae	1	1
3	Erasidae	1	1
4	Hersilidae	1	1
5	Lycosidae	2	2
6	Miturgidae	1	2
7	Oxyopidae	2	3
8	Pisauridae	3	3
9	Salticidae	9	14
10	Sparassidae	1	1
11	Tetragnathidae	2	4
12	Thomisidae	3	3
	Total	31	48

Table 2. Family wise distribution list of spider species from Charghad River basin Eco-system.

Sr. no	Family	Species
1	Araneidae	*Araneus species* (Male) *Araneus Praesignis* (Female) *Araneus diadematus* (Female) *Argiopaemula* (Female) *Lariniadirecta* (Female) *Neoscona bengalensis* (Female) *Neoscona bengalensis* (Male) *Neosconacrucifera* (Male) *Neoscona mukerjei* (Male) *Neoscona species* (Male) *Neoscona species* (Male) *Neoscona species* (Male) *Poltys* (Female)
2	Clubionidae	*Clubiona drassodes* (Female)
3	Erasidae	*Stegodyphus species* (Female)
4	Hersilidae	*Hersiliasavignyi* (Female)
5	Lycosidae	*Acantholycosa lignaria* (Female) *Hippasa holmerae* (Female)
6	Miturgidae	*Cheiracanthium inclusum* (Female) *Cheiracanthium insigne* (Female)
7	Oxyopidae	*Oxyopes bharatae* (Male) *Oxyopes pankaji* (Female) *Oxyopes pankaji* (Male) *Peucetia latikae* (Female)
8	Pisauridae	*Dolomedes species* (Female) *Pisaurinamira* (Female) *Pisaurinamira* (Male) *Thalassius marginellus* (Female)
9	Salticidae	*Euophrys frontalis* (Female) *Harmochirus brachiatus* (Female) *Hasarius adansoni* (Male) *Myrmarachne species* (Female) *Parahelpis species* (Female) *Phidippus species* (Male) *Phintella vittata* (Female) *Plexippus insulanus* (Male) *Plexippus paykulli* (Male) *Plexippus paykullii* (Female) *Plexippus petersi* (Female) *Plexippus species* (Female) *Plexippus species* (Male) *Plexippus species* (Female) *Telamonia dimidiate* (Female)
10	Sparassidae	*Heteropoda species* (Female)
11	Tetragnathidae	*Leucauge dorsotuberculata* (Female) *Tetragnathas species* (Female) *Tetragnathas species* (Male) *Tetragnathas species* (Male) *Tetragnathas species* (Male)
12	Thomisidae	*Misumena species* (Male) *Oxylate species* (Female) *Thomisus beautifularis* (Female)



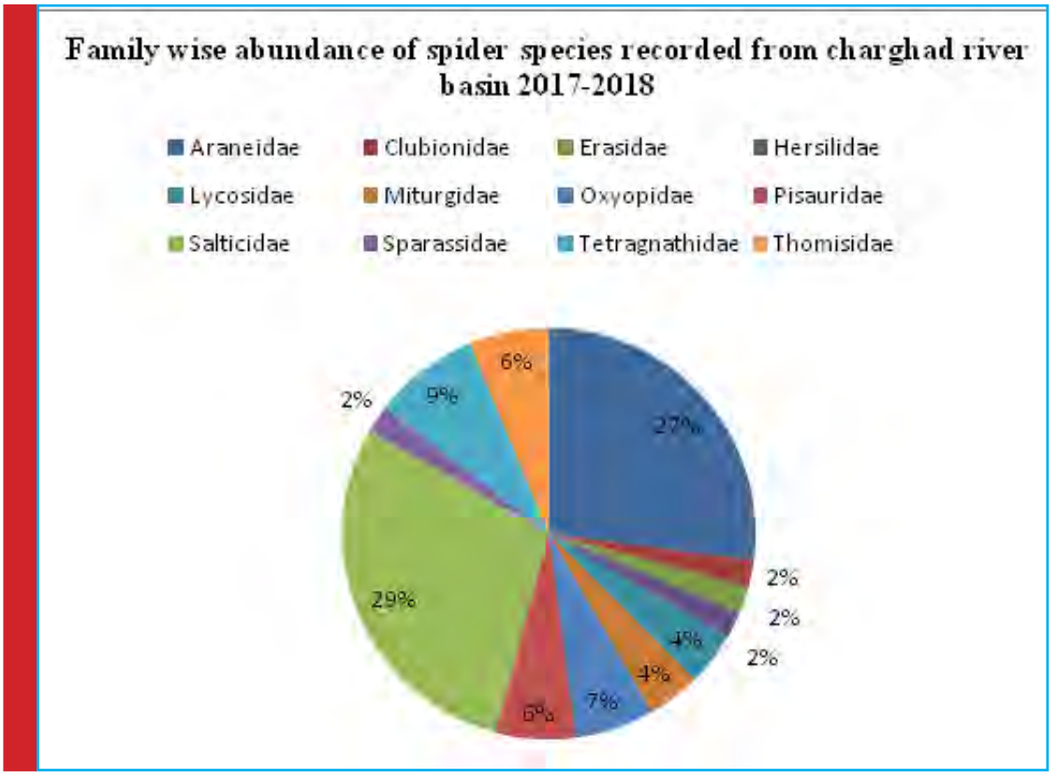
Xysticus minutes (Female)
Family : Thomisidae

Zosis sp. (Female)
Family : Uloboridae

that most abundant species belong to family Salticidae (29.03%) followed by family Araneidae (27.08%) comparatively moderate number of species from family Tetragnathidae (8.33%), Oxyopidae (6.25%), Thomisidae (6.25%), Pisauridae (6.25%), Lycosidae (4.16%), Miturgidae (4.16%) and lowest species diversity was found in the species belong to family Clubionidae (2.08%), Erasidae (2.08%), Hersilidae (2.08%), Sparassidae (2.08%).

Shannon Wiener diversity index (H) was '2'and Evenness of species was found to be '0.80'. The study suggests Charghad river basin has a rich diversified spider fauna. The Charghad River is a complex ecosystem for various arthropods. The diversity of speciesis different with respect to habitat, vegetation and prey base along the riverside. The diversity of specieswas found maximum in adjacent grasses and vegetation. Maximum species were recorded in November, December, January, February, it is due to fall in water level that increase the abundance of vegetation in the river bed and which also coincides with the life cycle of most insects including grasshoppers, aphids, millipedes, etc. Most of the spider species-belongs to family Salticidae and Oxyopidae were found abundant where grasses like *Cynodon dictylon* (hariali), *Ichamum sulcatum* (paonia), *Ichamum laxum* (sahada) and *Andropogon contorlus* (kusal) where dominant, where they can hide below thick foliage and have large prey base like Grasshopper nymph, larvae of various flies, millipedes, etc. Most of the spider species belonging to this family were recorded in winter and especially in November to January end.

The second most abundant family found in this ecosystem is Araneidae (19%) which was most abundant along riverside small trees and shrubs like *Zyzypheus vulgaris* (bor), *Bauhinia racemosa* (apta), Bamboo. Some of the orb-web spiders are dominant in thearea where big perennial plants found in large number.Where they are able toprepare websbetween the twigs for catching the small flying arthropods. *Larinia* species and *Neoscona* species were mostly observed in the night on shrubs and perennial plant with their orb webs, and found most abundant in the winter season. Spiders belong to family Thomisidae, Miturgidae, Clubonidae were found on flowering trees, shrubs and grasses, were they pray on small insects visit for nectar. Along withthe river stream



family Pisauridae and Tetragnathidae have rich diversity were they feed on small insect larva and small fishes. Spiders belonging to Hersilidae were abundant on the trunk of perennial trees.Followed by family Lycosidae, Erasidae, Sparassidae which are distributed over vegetation of the river bank. 48 species were recorded in the present study.Thus Charghad river basin contains rich spider fauna. However, this is not a final conclusion regarding species richness can't be drawn because the area of the river basin is unexplored.

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A Brief Note on Molluscan Diversity From Water Bodies of Amravati MS India

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ABSTRACT

Molluscs are the environment indicators and play a very important role in maintaining aquatic ecosystem by recycling nutrients and surviving as nutrition for certain aquatic organisms. Also they arean important source of food for other animals i.e. fishes, birds and mammals even for human being. In the age of global decline of biodiversity, it is necessary to study the present status of different biota and hence this attempt was made. The present paper deals with diversity of molluscan fauna fromfivefreshwater bodies i.e. Chatri Lake, Kekatpur Lake, Tapi River, Sipna River and Pedhi River of Amravati district in the period Jan 2015 to April 2017.A rapid survey method was used for careful visual estimation, handpick collection and recorded photographic evidences of molluscanspecies from selected habitats of the study area. A total of 30 molluscan species were reported and identified in this paper. These listed species belonging to 02 classes, 06 orders, 12 families and 17 genera. Out of 30 molluscan species 20 species belonging to class Gastropoda and 10 species belonging to class Bivalvia. Amongst the recorded Gastropodes 16 freshwater molluscan species and 04 were terrestrial molluscan including 01 slug and 03 snails. A bivalve was represents with 02 orders, 02 families and 03 genera. *Bellamya bengalensis, Lamellidens marginalis* species were more commonand widely distributed in all the waterbodies of study area. However, the few species *Lymnaea acuminate, Lymnaea leuteola* and *Indoplanorbis exustus* were found only in stagnant water, i.e. Lake Water.This study shows that the potential and importance of such habitats to diverse molluscan species and support many more species. It is a preliminary study on the molluscan diversity. Further studies are needed for detailed exploration of the molluscan fauna, its habitat and threats being experienced by these animals.

KEY WORDS: MOLLUSCS, DIVERSITY, AMRAVATI DISTRICT, MAHARASHTRA

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INTRODUCTION

The Phylum Mollusca is a second largest phylum in invertebrate. Molluscan are soft bodied animals with or without calcareous shell adapted to almost all habitats with varied ecology. Molluscs are divided into freshwater, marine and terrestrial forms.It includes snails, slugs, clams, oysters, mussels, scallops, cuttlefish, squid and octopus. All the molluscan comprises in three groups, Gastropods, Bivalves and Cephalopods. Gastropoda is extremely diverse group in Mollusca and adapted to all habitats,includes snails and slugs. Bivalves as a group have no head and it characterized by a shell that is divided from front to back into left and right valves. They include clams, oysters, mussels and number of families that live in freshwater (Subba Rao,1989; Patil and Talmale, 2005; Kumar and Vyas 2012; Tripathy and Mukhpadhyay, 2015).

Molluscans are the environment as well as bio-indicators and they play a very important role in maintaining aquatic ecosystem by recycling nutrients and surviving as nutrition for certain aquatic organisms. Freshwater molluscs play a significant role in aquatic ecosystem,and some of them are edible. Also, they serve an important source of food for other animals i.e. fishes, birds and mammals even for human being. In the age of global decline of biodiversity, it is necessary to study the present status of different biota.The taxonomic study of Indian fresh water molluscs has been done by Zoological Survey of India, Subba Rao (1989), Also in Maharashtra, freshwater Mollusca reported byRao (1925), Tonapi and Mulherkar (1963), Tonapi ((1971), (Subba Rao and Mitra (1975,1979), Surya et al. (2002), Patil and Talmale (2003, 2005). Tripathy and Mukhpadhyay, (2015), Magare et al. (2016), Kambale, (2018), Kumar et al., (2019).

As is evident from the available published literature from Vidarbha region of Maharashra,only few workers have made their contribution in study of Molluscan fauna in Vidarbha region.Occurrence of freshwater Bivalves in Pusad,Yavatmal district (Patil 2003), Freshwater Mollusca of Melghat Tiger Reserve studied(Patil 2005). Terrestrial snail diversity in Amravati city (Chavan *et al.,* 2015). But studies on diversity of molluscan from forest and water bodies are scarce, especially molluscan diversity in rivers and Lakes in Amravati district other than Melghat Tiger Reserve. Hence,the present study revealed that diversity of molluscan fauna from five freshwater bodies i.e. Chatri Lake, Kekatpur Lake, Pedhi River, Sipna River and Tapi River of Amravati district.

MATERIAL AND METHODS

Amravati district is a District of Maharashtra state in central India. The district is situated between 20°32' and 21°46' North latitudes and 76°37' and 78°27' East longitudes. The district occupies the geographical area of 12,235 km^2. There is Satpuda range towards the North of Amravati district.75% of Amravati district area is covered by Daccan trap while 25% area covered by Purna alluvium. Out of the total land of the district 30% covered by forest while 70% utilized for cultivation and human habitation. The climate of the district is hot and dry. The year can be divided into three clear seasons, winter season is from October to January, summer season from February to May and the monsoon season is from June to September. The area receives rainfall during southwest monsoon. The average rainfall is 800-1000 mm. Average temperature of the district ranges from minimum of 15^{o}C in winter to a maximum of 45^{o}C in summer with the humidity ranges from 10-15% to 60-95%.Melghat region is a part of the Satpuda Range of Hills in the Amravati district.The crests of this rangeattain an average elevation about 1000 meter. Melghat has Southern Tropical Dry Deciduous type of forest (Champian & Seth (1968)). Tapi, Sipna, Khapra, Khandu, Dolar, Khandu Chandarabhaga are the major rivers and many seasonal streams flows through Melghat. It experiences tropical climate with temperatures ranging between 13^{o}C and 22^{o}C during winter and between 23^{o}C and 42^{o}C during summer. In Melghat the annual rainfall ranges between 1000mm and 2000mm.

Molluscan Collection sites: Chatri Lake-It lies (N 20^0 53,684' and E 077^0 46,617' elevation 340m, covers an area of 111.231934m^2): it is the important water body for Local birds, migratory birds and Wildlife of Pohara–Malkhed Reserve Forest in Amravati district. It is onekm away from Amravati city on Amravati-Malkhed Road. **Kekatpur Lake-**It lies (N21 05,452'& E077 57,193', elevation 360 m): It is small fresh water body , located in Amravati district and about 20 km away from Amravati city towards North. It is surrounded by grass land, shrub forest and agricultural lands. This lake is one of the important wetland for residential as well as migratory bird fauna in Amravati district.

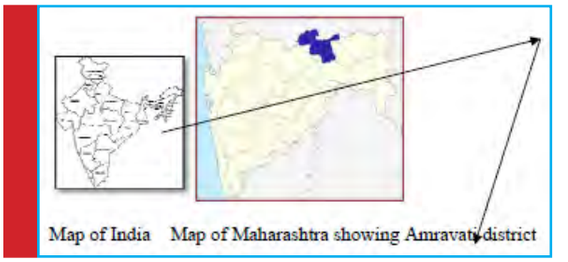
Pedi River-It originates from hills near Rithpur in Morshi tehsil of Amravati district.The Pedhi flows in easterly direction, after crossing the district it turns westwards and north-westwards to join the Purna river, Rithpur,Walgaon and Bhatkuli are few important villages at banks of the river. It is one of the water-supply source to the villages and agricultural land in some tehsils of Amravati district.Specimens werecollected from Pedi River Near Kund Village.((N2 57,29' & E07 40,17)

Sipana River in Melghat- It is one of the important river in Melghat. It originates from Melghat terrains and flows from central part of Melghat Tiger Reserve and finally joins to Tapi River. It serves lifeline to floral

and faunal diversity in Melghat tiger Reserve. Melghat Tiger Reserve (MTR) is one of the oldest tiger Reserve of Maharashtra and situated at the Northern part of Amravati district in Satpuda range. Specimens were collected from Sipana River Near Harisal Village. (21^{0}31' 21" N 077^0 07'39"E).

Tapi River in Melghat-Tapti River and the Gawilgad ridge of the Satpura Range forms the boundaries of the Melghat Tiger Reserve. The Tapi River flows through the Northern end of the Melghat Tiger Reserve, through a forest which lies within the catchment area of the river system and fed with other rivers like Sipna, Khandu and Gadga. Tapi is a major River in Central India, with a stretch of about 724 km, flowing from east to west; it is also one of the important rivers in Peninsular India. Specimens were collected from Tapi River near Rangubeli Village. (21^0 43' 08" N 077^0 08'14"E).

Molluscan Sampling: Present study was carried out on the basis of previous photographic collection during various visits and extensive survey during rainy season from June to September 2017 in the study area. The survey was performed at a weekly interval in all collection sites and microhabitats such as open land, cultivated field and forest during the rainy seasons. Specimens were collected by hand picking method from selected sites during the study period. Collected Molluscan washed properly and preserved in 5% formalin first and then transferred in 70 % alcohol. Photographs of the specimens were taken by Nikon camera D7000 and lens 60 mm micro for documentation and identification purpose. The specimens are identified as per Subba Rao (1989). The identification was confirmed by ZSI, Western Regional Center, Pune (Letter No.1548/MSI/2018/ Date 10-1-2018).



RESULT AND DISCUSSION

A total of 30 molluscan species were reported from the Amravati district. These listed species belonging to 02 classes, 06 orders,12 families and 17 genera. Out of 30 molluscan species 20s pecies belonging to class Gastropoda and 10 species belonging to class Bivalvia. Among the recorded Gastropodes 16 freshwater molluscan species and 04 were terrestrial molluscan including 01 slug and 03 terrestrial snails.Bivalves was representswith 02 orders, 02 families, 03 genera and 10 species. (Table 1 and Fig. 3).

The highest number of species recorded belonging to family Thiaridae followed by Viviparidae, Lymnaeidae, Arioplantidae, Bullinidae, Achatinnidae, Veronicellidae, Cerastuidae from gastropods and nine species reported belonging to family unionidae and only one species from corbiculidae of bivalves. Amongst the freshwater gastropods *Bellamya bengalensis* and *Melanoides tuberculata* were found more dominant, widely distributed and survival in varied aquatic habitats. The *Lymnaea*

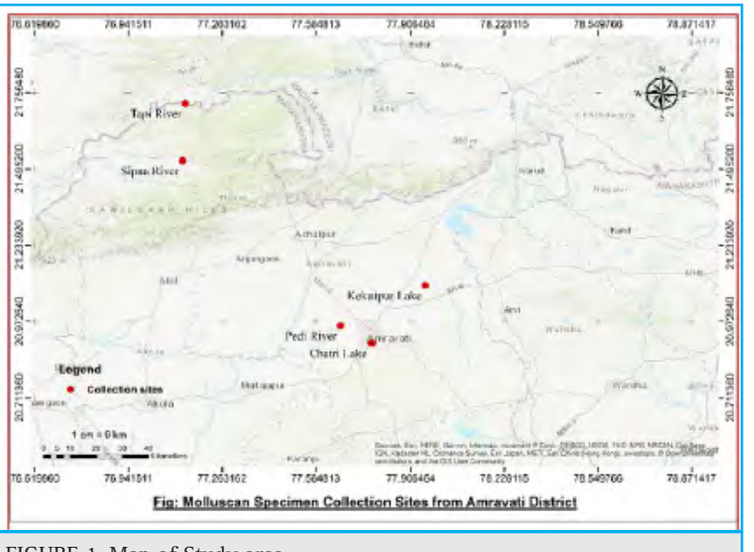


FIGURE 1. Map of Study area

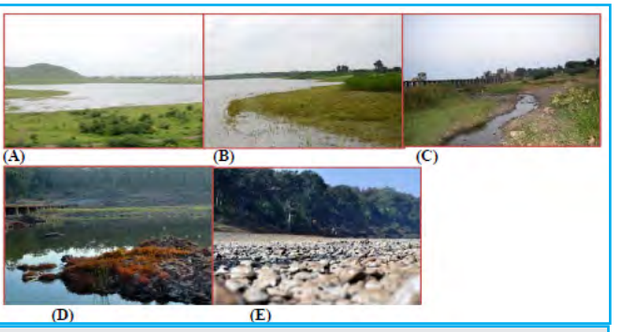


FIGURE 2. Specimens Collection sites: (A)- Chatri lake, (B)-Kekatpur Lake C)-Pedi River, (D)-Sipana River near Harisal and (E)-Tapi River near Rangubeli.

Table 1. List of Molluscan species reported from different ecological habitat in Amravati district.

Class	Family	Species	CL	KL	PR	SR	TR
Gastropoda	Viviparidae	*Bellamya bengalensis (Lamark)*	+	+	+	+	+
		*Bellamya bengalensis doliaris (Gould)**	-	-	-	-	-
		Bellamya dissimilis (Mueller)	-	-	-	+	+
	Pilidae	*Pila globus (Swainson)**	-	-	-	-	-
		*Pila viren(Lamark)**	-	-	-	-	-
	Thiaridae	*Thiara scabra(Mueller)*	+	-	-	+	-
		Thiara lineate (Gray)	+	+	+	+	+
		Melanoides tuberculata (Mueller)	+	+	+	+	+
		*Paludomus obesus(Philippi)**	-	-	-	-	-
	Lymnaeidae	*Lymnea acuminate(Lamark)*	+	-	-	-	-
		Lymnea leuteola(Lamark)	+	-	-	-	-
		*L.leuteola f.australis Annandale & Rao**	-	-	-	-	-
	Bullinidae	*Indoplanorbis exustus (Deshayes)*	+	+	-	-	-
		*Gyraulus convexiusculus (Hutton)**	-	-	-	-	-
	Ancylidae	*Ferrissia verruca(Benson)**	-	-	-	-	-
	Cerastuidae	*Rachis punctatus(Anton)*	+	-	-	-	-
	Arioplantidae	*Macrochlamys indica (Benson)*	+	+	-	-	-
		Cryptozona semirugata (Beck))	+	+	-	-	-
	Achatinnidae	*Achatina fulica(Bowdich)*	+	+	+	-	-
	Veronicellidae	*Laevicaulis altae(Ferussac)*	+	+	+	+	+
Bivalvia	Unionidae	*Lamellidens marginalis (Lamark)*	+	+	+	+	+
		Lamellidens corrianeus (Lea)	-	-	+	-	-
		*Lamellidens consobrinus (Lea)**	-	-	-	-	-
		Parreysia caurulea(Lea)	-	-	+	-	+
		*Parreysia fevidens(Benson)**	-	-	-	-	-
		*Parreysia annadalei(Preston)**	-	-	-	-	-
		*Parreysia corrugata(Mueller)**	-	-	-	-	-
		*P. corrugate laevirostris (Benson)**	-	-	-	-	-
		*P. cylindrical Annandale & Prashad**	-	-	-	-	-
	Corbiculidae	*Corbicula striatella (Deshayes)*	-	-	-	-	+

CL-Chatri Lake, KL-Kekatpur Lake, PR-Pedi River, SR-Sipana River, TR-Tapi River
*Not actually collected, earlier reported by Zoological Survey of India (Patil ,2005)

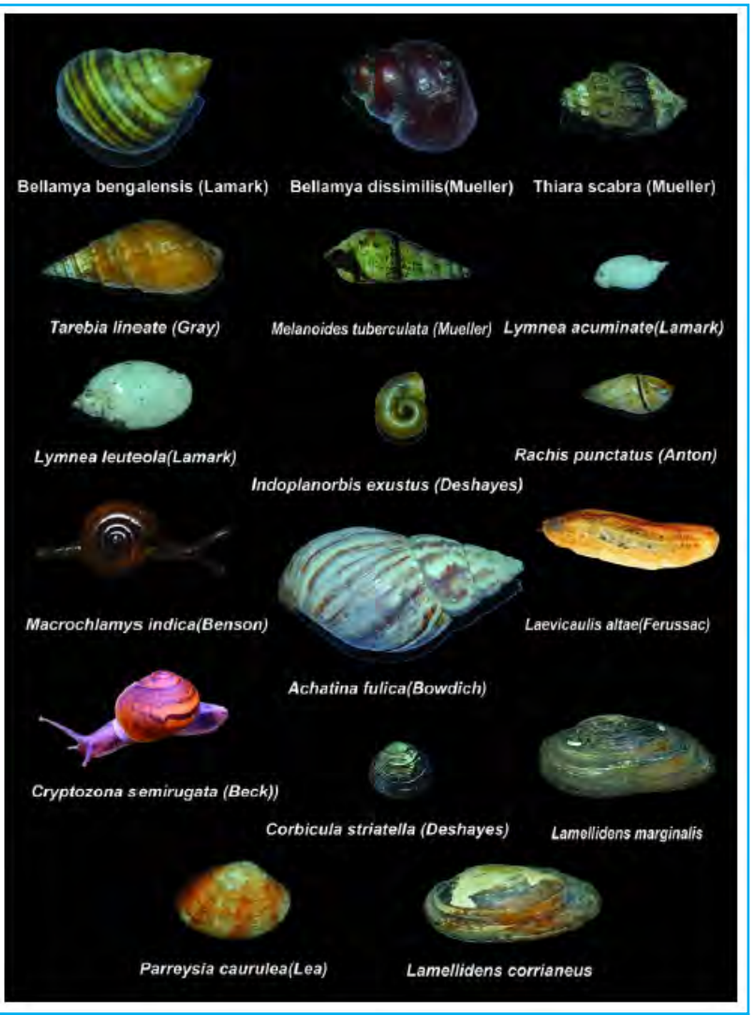


FIGURE 3. Collected and Identified molluscan specimens from Amravati District.

*acuminate, Lymnaea leuteola, Tarebia lineate,Thiara scabra*and *Indoplanorbis exustus* were highly habitat specific they proved to bio-indicator of ecologically diverse aquatic habitat. *Laevicaulis altae and Macrochlamys indica* were found dominant from terrestrial gastropods. Only *L. altae* species reported as a tropical land slug and *Macrochlamys indica*, *Cryptozona semirugata* and *Achatina fulica* reported as aterrestrial pulmonate snails. *Lamellidens marginalis* was found more common from bivalves in all selected sites, viz Lakes and Rivers. *Parreysia* speciesreported only from rivers of the Melghat.

Bellamya bengalensis doliaris (Gould), Pila globus (Swainson), Pila viren (Lamark), Paludomus obesus (Philippi), L.leuteola f.australis Annandale & Rao, Gyraulus convexiusculus (Hutton), Ferrissia verruca (Benson), *Lamellidens consobrinus (Lea), Parreysia fevidens (Benson), Parreysia annadalei (Preston), Parreysia corrugata (Mueller), P. corrugate laevirostris (Benson)* and *P. cylindrical Annandale & Prashad* were not reported during present study, but these species were earlier reported from Melghat and Purna river by ZSI scientist (Patil, 2005). Molluscan are good indicators of localized condition indicating water quality. Freshwater molluscs play a significant role in the aquatic ecosystem structure and biodiversity. Also, they serve an important source of food for other animals i.e. fishes, birds and mammals even for human being. The existence of molluscan is highly necessary because they constitute food for many aquatic organisms (Subha, 2003). This study shows that the potential and importance of such habitats to diverse molluscan species and support many

more species. It is a preliminary study on the molluscan diversity in Amravati district. Further studies are needed for detailed exploration of the molluscan fauna and its microhabitat and threats being experienced by these benthic macro fauna.

CONCLUSION

In this paper a total of 30 molluscan species were reported from the Amravati district.This paper is shows the first list of freshwater and land molluscs from Lakes, Rivers, forests and agricultural lands of Amravati district. The present study provides the base line data for the molluscan diversity in Amravati district. Further long term research is needed to explore the diversity of molluscan, population estimation, its habitat, seasonal variations and threats being experienced by these animals.

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Antiangiogenic potential of endophytic fungi *Alternaria alternata* isolated from *Lawsonia inermis* Linn

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ABSTRACT

Antiangiogenesis is the complex mechanism used for the inhibition of growth of blood vessels from the pre-existing vasculature. Blood vessel is known to perform a crucial role in development of tumor. Blocking of angiogenesis through the control over the action of the cytokine VEGF could be possible mechanism in cancer therapy. This is very important mechanism used to prevent the proliferation of blood vessels towards growth of tumors. It has proved to be potentially attractive approach in case of controlling dreadful disease like Cancer .Knowing the importance of this process, for the first time,the study was aimed to investigate the antiangiogenic potential of endophytic fungi from the medicinal plant *Lawsonia inermis linn*. We successfully isolated *Alternaria alternate* from the leaves of *Lawsonia inermis Linn* and identified microscopically as well as by 18srRNA and ITS sequence analysis. The GC-MS analysis revealed the presence of wide range of bioactive secondary metabolites showing significant antimicrobial property against four human bacterial pathogens .The ethyl acetate extract of *Alternaria alternata* showed maximum zone of inhibiton of 21.8±0.8mm against *Pseudomonas aeroginosa*. After these analysis, antiangiogeneic property of *Alternaria alternata* ethyl acetate extract was studied by Chick Chorioallantoic membrane assay (CAM) which showed the treated CAM has (84±2.60) less no. of tertiary blood vessels as compared to the control (124± 2.64). Therefore the results suggest that *Alternaria alternata* can be considered as a potential source of antiangiogenic agent. Further investigation of characterization and structure elucidation of active compounds from this extract is needed to know the exact antiangiogenic component.

KEY WORDS: ANGIOGENESIS, ANGIOGENESIS, VEGF, ENDOPHYTIC FUNGI,SECONDARY METABOLITES CHORIOALLANTOIC MEMBRANE (CAM) ASSAY, LAWSONIA

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INTRODUCTION

Angiogenesis is the dynamically regulated process where new blood vessels are escalated from the previously formed blood vasculature. It is known to be essential for normal physiological process like embryonic development, organ formation, reproductive system, cyclical ovarian function, tissue renewal, and wound healing. Also it also play a vital role in pathological conditions like rheumatoid arthritis, arteriosclerosis, myocardial infarction, ischaemic diseases, diabetic retinopathy and cancer. The later pathological disease called Cancer has drawn more attention of researchers some few year back. The reason behind the cancer being the most dreadful disease is the unending requirement of oxygen and nutrients supply from the growing blood vessels which later on make it critical, metastatic and leads to death of the person.

According to Folkman (1971) tumor growth is known to depend on angiogenesis where it is triggered by chemical signals from tumor cells in a phase of rapid growth. Tumors do not grow progressively unless they induce a blood supply from the surrounding stroma. Cancers that lack angiogenesis remain dormant. Therefore in order to get control over the tumor cells blood vessels supply to cancer cells should be inhibited through process called antiangiogenesis.,Since in the absence of vascular support tumors become necrotic, or even apoptotic, (Holmgren *et al* 1995). As per Judah Folkman, the main reason behind the excessive formation of blood vessels around the tumor is due to the Vascular Endothelial Growth Factor-A (VEGF-A) is supposed to be a potent angiogenic mitogen inducing migration and proliferation of endothelial cells. Blockade of VEGF has proven to be effective way of inhibiting tumor angiogenesis. Therefore Folkman and his colleagues were the first to propose using inhibition of tumor vasculature formation as anticancer therapy (Folkman 2003). Of late researchers are now actively involved in the development of novel anti cancerous drugs throughout the world .According to some reports, many of the available anticancer drugs are exhibiting toxicity to proliferating normal cells along with the cancer cells also the repetitive dosage is enhancing its resistance which results in increasing the the need for bioactive components from natural products, (Remesh 2017).

Natural products are naturally derived metabolites and by products obtained from microorganisms, fungi, plants or animals, (Baker *et al* 2000). According to Schulz (2001), novel secondary metabolites are derived from organisms that occupy unique biotopes with unusual environment are known to be prolific producers of bioactive metabolites. Endophytes are one of those microbes that have the ability to thrive in the specific biotopes like higher plants which are traditionally been exploited for medical, industrial and agricultural use. Endophytic fungi are the microbe that colonize the internal tissues of plant without causing any infection or harm to them, (Bacon and White 2000, Remesh 2017).

In recent advances of research, endophytes are viewed as a excellent source of bioactive natural product as they are known to possess therapeutic value for the prevention and treatment of various types and stages of cancer. As per the estimation Dreyfuss and Chapela Rossman, there are as many as 1 million species of endophytic fungi unexplored and undescribed. There are very few research has been reported on the study of angiogenesis and antiangiogenesis property of endophytic fungi using Chick chorioallantoic membrane assay. it was found that Hulikere *et al*, (2016) studied the antiangiogenic and antioxidant activity of endophytic fungus *Penicillium citrinum* and *Cladosporium Cladosporoides* from seaweed (*Sargassum wightii*). This research work has created impetus in us to investigate the antiangiogenic potential of bioactive secondary metabolites of endophytic fungi isolated from medicinal plant *Lawsonia inermis* Linn (Mehndi) located in Satara District, (Maharashtra), India, Using chick Chorioallantoic membrane assay (CAM).

Chick embryo chorioallantoic membrane in vivo assay is one of the best established and most popularly used model for angiogenesis and cancer studies. As this method is Cost effective, less time consuming, easy to perform, reproducible and has visible vasculature which make this useful for both intravascular and topical administration of study agent, relatively rapid assay method and can be adopted very easily to study tumor growth, (Ribatti *et al*, 2001). Therefore in order to discover alternative treatment for cancer chemoprevention, the chorioallantoic membrane assay is used to analyse various natural compounds that could reduce or inhibit several pathways involving malignancies and excessive angiogenesis related diseases.

MATERIAL AND METHODS

Collection of plant material: In order to obtain endophytes, *L. inermis* has been selected on the basis of their unique biology, age, endemism, ethnobotanical history, or environmental setting. It seems that endemic plants growing in moist, warm climates or in areas of great biodiversity are among the first choices for study, so accordingly *L. inermis* is collected from Satara district in Northern Western Ghats. The properly identified and authenticated plant was used in the current study. Healthy and mature leaves were collected from the field grown plants were brought to laboratory and processed within 24 hrs of collection or stored in icebox.

Isolation of endophytic fungi from *Lawsonia inermis*: To isolate endophytic fungi from leaves of *Lawsonia inermis* Linn, Collected leaves were washed thoroughly with water to expel the unwanted material from the surface. Each leaf was surface sterilized by using the method described by Arnold et al (2007) with minor changes. For sterilization of surface of leaves, leave were immersed in 70 % ethanol for 1 min, further dipped in 0.1% $HgCl_2$ for 1 min and later washed in autoclaved distilled water, (Bisht et al 2016) and blot dried on filter paper. Finally the leaves were cut aseptically into 1 cm long segments and placed on Potato dextrose agar plate supplied with 50ug for /ml of streptomycin to avoid any bacterial contamination .later on the plates were sealed with parafilm and incubated at 28 ± 2^0c for 8-10 days in incubator. After the respective days, the hyphal tips of fungi emerging from leaves were transferred aseptically to fresh PDA slants to get pure culture for fungi.

Identification of Isolated fungi- The fungal isolates mounted on the sterile slides then it was stained with lactophenol cotton blue and then examined in 100x light microscopy. Morphological identification of the isolates was carried out on the basis of surface texture, pigmentation, and spores at the hyphal tips which helped to identify the endophytic fungi at the species level. The authorized identification of endophytic fungi was done from National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, and Pune, India. The identified fungal strains were isolated and then sub-cultured in a Petri dish which contains sterile PDA media. To preserve as a pure culture, the endophytic fungi were inoculated in PDA slant.

Molecular identification: Fungal genomic DNA was isolated using Qiagen DNeasy kit. Fungal ITS region gene was amplified using standard PCR reaction. The primer pair ITS1 and ITS4 was used in a PCR reaction with an annealing temperature of 54°C. After amplification, products were purified by using Invitrogen PCR product purification kit (Life technologies, USA) and were directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at Blast N site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST).

Fermentation and extraction of secondary metabolites: Erlenmeyer flasks (250 ml) containing 100 mL potato dextrose broth were used for cultivation of fungal isolates. The broth was inoculated with two loops of fungal isolates and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. The extraction was carried out according to the method described by Radji *et al* (2011). The broth culture was filtered by double-layered muslin cloth to separate the mycelia and filtrate. To the filtrate equal volume of ethyl acetate (1:1) was added, mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers formed. Then the upper layer of ethyl acetate was collected using separating funnel as it contained the extracted compounds. The mycelium was ground properly in a pestle and mortar using ethyl acetate as a solvent and then it was filtered using cheesecloth. Both mycelia and culture filtrate extracts were pooled together and evaporated to dryness in the hot air oven. The extract residue was dissolved in Dimethyl Sulfoxide (DMSO) and stored at 4°C to be used as a stock solution for spectroscopic characterization and antimicrobial assay.

Antimicrobial screening by Agar Well diffusion assay: Agar well diffusion assay method described by Magaldi *et al* with little modification has been used for the evaluation of antimicrobial property. The crude extracts of fungal endophytes were used and dissolved in DMSO. The test organisms viz. *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* were obtained from the Department of Microbiology, Yashavantrao Chavan Institute of Science, Satara, India. The wells of 1cm in diameter were bored in the media with the help of sterile cork borer. The well was filled with 40 µl of crude extracts of different fungi. For negative control, one well was filled with only 40 µl DMSO. These plates were then refrigerated at 4 °C for 4-6 hrs for antimicrobial diffusion and then incubated at 37°c for 24 hrs. The clear zones of inhibition formed around the wells were measured by counting the diameter of a circle in millimeters. The test was performed in triplicate with each bacterial strain and mean zone of inhibition was recorded.

Gas Chromatographic Mass Spectroscopic profile: To identify the compounds from the fungal extract, GC-MS analysis was carried out using Shimadzu model QP-2010 with a nonpolar 60 M RTX 5MS column. The carrier gas used was Helium and the temperature programme was adjusted to initial oven temperature at 40 °C for 3 min and the final temperature of oven was 480 °C with rate at 10 °C. One microliters (1 µl) sample was injected using a split less mode. Mass spectra was recorded over 35–650 amu range with electron impact ionization energy 70 eV. The total time required to run the sample was 45 min. Relative quantitative determinations were made by relating respective peak areas to total ion chromatogram (TIC). Unknown components compared with known mass spectra of National Institute of Standards (NIST) for molecular identification of compounds. Name, molecular weight, retention time and peak area percentage of the test material was tentatively determined.

Chick Chorioallantoic membrane (CAM) *In vivo* assay: The antiangiogenic potential was detected by using CAM

assay model as described by Domenico R. *et al* (1996). Fertilized eggs of *Gallus gallus* were purchased from Satara Poultry hatchery, then were cleaned and disinfected with 70% alcohol and divided in three groups for 48 hrs, 72 hrs and 96 hrs. The eggs were incubated in an aseptic incubator in vertical position such as blunt end of egg faced upward and was maintained at 37^0C temperature and humidity at 70%.

Dose administration - For the dose administration Hanks Balanced Salt Solution (HBBS) was used as saline. Endophytic fungal extract was prepared in ethyl acetate, of which 20 ug/ml of extract was dissolved in 1ml DMSO. All treatments were given in final volume of HBSS (mg/ml). At different developmental stages (48 hrs 72 hrs 96 hrs) dose was initiated by making a small window at the blunt end of egg. With the help of insulin syringe of 0.2 ml of dose was injected on to the chorioallantoic membrane and then sealed with surgical adhesive tape. The development was continued up to 144 hrs. (Korn & Cramer, 2009). After 144 hrs The eggs were then opened for morphometric evaluation. Later the CAM samples were fixed in CAF (Calcium Acetate Formalin) fixative to carry out further histopathological process and finally stained with Haematoxylin and eosin for microscopic evaluation.

RESULTS AND DISCUSSION

From the present study, after following the standard isolation procedure of endophytic fungi from the leaves of medicinal plant *Lawsonia inermis* Linn, *Alternaria spp*. was isolated successfully. Pure culture was maintained by sub culturing it at least three times.

From the preliminary identification of fungi it was observed that the fungal colony had olivaceous black or dark gray color with white margin with woolly texture (Fig:1) while features like elongated chains of septate brownish conidia with short beak and smooth surface were observed in the microscopic examinations which were confirmed from National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute (ARI) Pune. Therefore as per the morphological and microscopic observations the isolate was identified as *Alternaria* spp, further confirmation up to species level was

FIGURE 1. A) Collected plant sample of *Lawsonia inermis* Linn, B) emergence of mycelia from cut leaf sections, C) colony morphology of *Alternaria alternata*, D) microscopic view

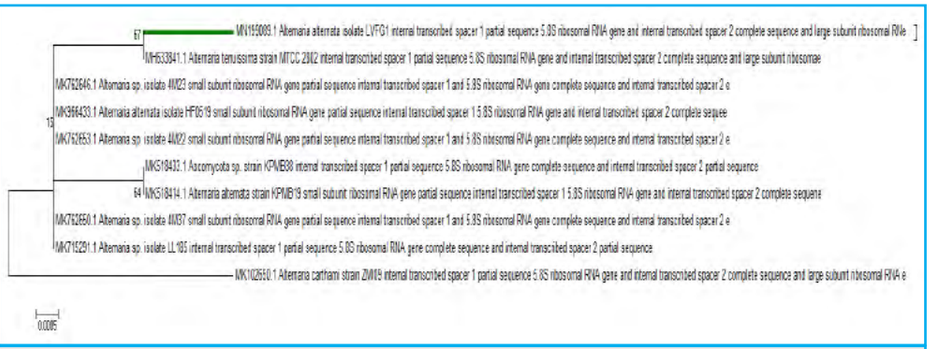

FIGURE 2. Evolutionary relationships of taxa

confirmed by molecular analysis. Molecular identification was done with the help of 18s rRNA gene and Internal transcribed Spacer (ITS) region which identified the *Alternaria* spp as *Alternaria alternata* based on BLAST results showing 100 percent identity, 100 % query coverage with nucleotide homology and phylogenetics analysis The information regarding close homologs is represented in the alignment view (fig: 2).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei (1987). The optimal tree with the sum of branch length = 0.01238757 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches, (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura M. 1980) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 486 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al* 2013).

The Agar well diffusion assay was performed to evaluate the antimicrobial property of the *Alternaria alternata* ethyl acetate extract. In this, it showed promising inhibition of four human bacterial pathogens (table 1). In this assay 10 µl of extract was dissolved in DMSO which also was used as a negative control to state whether the inhibition was not occurred due to solvent. The evaluation was carried out on the basis of classification as (0-6mm)- no activity, (7-9mm)-not significant, (10-12mm)- poor activity (13-15mm)- low activity (16-18 mm)- good activity, and above 18 mm significant activity. From this it was observed that the extract showed significant activity with maximum zone of inhibition against *Pseudomonas aeruginosa* (21.8mm), while *Bacillus subtilis* showed good activity with (17mm) inhibition while *S.aureus* showed poor activity with (13.2mm) inhibition and E.coli showed low activity with 9.4 mm inhibition (table no.1). Thus the above results indicates that the endophytic fungi *Alternaria alternata* possess significant antimicrobial property which was then further characterized for secondary metabolite profile and investigation of antiangiogenic potential.

Table 1. Zone of inhibition (in mm) of fungal endophytes crude extracts by Agar well diffusion method

Name of organism	Diameter of zone of inhibition (mm)
P. aerogenusa	21.8±0.8
B . subtilis	17±0.6
E.coli	9.4±0.2
S.aureus	13.2±1.3
DMSO	--

(--) –No inhibition, DMSO – Dimethyl Sulfoxide

Antiangiogenic activity was studied by Chick chorioallantoic Membrane assay using Window method. After 144 hrs, the eggs were opened for morphometric and histological analysis. For morphometric study, firstly the CAM area was measured as described by Melkonian *et al* (2002), from the (fig :3) and (table no. 2), it can observed that there has been 24.6 % decrease in the total CAM area of eggs treated with *A. alternata* extract than HBSS control eggs. Also the tertiary blood vessels on CAM area were counted on manually on Computer by considering the sprouting points, it was observed that there was significant decrease in the no. of tertiary blood vessels showing 32.7% inhibition while normal and HBSS (Hanks balanced Salt Solution) control showed increase in the number of blood vessel and branches.These suppression of blood vessels were confirmed by histological section stained with Haematoxylin and eosin show-

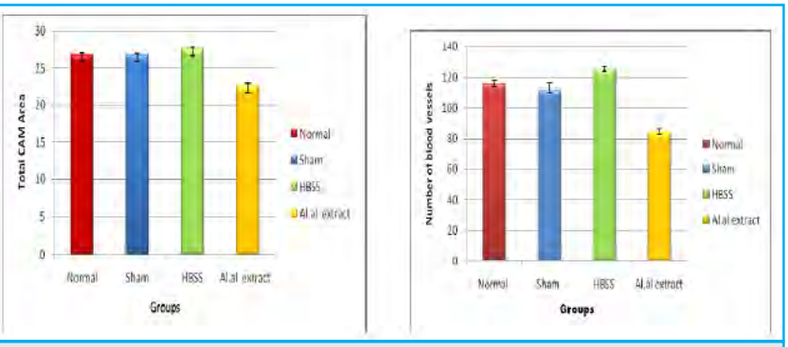

FIGURE 3. Alterations in area of CAM and number of tertiary blood vessels by *Alternaria alternata* extract.

Table 2. Influence of endophytic fungi *Alternaria alternata* methanolic extract on number of blood vessels.

Group	Total Number Tertiary blood vessels	Total CAM area
Normal	115 ±2.9	26.8±0.2
Sham	111±5.5	26.8±0.1
HBSS	124±2.6	27.6±0.1
Al.al extract	84±2.6	22.6±0.4

(HBSS-Hanks Balanced salt Solution, Al. al –*Alternaria alternata*) Results expressed in mean±S.E,P-value <0.05 considererd most significant value

ing inhibition of proliferative vessel and reduction in capillary plexus of treated vessels as compared to normal. (Fig:4) For morphometric analysis- CAM area was measured according to method described by Melkonian *et al* (2002) as, CAM area = (1/2A) x(1/2B)x π, where, A=Longest length,B=Longest width, and π=3.14. For Histological analysis- Normal, controlled and treated CAMs were fixed in CAF Fixative, a section of tertiary blood vessels was cut and processed in alcohol grades for dehydration and then embedded in paraffin. The paraffin blocks were then sectioned of 6µ size using rotary microtome. these vertical sections were stained with

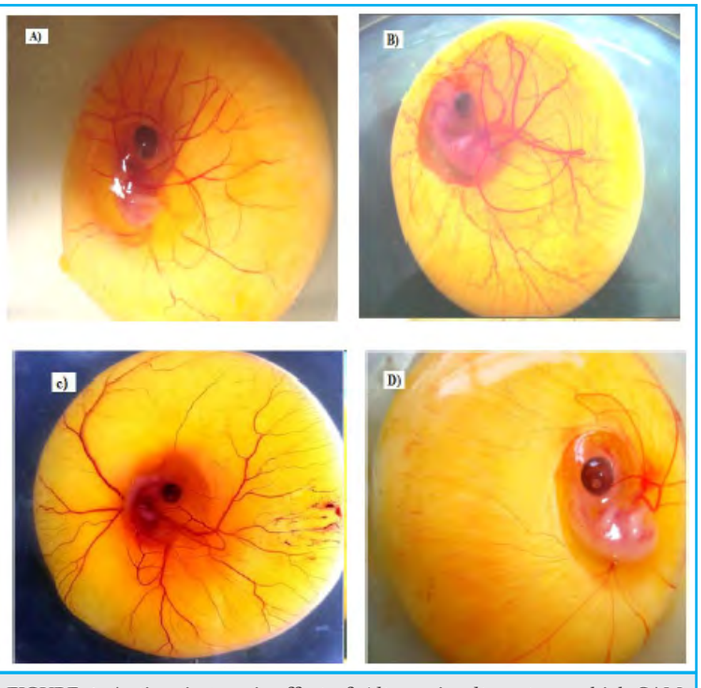

FIGURE 4. Antiangiogenesis effect of *Alternaria alternata* on chick CAM A) Normal, B) Sham control, C) HBSS control, D) Treated CAM

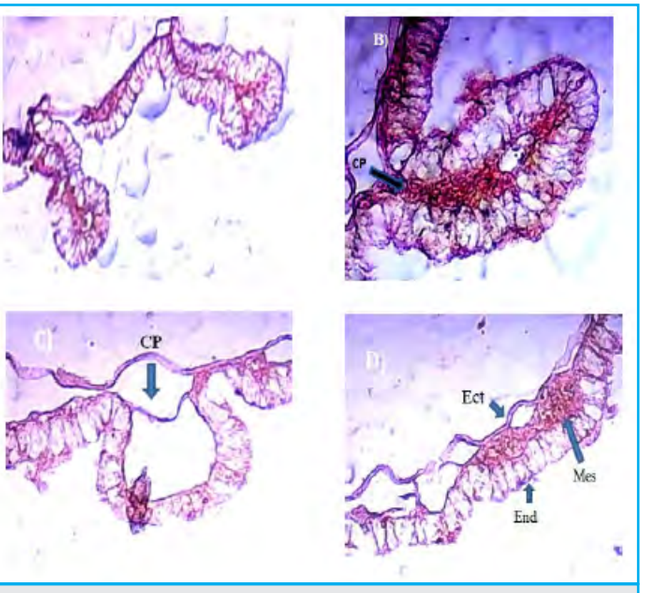

FIGURE 5. T.S of Chick CAM blood vessels H-E stained (40x) A) Normal B) Sham C) HBSS D) Treated

H-E technique. Finally the slides were observed under light microscope for histological changes in structure of blood vessels.

Gas Chromatographic –Mass Spectroscopic analysis- The GC-MS analysis was carried out for fungal extract that showed the potential antimicrobial property. Therefore the ethyl acetate fungal crude extract of *Alternaria alternata* showed total twelve peaks indicating the presence of twelve compounds (Fig. 6). Among them Hexadecanoic acid, methyl ester (35.73) showed highest percentage of area followed by 9-Octadecenoic acid, methyl ester(E)- (24.55), 2-Fluorobenzoic acid, Heptadecyl ester (12.79), 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (3.66), Methyl tetradecanohate (2.19), 2-Ethyl-1hexanol (1.94), Methyl hexadec-9-enoate (1.85), 1,2 Benzenedicarboxylic acid (0.93), Nonanedioic acid, dimethyl ester (0.92), 1-Nonadecene (0.91), and Heneicosane (0.82) (Table 3).

Compounds detected were predominantly derived from the ethyl extract especially hexadecanoic acid methyl ester which is an aliphatic acid ester reported to cause growth inhibition and apoptosis induction in human gastric cancer cells (Yu *et al* 2005). Number of compounds detected including 9-Octadecenoic acid, methyl ester (E)- (24.55) are to possess anti-inflammatory and cancer preventive properties.

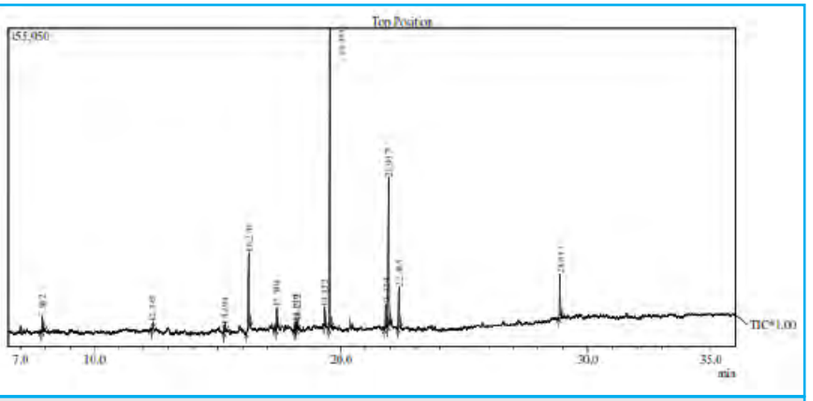

FIGURE 6. GC-MS analysis of ethyl acetate extract of *A. alternata*

Table 3. Phytoconstituents in *Alternaria alternata* ethyl acetate extract

S. No	Name	Peak	R.T	I.T	F.T	Area	Area (%)
1.	2-Ethyl-1-hexanol	1	7.892	7.845	7.955	41804	1.94
2.	1,2-Benzenedicarboxylic acid	2	12.345	12.300	12.395	20046	0.93
3.	Nonanedioic acid, dimethyl ester	3	15.294	15.260	15.325	19804	0.92
4.	2-Fluorobenzoicacid, heptadecyl ester	4	16.270	16.160	16.335	275250	12.79
5.	Methyl tetradecanoate	5	17.389	17.345	17.420	47222	2.19
6.	1-Nonadecene	6	18.149	18.100	18.180	19606	0.91
7.	Heneicosane	7	18.222	18.180	18.255	17585	0.82
8.	Methyl hexadec-9-enoate	8	19.322	19.280	19.360	39844	1.85
9.	Hexadecanoic acid, methyl ester	9	19.555	19.495	19.610	769183	35.73
10.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	10	21.824	21.765	21.870	78770	3.66
11.	9-Octadecenoic acid, methyl ester, (E)	11	21.937	21.885	21.980	528586	24.55
12.	Methyl stearate	12	22.365	22.305	22.415	148763	6.91

CONCLUSION

Many reports suggested the various medicinal properties of endophytic fungi *Alternaria alternata* as antimicrobial, antifungal, antidiabetic, anticancerous but this study has first time reported the anitangiogenic potential of terrestrial endophytic fungi *Alternaria alternata* isolated from leaves of medicinal plant *Lawsonia inermis*. The GC-MS analysis revealed the presence of 12 bioactive compounds which showed significant antimicrobial property against four bacterial pathogens showing significant zone of inhibition (21.8±0.8mm) against *Ps.aeruginosa*. Due to presence of this bioactive metabolites, we were able to evaluate the antiangiogenic potential of *Alternaria alternata* ethyl acetate extract by observing the maximum inhibition of blood vessels in treated CAM than the control one. This research work defines the very important biological potential which can be considered as potential candidate for antiangiogeneic treatment.

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