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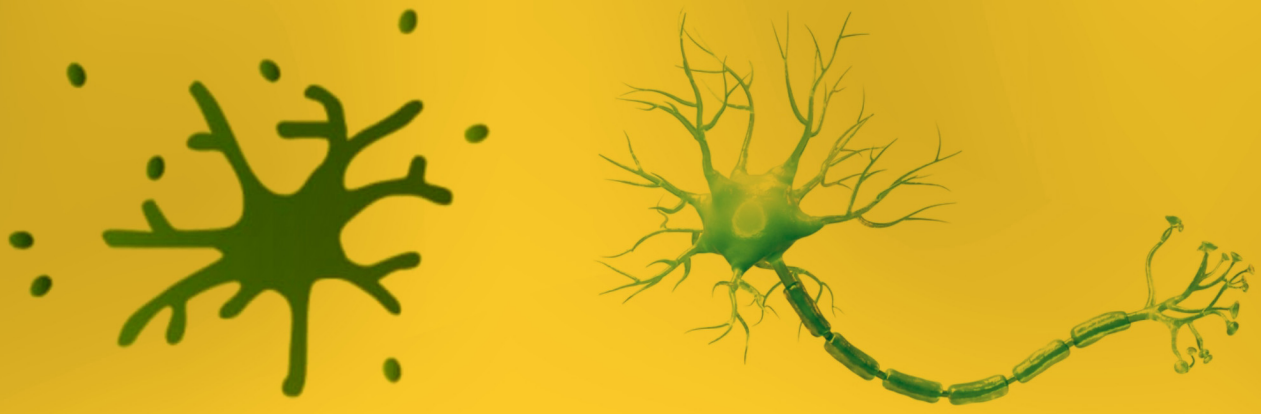

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RESEARCH ARTICLES

Bioscience Biotechnology Research Communications

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

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

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

Biosc Biotech Res Comm is an open-access international platform for publication of original research articles, exciting meta-reviews, case histories, novel perspectives and opinions in applied areas of biomedical sciences. It aims to promote global scientific research and development, via interactive and productive communications in these areas, helping scholars to present their cherished fruits of research grown on toiled and tilled trees of hard work in life sciences. Being the publication of a non-profit academic Society for Science and Nature, Bhopal India, since 2008, *Biosc Biotech Res Comm* strongly believes in maintaining high standards of ethical and quality publication.

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

Sharique A. Ali, PhD
Editor-in-Chief

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An analysis of the Clinical Parameters of Implant Site Grafting Using Allografts and Xenografts in Dental Implant Therapy

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ABSTRACT

Prior to the implantation of dental implants, grafting influences the periodontal clinical parameters. This study looked into and contrasted several clinical parameters in dental implants using allografts and xenografts. In this cross-sectional study, a total of 188 patients', male 103 (54.8%) and 85 (45.2%) female aged between 19 to 79 years (mean age=47.7±8.6 years) were included, at King Saud University's Dental College in Saudi Arabia, were carried out. Dental appointments were scheduled for patients. A thorough clinical and radiological evaluation of the implants was completed throughout these sessions. Analysing and contrasting the clinical parameters Allograft and Xenograft bleeding on probing, Probing depth, and Attached gingiva in the two types of grafting procedures, Bone Level and Tissue Level implants, when the two types of final restorations (cement retained and screw retained) were placed, the four types of grafting materials (Bioss, Cortical, Cancellous, and Mixed), and the three oral hygiene groups (fair, good, and poor) of the patients did not exhibit statistically significant differences in mean values. Based on the frequency of implant thread exposures observed in the radiographs, patients treated with xenografts (35.1%) and allografts (31.8%), respectively, showed one to four thread exposures. Allografts and Xenografts materials resulted in predictable outcomes as effective graft materials in terms of dental implant site development. The study found no significant differences in clinical parameters such as bleeding on probing, probing depth, attached gingiva, and oral hygiene between allografts, bone level, tissue level, cement retained, and screw retained restorations.

KEY WORDS: PERI-IMPLANTITIS; DENTAL IMPLANTS; IMPLANTS; IMPLANT PROSTHESIS; IMPLANT HEALTH; IMPLANT PARAMETERS.,

INTRODUCTION

Because of their excellent success rate, dental implants are becoming a more common and widely accepted choice for replacing lost teeth in professional dentistry practices. Nevertheless, peri-implant tissue health is being jeopardized by certain risk factors that result in peri-implantitis, which has led to an increase in implant failure rates recently (Beschnidt et al., 2018). Peri-implant mucositis and peri-implantitis are problems that reduce the overall success rate of dental implants.

Numerous factors affect the quality and health of the soft tissues surrounding implants. The primary factor influencing the soft tissue health surrounding an implant is the amount of keratinized mucosa surrounding it, (Benedek et al., 2024).

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Since peri-implantitis is still a condition that is difficult to cure and is not well understood, it is imperative that treatment phases be carefully controlled during the whole course of therapy in order to better prevent the condition. The significance of implant-supported appliances for oral restoration is increasing (Rokaya et al., 2020). Over the past ten years, the survival rate of these implants has increased dramatically to >90% at 10 years after implant therapy, which has had a beneficial effect on patient satisfaction. However, implant success rates are hampered by inadequate bone volume and quality. Dental implant delivery might be complicated by bone loss brought on by numerous jaw diseases, periodontitis, and trauma (van Velzen et al., 2015).

To solve this problem and raise the success rate of treatment, bone augmentation is usually recommended before implant insertion. Allografts, xenografts, autogenous bone grafts, and alloplastic materials are utilized in bone grafting. Each has benefits and drawbacks of its own. Because autogenous bone

has osteoconductivity, osteoinductivity, and osteogenecity, it is the most commonly utilized and gold standard material (Ferraz, 2023).

Osteoinductive and osteoconductive allografts are obtained from donors within the same species. Donors for xenografts come from several animals, and they have osteoconductive qualities. Alloplastic sources are solely osteoconductive and can come from natural or manmade materials such calcium sulfate, hydroxyapatite, tricalcium phosphate, or ceramics. In dentistry, bone regeneration is frequently performed, particularly to support the growth of dental implant sites (Möhlhenrich et al., 2021, Ashfaq et al 2024).

The risk factors linked to implant failures must be taken into account and assessed both before and after implant loading in order to reduce the possibility of peri-implant illnesses. As far as we are aware, not many research have examined the two distinct graft types-allografts and xenografts-in relation to implant site development (Kochar et al., 2022; DO TA et al., 2020). We hypothesize that there are no appreciable variations in the clinical parameters of dental implant therapy when applying the two kinds of grafts to the development of implant sites.

Probing-induced bleeding, periodontal pocket depth, and connected gingiva were among the clinical parameters that were investigated. Consequently, the purpose of this retrospective secondary analysis was to examine how dental implant therapy was affected by allografts and xenografts by tracking changes in a number of periodontal parameters over the course of two to three years following implantation and functional loading.

MATERIAL AND METHODS

Patients who had titanium dental implants (Straumann, Switzerland) at King Saud University's College of Dentistry in Riyadh, Saudi Arabia, between 2015 and 2018 were the subjects of the study. Periodontal clinical parameters were gathered during routine implant maintenance visits from June 2018 to September 2019 for the patients who were selected at random and made dental appointments. To further rule out any operator-dependent bias, each clinical measurement was performed by a single, blinded, trained, and calibrated researcher.

Patients in the study were systemically healthy but partially edentulous, with one or more missing teeth being replaced by a single crown implant-supported restoration that required intraoral care for a minimum of one to three years at the time of the evaluation session. The same facility was used for prosthesis restorations as well as implant surgery. The exclusion standards were; (1) uncontrolled systemic diseases (e.g., diabetes [HbA1c > 7], osteoporosis); (2) smoking; (3) pregnancy or nursing in women; (4) medication intake that affects bone turnover and mucosal healing (e.g., steroids, anti-resorptive therapy); (5) use of antibiotics for medical or dental purposes within two months of the examination; (6) any restorations that do not allow for the calculation of periodontal pocket depths (PPD); (7) inability or refusal to sign the informed consent form and (8) lack of base line radiographs taken at the time of implant or final crown.

The study variables assessed at the time of examination were; (1) gender., (2) Implant type (Bone Level vs Tissue Level), (3) Type of Graft (Allograft vs Xenograft), (4) Restoration Type (Cement Retained vs Screw Retained), (5) Subtype of Graft (Bioss, Cancellous, Cortical, Mixed), (6) Oral Hygiene (Fair, Good, Poor), and (7) Timing of Graft (With Implant, 2 months before, 4 months before, 5 months before, 6 months before, 7 months before, 12 months before).

The following clinical parameters were evaluated for every implant: Bleeding on probing (BOP) was measured by looking for bleeding at the probing site immediately following the evaluation of periodontal pocket depth, and classifying it as either (+) or (−). Using a plastic probe (11 Colorvue Probe, Hu-Friedy), the periodontal probing depth (PPD) was measured by gently applying pressure (less than 0.25 Ncm) around the neck of an implant at three buccal and three lingual points. The probe was positioned parallel to the implant's crown at the mid-buccal and mid-lingual points, and tilted inward by 10 degrees at the proximal points to the nearest mm.

From the peri-implant marginal mucosa to the muco-gingival junction (MGJ) at the buccal and lingual side of each implant, attached gingiva was evaluated. The attached gingiva was measured using a periodontal probe around the implant. The measurement was made by subtracting the probing depth from the distance between the buccal and lingual marginal portions of the implant's mucosa and the peri-implant marginal mucosa at the muco-gingival junction (MGJ).

In order to confirm the bone level at the same facility, standardized periapical radiographs were taken during the clinical evaluation using the long cone paralleling technique and film holders. These radiographs were then compared to a baseline radiograph taken during prosthesis installation. In order to account for any radiography distortion, the implant's length was used as a fixed reference point during the pixel/mm ratio calibration process. The radiographic distance measured mesially and distally, parallel to the implant's long axis, between the implant shoulder level and the highest coronal bone-to-implant contact level was used to estimate bone loss. The radiography measures were all done by the same blinded examiner.

Data Analysis: The software SPSS 24.0 (IBM Inc., Chicago, USA) was used to analyze the data that was obtained. The variables bleeding on probing, probing depth, and attached gingiva, as well as other categorical study variables like patients, gender, implant type, type of graft, restoration type, subtype of graft, oral hygiene, and timing of graft, were described using descriptive statistics like mean, standard deviation, frequencies, and percentages. The means of the quantitative outcome variable were compared to the categorical study variables using the Student's t-test

for independent samples and one-way ANOVA. The results have been reported with statistical significance at a P-value of less than 0.05.

Ethical Statement / Informed consent: The study was approved by the Institutional Review Board of King Saud University Medical City, Riyadh, KSA (IRB Approval of Research Project No E:87-563).

RESULTS

A total of 188 patients, 103 male (54.8%) patients and 85 (45.2%) female patients aged between 19 to 79 years (mean age=47.7±8.6 years) were included due to sufficient clinical data available. Amongst these 188 patients, total of 151 (80.3%) patients received Allografts and 37 (19.7%) patients received Xenografts. Amongst these selected patients, total of 177 dental implants including 145 (81.9%) implants were Bone level implants and 32 (18%) implants were Tissue level implants. The restoration type placed included 42 (29.3%) as Cement retained and 101 (70.6%) cases had Screw retained final restorations. For the rest of the cases the data was not available regarding the restoration type. Regarding the Sub type of graft materials, Oral hygiene status and Timing of the placement of graft materials are presented in Table 1.

Table 1. Demographic data of the participants of the study.

Gender	Male			Female			Total
	85 (45.2%)			103 (54.8%)			188 (100%)
Implant Type	Bone level			Tissue level			Total
	145 (81.9%)			32 (18.0%)			177 (100%)
Type of Graft	Allograft			Xenograft			Total
	151 (80.3%)			37 (19.7%)			188 (100%)
Restoration type	Cement Retained			Screw Retained			Total
	42 (29.3%)			101 (70.6%)			143 (100%)
Sub Type of Graft	Bioss	Cancellous		Cortical	Mixed		Total
	54 (30.5%)	14 (7.9%)		17 (9.6%)	92 (51.9%)		177 (100%)
Oral Hygiene	Fair	Good		Poor			Total
	75 (39.9%)	103 (54.8%)		10 (5.3%)			188 (100%)
Timing of Graft	With Implant	2 months before	4 months before	5 months before	6 months before	7 months before	12 months before	Total
	62 (33.0%)	7 (3.7%)	9 (4.8%)	1 (.5%)	50 (26.6%)	46 (24.5%)	13 (6.9%)	188 (100%)

Comparison of the clinical parameters, Bleeding on probing (P=0.062), Probing depth (P=0.225) and Attached gingiva (P=0.835) within the two types of grafting procedures (Allograft and Xenograft), by independent samples T test revealed non-significant differences among their means values (Table 2).

The independent samples T test revealed non-significant differences between the means values of the clinical parameters, bleeding on probing (P=0.921), probing depth (P=0.889), and attached gingiva (P=0.906), within the two types of implants (Bone Level and Tissue Level) (Table 3).

When the two types of final restorations (cement retained and screw retained) were compared using the independent samples T test, non-significant differences were found between the means of the clinical parameters of bleeding on probing (P=0.177), probing depth (P=0.897), and attached gingiva (P=0.629) (Table 4).

Comparison of the clinical parameters, Bleeding on probing (P=0.018), Probing depth (P=0.027) within the four types of grafting materials used (Bioss, Cortical, Cancellous and Mixed), by One way Anova test revealed significant differences among their means values. While for the Attached gingiva (P=0.896) the statistical differences were non-significant (Table 5).

A one-way Anova test comparing the clinical parameter, bleeding on probing (P=0.831), among the patients' three oral hygiene groups (fair, good, and poor) showed no

statistically significant variations in mean values. However, there were statistically significant differences for the probing depth (P=0.000) and attached gingiva (P=0.000) (Table 6).

Table 2. Comparison of the clinical parameters within the type of graft.

	Type of Graft	N	Mean	Std. Deviation	Std. Mean Error	Mean Difference	*Sig. (2-tailed)
Bleeding on Probing	Allograft	138	1.40	.884	.075	-0.304	0.062
	Xenograft	37	1.70	.845	.139		
Probing Depth	Allograft	151	4.56	2.087	.170	0.455	0.225
	Xenograft	37	4.11	1.807	.297		
Attached Gingiva	Allograft	148	1.62	.936	.077	-0.036	0.835
	Xenograft	35	1.66	.765	.129		

*P value was significant at P<0.05.

Table 3. Comparison of the clinical parameters within the type of Implant.

Type	Implant	N	Mean	Std. Deviation	Std. Error Mean	Mean Difference	*Sig. (2-tailed)
Bleeding on Probing	Bone Level	135	1.46	.870	.075	-0.016	0.921
	Tissue Level	40	1.48	.933	.148		
Probing Depth	Bone Level	145	4.46	2.075	.172	-0.050	0.889
	Tissue Level	43	4.51	1.932	.295		
Attached Gingiva	Bone Level	141	1.62	.899	.076	-0.019	0.906
	Tissue Level	42	1.64	.932	.144		

*P value was significant at P<0.05.

Table 4. Comparison of the clinical parameters within the type of Final Restoration.

	Type of Restoration	N	Mean	Std. Deviation	Std. Error Mean	Mean Difference	*Sig. (2-tailed)
Bleeding on Probing	Cement Retained	84	1.37	.954	.104	-0.180	0.177
	Screw Retained	91	1.55	.806	.085		
Probing Depth	Cement Retained	87	4.49	2.085	.224	0.039	0.897
	Screw Retained	101	4.46	2.008	.200		
Attached Gingiva	Cement Retained	86	1.66	.835	.090	0.065	0.629
	Screw Retained	97	1.60	.965	.098		

*P value was significant at P<0.05.

According to the frequency of implant thread exposures (Table 7) found in the radiographs, patients treated with allografts (31.8%) and xenografts (35.1%), respectively, exhibited thread exposures ranging from one to four. The findings were essentially identical, with 4 (10.8%) cases exhibiting 3 thread exposures in xenografts and 14 (9.3%) cases in allografts.

DISCUSSION

For oral rehabilitation operations including implants in edentulous parts of the dental arches, the bone grafting technique is considered the gold standard (Debbarma, 2024; Schwarz et al., 2021). Various graft materials, including autogenous graft, allograft, and xenograft, are available for this purpose. Each graft material has special qualities

of its own, and the materials work well for the intended use depending on the specifications. Nonetheless, the long-term prognosis of the procedure varies depending on the grafting material used (Ferraz, 2023; Gallo et al., 2022). Numerous investigations have been carried out to evaluate the effectiveness and efficiency of different graft materials in relation to implant survival and success rate (Win et al., 2024).

Table 5. Comparison of the clinical parameters within the sub type of Graft material.

	Sub Type of Graft	N	Mean	Std. Deviation	Std. Error Mean	*Sig.
Bleeding on Probing	Bioss	41	1.61	.862	.135	0.018
	Cortical	20	.95	.945	.211	
	Cancellous	22	1.73	.827	.176	
	Mixed	92	1.45	.856	.089	
Probing Depth	Bioss	54	3.93	1.703	.232	0.027
	Cortical	20	3.90	2.174	.486	
	Cancellous	22	4.77	2.137	.456	
	Mixed	92	4.85	2.096	.219	
Attached Gingiva	Bioss	52	1.62	.867	.120	0.896
	Cortical	19	1.68	.946	.217	
	Cancellous	22	1.50	1.058	.226	
	Mixed	90	1.66	.889	.094	
*P value was significant at P<0.05.						

Table 6. Comparison of the clinical parameters for the Oral Hygiene of the participants.

	Oral Hygiene	N	Mean	Std. Deviation	Std. Error Mean	*Sig.
Bleeding on Probing	Fair	69	1.46	.867	.104	0.831
	Good	96	1.48	.894	.091	
	Poor	10	1.30	.949	.300	
Probing Depth	Fair	75	6.03	2.033	.235	0.000
	Good	103	3.27	1.148	.113	
	Poor	10	5.20	.422	.133	
Attached Gingiva	Fair	73	1.44	1.080	.126	0.000
	Good	103	1.67	.692	.068	
	Poor	7	3.00	.000	.000	
*P value was significant at P<0.05.						

188 patients who received allografts and xenografts prior to implant placement at various intervals and who had their implants serviced for two to three years were enrolled in the current retrospective research study. The patients were screened, examined, and compared for the effects of these graft materials on some of the critical clinical parameters, such as bleeding on probing, probing depth, and attached gingiva, which are important to the success of these dental implants.

For bone regeneration, graft materials such as allografts and xenografts are utilized. These two biomaterials have benefits as well as drawbacks (Ferraz, 2023; Gallo et al., 2022). The two types of freeze-dried bone allografts that are most commonly used are demineralized (DFDBA) and freeze-dried bone allografts (FDBAs) (Win et al., 2024).

Because DFDBAs are demineralized, it is believed that they contain bone morphogenetic proteins, which may have osteoinductive qualities (Grassi et al., 2020). Xenografts, which are derived from non-human animals, include osteoconductive qualities. Deproteinized bovine bone mineral (DBBM) is a frequently utilized xenograft, as it has a gradual replacement rate that helps preserve tissue volume during bone regrowth (Li et al., 2000; Rodriguez & Nowzari, 2019). Allografts possess osteogenic, osteoinductive, and osteoconductive properties; nevertheless, if utilized exclusively, bone remodelling may be substantial.

Additionally, because there are two surgical sites, there is a larger risk of morbidity, and there may be limited bone supply (Baldwin et al., 2019). Consequently, a few of the physicians combine various kinds of bone grafts. The most popular combination combines the biological characteristics of an autogenous graft with the gradual resorption of mineralized xenografts; this is achieved by using mixed autogenous bone and xenograft material (Janjua et al., 2022).

In the present study, the clinical outcomes and radiographic findings acquired using the two types of allografts i.e., allografts and xenografts in patients who received dental implants revealed comparable results. This has also been reported in some previous research studies (Ferraz, 2023; Gallo et al., 2022; Janjua et al., 2022; Zhao et al., 2021). However, some studies have reported more new bone formation and less residual graft for allografts as compared to the xenografts cases. The difference between the allografts as compared to the xenografts resorption depends on the pore size, pore morphology, pore percentage, connection between pores, pore connectivity, and granulometry (Janjua et al., 2022; Zhao et al., 2021).

When compared with naturally healed sockets, grafting bone decreased the relative proportion of vital bone, but enhanced new bone formation. It is possible to shorten the treatment time between bone grafting and prosthetic completion in implant-site development using grafted bone (Chang, 2021).

Recently performed randomized controlled clinical trials (RCTs) using allografts for dental implants revealed a great regenerative potential and resulted in 38.42% of newly formed mineralized tissue in sites treated with demineralized freeze-dried bone allograft (DFDBA) (Stumbras et al., 2020). Meanwhile the same study using mineralized freeze-dried bone allograft (FDBA) for post-extraction sockets resulted in 24.63% of newly formed bone months after grafting. Another RCT analyzing FDBA with 38 extraction sockets showed favorable and similar regenerated bone results after 6 months (Stumbras et al., 2020).

In the present study three and a half percent of the patients treated with allografts and xenografts, respectively, had implant thread exposures ranging from one to four. This information was obtained from the frequency of implant thread exposures detected using the radiographs. With 14 (9.3%) cases in allografts and 4 (10.8%) cases in xenografts exhibiting 3 thread exposures, the results were almost the same. However, more research is needed to compare the correlation between different grafting materials and the thread exposure of the dental implants (Azadi et al., 2025).

The current study has a number of limitations, the most significant of which are the inherent restrictions of retrospective research and the uneven outcomes resulting from the use of various methodology tools and indices. Furthermore, the absence of surveillance resulting from the retrospective design may impede the identification of a genuine correlation between the variables under investigation and the outcomes being assessed. Further limitations can be attributed to the fact that the current sample was drawn from a single institution, making it impractical to extrapolate the results to the entire implanted patient population. The analysis might have been impacted by the various grafting materials used. Lastly, there were no data on bone morphology, which could have influenced how the implant site remodeled during grafting and, ultimately, how the implant procedure turned out. Future research should also look into the impact of additional variables on implant site development when employing xenografts of different origins. Additionally, more research is required to determine how the variations found in this study would affect the longevity of dental implants.

CONCLUSION

Both grafting materials i.e., Allografts and Xenografts, are suitable graft materials for grafting of the implant site before implant placement. This means that both graft materials resulted in predictable outcomes and effective graft materials in terms of dental implant site development. Analysing and contrasting the clinical parameters bleeding on probing, probing depth, and attached gingiva in Allograft and Xenograft, Bone Level and Tissue Level implants, cement retained and screw retained final restorations, four types of grafting materials (Bios, Cortical, Cancellous, and Mixed), and the three oral hygiene groups (fair, good, and poor) of the patients did not exhibit statistically significant differences in mean values.\\Nevertheless, given the limitations of this retrospective investigation, these findings should be interpreted cautiously. In the future, a clinical trial will need to be used to investigate the connection between additional variables and transplant materials. By lowering the risk of peri-implant illness, we can provide a satisfactory implant outcome through meticulous treatment planning, ideal restoration design, and frequent follow-up visits.

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Global Change Driven Effects of Climate on Food Security of the Indigenous Population of India: A Quality Systematic Review

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ABSTRACT

This qualitative systematic review aims to understand the effect of global climate changes on the food security of the indigenous people (IP) of some areas of India. In India, there are 705 official and recognized ethnic groups, however, it is estimated that there are more ethnic groups that would qualify for the status of Scheduled Tribes but have not been officially recognized yet. Since the beginning of civilization, ethnic groups have helped protect biodiversity in and around their natural habitats in significant ways. As part of in situ biodiversity conservation and ecological restoration, endemic tribal people keep endemic plants and wild types in sacred groves that were once used for shifting agriculture but are now empty. Indigenous and ethnic peoples have adapted to the worst natural conditions. It has been found that the most interesting thing about these indigenous and ethnic groups is that they live in places with rich biodiversity. Moreover, indigenous people's livelihood and food security are climate- driven and they are keen observers of any change in the same. Climate change and severe events may exacerbate the vulnerability of indigenous populations. It is worth mentioning that the main challenges that are already encountered by the poor IP include economic and political exclusion, abuse of human rights, unemployment, discrimination, and resource depletion. A qualitative systematic review has been performed in this study. The search strategy, PICO model, inclusion and exclusion criteria, and data extraction table have been used for a methodological analysis of the research. The impact of climate change on land, wildlife, and water can be detrimental to the IP's traditional ways of livelihood, and food security. The residents of this specific community were further compelled to transfer and live in the areas where their ancestors had a site for thousands of years to minimize the detrimental effects. It affects their social capital, food security, water supply, sanitary conditions, mental health, ability to fight off infectious diseases and injuries, and availability of healthcare directly or indirectly. These people have a close interaction with the environment and its resources therefore, they are the first to experience the direct effect of climate change. It can be concluded that any change in climate affects the food security of IP in India.

KEY WORDS: GLOBAL, CLIMATE, CHANGE, FOOD-SECURITY, INDIGENOUS-PEOPLE ,

INTRODUCTION

Indigenous people (IP) are separate cultural and societal groups that have common ancestral ties to the natural resources and lands on which they currently reside, were formerly displaced from, or both. The Indigenous food system is based on subsistence farming and food sovereignty. It includes the cultivation, processing, and consumption of food crops that are specific to a geographic location and its origin predates industrial agriculture. The lives of indigenous people are intertwined with the ecology in which they reside. They form symbiotic relationships with the soil, water, and natural resources. Any possible danger to nature exposes species to the threat of food insecurity and existence (Saxena, 2021).

Indigenous people make up approximately 6% of the world's population and they account for approximately 90% of the extremely poor (World Bank Report 2024). The scenario is no different in India according to the census of India, 2011 tribal population constitutes 8.6% of the country's population and National Family Health Survey data, 2015-1016 says 45.6 percent of indigenous members live in the lowest wealth bracket. It has been found that natural resources and land on which the IP depend are indistinguishably linked with their cultural livelihood identities along with their spiritual and physical well-being. Indigenous peoples are communities

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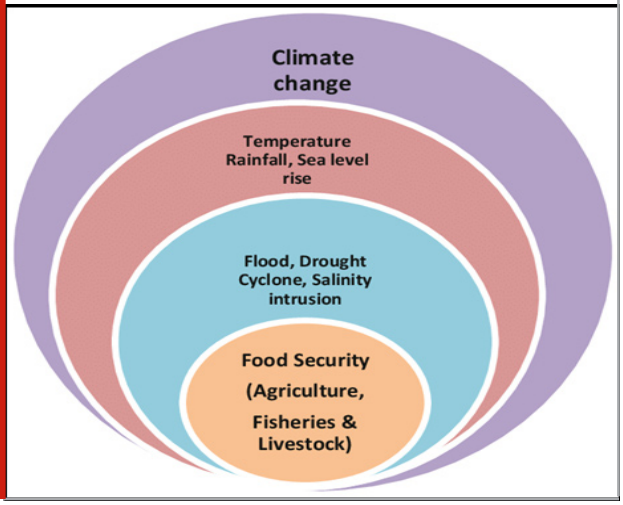
with their languages, cultures, and beliefs, who have a special connection to the land and natural resources where they live. They are often distinct from the dominant societies in which they live.

Indigenous people often have their own language, which is different from the official language of the region. They have unique knowledge systems and practices for managing natural resources sustainably. Interestingly, indigenous peoples have a special connection to their ancestral land, which is important for their survival; they have a historical connection to the region they live in, dating back to before colonization. The United Nations has indicated a "code-red" making climate change an existential threat to humanity (Intergovernmental Panel on Climate Change, Sixth Assessment Report, 2021).

The world is rapidly warming and the consequence manifests in an intensification of floods, droughts wildfires, and other traumatic exposures. The marginalized and vulnerable sections like the Indigenous people have contributed the least to climate change but historical discrimination, political disenfranchisement make surviving climate change and thriving within it more challenging (Barnwell et al.,2022).

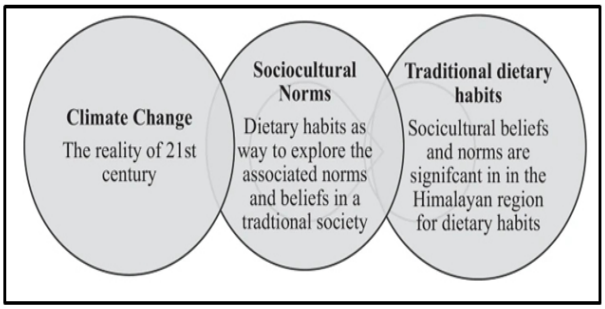
As the IP have their dependency and close interaction with the environment and its resources, they come the first to feel the direct effect of climate variation. It is worth mentioning that the main challenges which are encountered by the poor IP include economic and political exclusion, abuse of human rights, unemployment, discrimination, and resource depletion. As per the observation of Thomas et al., (2019), extreme weather occurrences are a prime example of how unevenly people are affected by climate change, even within relatively limited geographic areas. These challenges become worse due to climate change. It is worth mentioning that climate change causes danger and threat to the survival of the IP of India even though they contribute little to no greenhouse gas emission.

Figure 1: Link of Climate Variation and Food Security (Source: Fanzo et al., 2018)



According to the report of Global Food Policy 2022, a warning has been issued stating that the climate change will push the Indian people towards hunger by the end of 2030 due to the decline in agricultural production and food supply chain disruption. According to the findings of Fanzo et al., (2018), the climatic change significantly impacts the food system because it affects the way of food production and consumption. The main aim of food security is to ensure that people at all times have social physical and economic access to safe nutritious and sufficient food which made their dietary needs and food preference for a healthy and active life. In the context of IP of India, they eat nuts, fruits, roots, seeds, vegetables, and grasses.

Figure 2: Impact of Climate Change on socio-economic norms and traditional dietary habits (Source: Das and Mishra, 2022)



Problems faced by the tribals of India due to climate change: One of the greatest problems of modern society is climate change. It threatens the security and lives of the vulnerable sections of societies worldwide. Carroll and Noss (2021), in the context of India, stated that the local population specifically those who live near the high-risk prone zones such as mountain regions and coastal areas are expected to face high unpredictability due to the change in the climate. It is worth mentioning that the local health beliefs and Indigenous knowledge act as a shelter which helps to prohibit and prompt the responsiveness and sensitivity towards climate instability. Henceforth, ensuring nutritional and food security is one of the primary tasks of the strategy makers and the government of India.

In India, there are 705 official and recognized ethnic groups, however, it is estimated that there are more ethnic groups that would qualify for the status of Scheduled Tribes but have not been officially recognized yet. It is estimated that many IPs keep a language distance from the original languages or languages of the region of the country in which they reside. However, many have either lost their native languages or are in danger of going extinct because of being uprooted from their homes and/or relocated to other areas.

It has been estimated that the dietary habits of the tribal community in the Himalayan region are changing because of climate change. It is a new norm in the 21st century. According to many studies conducted both natural and anthropomorphic factors are responsible for climate and environmental change. In addition, the anticipated impact of climate variability creates a severe impact

on the food security of the vulnerable communities or IPs who are deciding near the mountain areas and coastal regions. It has also been estimated that the change in climate on the natural ecosystem creates a higher possibility of increased incidence of inadequate rainfall, unpredictable change in temperature, and forest fire. It is worth mentioning that a prolonged period of climatic change might result in disrupting the seasonal precipitation rates and water availability, impact the mountain ecology, and affect food security.

It has been found that the impact of climate variability differs among different and unprivileged communities and distinct social groups. The distinct social groups and unprivileged communities are the most vulnerable ones. According to the research of Das and Mishra (2022), in the context of Indian IP, it has been found that most of the Himalayan population lives in rural areas and with lack of proper communication and connectivity with the real world. It has been identified that most food security gets heightened at times of drastic climate changes and adverese environmental events. It highly creates a negative impact on the food and nutritional intake of these IP communities in India.

Climate change not only impacts food production but also reduces freshwater resources. According to Rizal and Anna (2019), climate changes that are induced by natural calamities can result in food insecurity. Some of its examples are an increase in drought, agricultural labor migration, joblessness, and high food insecurity. Moreover, the lifestyle of indigenous people can be severely impacted by environmental change. For example, the declining knowledge of ethnobotanical species has resulted in a reckless increase in the use of natural resources.

Similarly, Das and Mishra (2022), stated that climate change can also impact the traditional living way of tribal and vulnerable communities such as animal herding. Animal herding has been considered to be one of the most common customary practices in the herding community and exists in Mountain regions. All of these factors and understanding that the change in climate and environmental changes directly impact the food security of the vulnerable section or tribal communities of India.

Impact of Climate Change on the food security of Indian IP: The tribal community and IP of India face a substantial ecological issue caused by the change in the climate. The change also includes alteration in the populations of species that are important to their culture and the possibility of entire human settlements being forced to migrate. According to the findings of Bang et al., (2018), these challenges the tribal people to remain concerned for collective continuance. Collective continuous refers to the capacity of the community to adapt different sufficient ways to flourish the livelihood of its members into the future. Moreover, adaptation refers to the adjustment made by the population in response to the anticipated changes.

According to Nursey-Bray et al., (2018), it has been considered that the indigenous people are the worst affected society by climate change. The effects of changing climates such as frequent droughts, hurricanes, burning temperatures, plagues and diseases, and floods make the survival of IP difficult. At the same time, it also highly influences their food security because climate change directly impacts the agriculture and availability of food. As the IP is mostly dependent on Natural sources therefore their food chain is highly interrupted by climate or environmental changes.

However the failure to understand and recognize the responses and challenges faced by them exacerbates their suffering. Moreover, as per the observation of Patil (2019), every step of their lives is impacted by disadvantages and prejudice regarding climate change. It is worth mentioning that the close relationship of IP with the natural environment and resources makes them highly sensitive to the effects of global warming (Kumar et al., 2020). In some cases, it has been identified that the existence of many people and tribal communities is threatened by climate change and by the fast-expanding production of biofuels, which are being promoted as a "solution" to the challenge.

The Bhil has been the largest tribal group in India. This ethnic group is found in Gujarat, Chhattisgarh, Karnataka, Madhya Pradesh, Rajasthan, and Andhra Pradesh. As per Saxena et al., (2019), the major climate change that is faced by this community is a drought. In addition to the context, it has been found that in many parts of India, the lack of monsoon creates a water shortage, which results in poor yields. The major drought-prone areas of India are found to be Northern Karnataka, Andhra Pradesh, Telangana, Gujarat, Odisha, Southeastern Maharashtra, and Rajasthan.

According to D'Cruz et al., (2022), it has been found that most of the Bhils are farmers. However, the continuous pressure of marginal land holdings, subsistence agriculture, debt burden and frequent drought has forced many of these indigenous people to leave their land and switch occupations. Drought causes depletion of water availability in soil which directly impacts livestock productivity. As per Sharma et al., (2020), as most of the people in this community survive on farming therefore drought directly impacts their food accessibility and availability. The lack of food created hunger issues and led the people to starve for many days.

How does IP respond to such climatic change and its negative impact on food security?: The cultural and natural resources of indigenous people in India are impacted by human cause changes and climate changes. As its effect starts to get worse the traditional ecological knowledge system which enables both indigenous and non-indigenous people to discover and adopt various methods to understand the ecological changes. According to the research of McCunn (2021), it is worth mentioning that the Indigenous people tend to live close to the environment and nature rather than people who are growing up in cities. It has been found that the people who live in cities make more food products than they require for survival.

According to the research of Gartaula et al., (2020), this provides the IP section of India with an extraordinarily intimate sense of knowledge of local weather animal and plant life. Over many

centuries, traditional knowledge has been accumulated by the Indigenous people on subjects like when to grow crops and where to go hunting for food. However, as the climate changes, some of this knowledge is now proving to be outdated. Some civilizations are in danger of extinction due to climate change and the rapidly expanding area being planted in biofuel crops.

It is worth mentioning that the capacity of indigenous people to assess and monitor their environment and make decisions, such as whether to plant crops, has also been hampered by the increasingly unpredictable weather. As per the observation of Etchart (2022), they have grown increasingly interested in finding other ways to survive, such as assisting drug traffickers or letting loggers and gold prospectors enter the jungle. The indigenous and peasant groups of India who live in forests are being negatively impacted by the biofuel crops. It has been found that biofuel crops are promoted as a part of the solution to climate change. The land rights, traditional ways of life, and even survival of indigenous community people are threatened or destroyed.

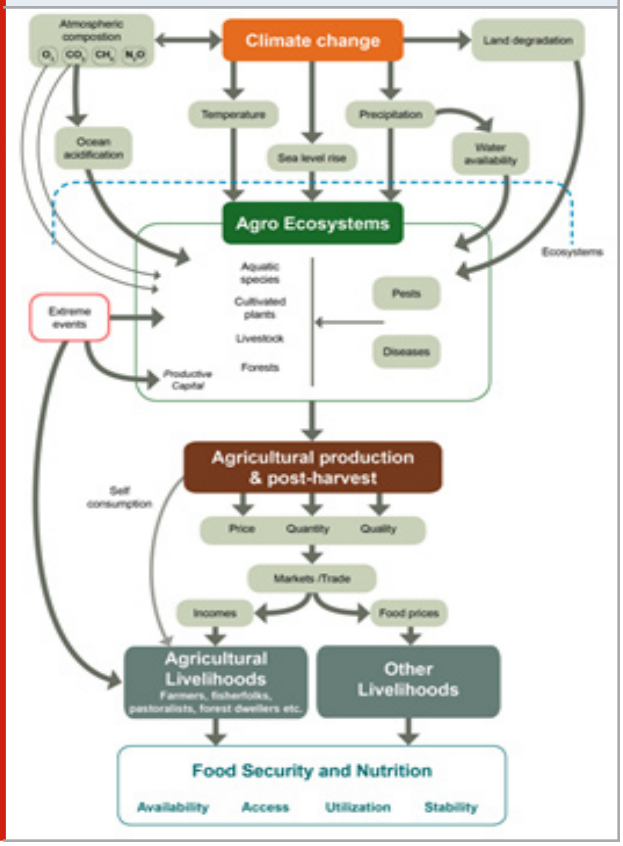
The reason behind persisting climate change-related food security problems: Climate change has been a major reason for the food security in the Indian economy. In addition to this, in referring to, Figure 2 keeping in mind the 'availability', 'ability', 'utilization' as well as 'sustainability', the formidable problems are still prevailing thus, sustainable practice of agriculture is been carried out by putting greater emphasis on the public health and food security. According to the perspective of Duchenne-Moutien and Neetoo, (2021), "Sustainable development goals" adopted by the Indian government are targeting ending hunger and achieving food security by improving nutrition. Climate change has had a huge influence on our food chain. It has an impact on how we produce and consume food. This influence is magnified in a primarily agricultural country such as India, causing ripple effects across the food system. India is seeing its warmest days in recent memory. The continuing heatwave has caused considerable harm to agriculture as well as food security (Outlookindia.com.,2022).

It has harmed the wheat crop therefore harming food production, making the cost of wheat flour to skyrocket. Furthermore, it should be viewed despite the reality that food security seems all about quantity but about nutritional quality. However, the Indian economy failed to achieve the development goals for the millennium and resulting in a proportion of individuals suffering from hunger.

Additionally, the associated problems related to climate change are increasing gradually and calls out for urgent action to be taken for allowing enough time to build resilience in the food production system of India. As seen in the figure above, climate change poses a continuum of threats to agribusiness, agricultural productivity, economically and social ramifications, including, national food security.

According to Munaweera et al., (2022), on the farm, the production of the field crop might rise, or decline based on herbicides fighting for nutrients and water, as well as corrective farming measures. Pests, as well as illnesses, are anticipated to spread because of climate change, emerging in places that are less equipped for them, both biologically as well as procedurally, with possibly greater terrible impacts (Fao.org., 2019). These increased hazards to farm productivity immediately result in increased threats to the achieving food security of those who depend on traditional agriculture for food as well as a living. Thus, these individuals can influence faraway communities' food security as well as nutrition via price fluctuations along with interrupting commerce.

Figure 3. The cascading consequences of climate change on food security as well as nutrition are depicted schematically (Source: Fao.org., 2019)



Drawbacks and Difficulties faced by the IP while responding to climate change and food security: Concern regarding ensuring food security, especially for the vulnerable communities has grown as a result of the threat of climatic change and global environmental changes such as land cover, change in availability of water, cycling, and altered availability of nitrogen. According to the views of George and McKay (2019), it is worth mentioning that there is also growing worry that meeting the cost of food demand from a rise in population and shift of that reference would result in more environmental degradation, native vegetation loss, and more agricultural intensification. This might also impact the food security and food system of the indigenous people. As per the observation of Zurayk (2020), food security is not only concerned with the availability of food but also the accessibility and utilization of food. However, it has been found that climatic change can impact food security and food availability. As indigenous

people are the closest to the environment and nature therefore their food security is highly impacted by climatic change.

In addition, it has been found that short-term adaptation activities are underway, but capacity, constraints, and resources limit the implementation of the long-term strategies. It is important to note that some mitigation measures have unpleasant direct and indirect consequences for the IP community. For illustration, some initiatives for agriculture may reduce the emission of greenhouse gases but also lead to an increase in the plantation of monoculture crops and have been identified to be linked with the decline in food security and biodiversity.

As per the views of Mukhopadhyay et al., (2021), it can be said that to prevent these plans from having a negative impact on IP communities, it is essential that these groups fully and effectively participate in the development of the mitigation measures that the state develops.

As climate change direct impact, the food security of the indigenous people, an issue of child and maternal nutrition in these community suffers. According to the observation of Noll and Murdock (2020) food security and food sovereignty for the IP includes the principles of effective management and proper use of nutrition and food resources. It is important to note that in order for this to happen, traditions and cultural values are highly required to be understood for safeguarding local food systems and ensuring that vulnerable women and children receive enough nourishment.

As per the views of Drost (2019) when Indigenous values for holistic health are honored, including the emotional, spiritual, and mental components, as well as physical health status, this successfully happens with self-determination and can result in health improvement. It has been observed that the traditional values of IP recognize that the ecosystem food diversity and gender parity contribute to the provisioning of food security. Similarly, according to the findings of Domingo et al., (2021), the value for nutrition, protection, and care of kin and their natural resources are a consistent thread in the indigenous society is a special issue. On this note, it can be said that policies to protect and recognize the right of the IP to their food security and land will benefit human society.

Nalau et al., (2018) found that IP play an important role in many ecosystems as they stay active in their territories and land and thus help to enhance the resilience of these ecosystems. In addition, the IP of India understands and responds to the effect of Climate Change in very innovative ways such as relying on their traditional piece of knowledge and technological interventions to find reliable solutions that might help the Indian society to cope with the challenge. Due to food insecurity, the IP was forced and chose to migrate away from their traditional, and due to this they often face double discrimination as indigenous people and migrants.

According to Agarwal (2018), one of the biggest drawbacks of climate change and food insecurity is that indigenous people become more vulnerable to irregular migration such as due to abrupt displacement caused by a catastrophic or climate change event, limited legal migration alternatives, and little opportunity to make educated decisions, people of this community are more susceptible to crimes like trafficking and smuggling. It has been observed that people who belong to the indigenous communities are being forced to move to cities for economic reasons by deforestation, particularly in emerging nations. These families frequently end up in urban slums.

The aim and objectives of this research is to understand the effect of variations in climate on the food security of the IP of India. The objectives that are set by the research analyst for this study are defined below: To understand the major problems faced by the Indigenous people of India due to climate change, to analyze the impact of Climate Change on the food security of Indian Indigenous people? to investigate how Indigenous people respond to such climatic change and its negative impact on food security and to find out the drawbacks and difficulties faced by the Indigenous people while responding to climate change and food insecurity.

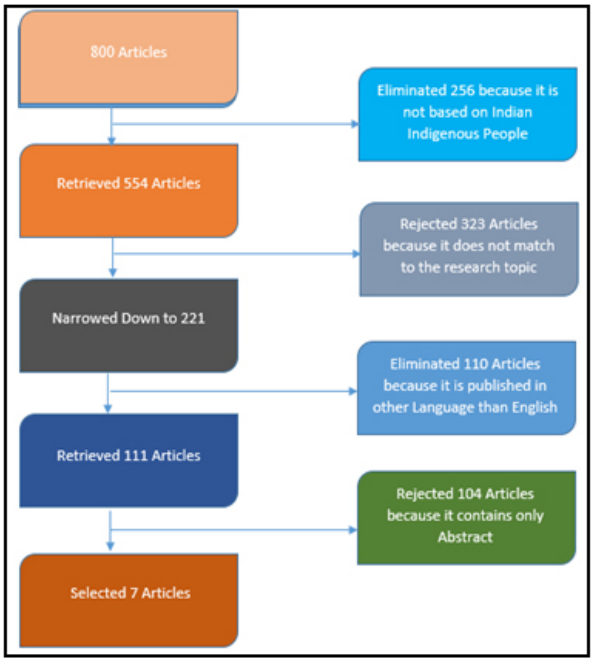
This systematic review further aims to bring together all the sources of evidence on the effect of Climate Change on the food security of Indian IP. From this systematic review, readers can expect to gain an in-depth analysis of the major impact of food insecurity caused by climate change on the IP of India. Moreover, it also had a special emphasis on understanding the major factors and challenges faced by the local tribes or communities and Adivasis to survive the drastic change and the hit on their food security.

METHODOLOGY

According to the research subject or study area, the researcher has initially gathered a total of 800 publications. Following an analysis of these 800 studies, it was discovered that 256 of them did not consider the indigenous populations of India. As the studies that focused on India's indigenous people were expressly excluded from the selection criteria, the researcher disregarded these 256 papers. Out of 800 items, 256 were discarded, leaving 554 behind. However, the analysis of these 554 papers revealed that 323 did not correspond to the research subject. These kinds of articles are eliminated for this specific purpose, leaving only 221 items in total.

Following a study of the remaining articles, it was determined that 110 of them did not meet the selection criteria since some of them were not written in English. These 110 articles have been removed from the study to preserve the validity and trustworthiness of the research. The fundamental goal of this elimination was to guarantee the veracity and accuracy of the outcomes. The total number of studies has now been reduced to 111 publications, although 104 of them simply have abstracts and do not provide a thorough understanding of the study. This specific reason led to the elimination of these 104 papers from the research as well. Consequently, just 7 publications were left on which to base the findings, as shown in the Prizma Flow Chart (Fig No 4).

Figure 4. PRISMA Flowchart



RESULTS

Das and Mishra, (2022): This study includes the participants of the indigenous people who live in the Himalayas. This research examines the eating practices of the indigenous people during climate-related catastrophes. It is worth mentioning that the goal of this systematic review is to evaluate indigenous eating habits that are significant to the culture and socio-ecological dynamics to promote sustainable consumption patterns (Das and Mishra, 2022).

The study revealed that man-made activities such as overpopulation, pollution, burning fossil fuel and deforestation have a high impact on the degradation of Himalayan areas which makes the residents of that vertical area more susceptible to climate variations or changes. This study also replied that a complete understanding of the local socio-cultural attitudes related to food choices is necessary for inclusive and responsive climate change initiatives (Das and Mishra, 2022).

El Bilali et al., (2020): This study has been conducted with the view of understanding the impact of Climate variation on the food security of vulnerable people. The study highlights the challenges faced by indigenous people due to the continuous chemical changes. The author reveals that climate change has a negative impact on the food security and food availability of the vulnerable people of India. It is a qualitative study that reflects that the accessibility of food has been impacted by factors like food availability, food production, and effects of extreme weather occurrences (El Bilali et al., 2020).

It is worth mentioning that the food usage and dietary habits of the indigenous group change due to the variation made in the production system brought by the change in the climate. At the same time, long-term food security has also been found to be impacted by the impact of changing climate on the stability of the food system and its resilience. Additionally, the pursuit of food security increases greenhouse gas emissions from deforestation and land use changes by increasing agricultural intensification and agricultural area expansion (El Bilali et al., 2020). Because of the complex relationship between food security and climate change, there is a need for integrated policies that maximize co-benefits while resolving trade-offs.

This study by Bilali reveals that the indigenous people who live in such conditions has a high rate of poverty and hunger because of the adverse effect of Climate variation on food security. The author has also reflected that the degradation of climate and the utilization of food has also impacted the nutritional status of the population, especially in the context of poor and Indigenous people (El Bilali et al., 2020). The change in climate results in increasing temperature, reducing the quality of water, and hygiene habits special in semi-arid and arid areas. It also increases the burden of diseases such as diarrhea among the children of the indigenous community. This qualitative research has also stated that global warming is one of the causes of malnutrition because it negatively impacts the nutrition and food security of vulnerable groups such as children and women.

Patil, (2019): This study was conducted on the tribal population living in the Satpuda Mountain Region of Jalgaon District. This particular research has been conducted by Patil to understand the issues of food security faced by the IP who resides in the Satpuda Mountain Region of Jalgaon District. The results of the study are based on both primary and secondary results. The study reveals that this area is not able to produce enough food to suit the needs of the local people. Food availability has generally been dropping in the study region. Due to a decreased percentage of land being used for agriculture and a growing population, there is less food available in the Satpuda region (Patil, 2019).

Food security is severely hampered by the significant yearly variations in the supply of food grains brought on by the frequent occurrence of droughts and the uneven distribution of rainfall across regions. This research states that the government must protect the tribes' agricultural land to ensure their food security. It can be said that the primary source of the poor tribal people's food security is the land. They should not lose their government, as this would harm their economic situation. It is worth mentioning that if they are required to be relocated, then they must be given the same quality of land and housing (Patil, 2019). It has been found that the Public Distribution System (PDS) cannot provide enough food and all forms of nourishment on its own. As a result, the government need to make the necessary provisions so that people can produce their own food close to home and save money on transportation.

Sebastian (2019): This study is based on the Baiga Tribe in India. The primary aim of the study is to understand the use of Sustainable resource management with the help of indigenous knowledge and practices on the food security of the Baiga Tribe in India. It is a qualitative study and the main aim of the study is to prove that indigenous knowledge and practices are a key to sustainable food security.

The study defines that India has a long history of advanced civilization that can be traced back to its ancient past and ancient Indian village system. At last, it can be said that this research provides insight into the value of indigenous knowledge in various tribal and rural people's efforts to manage forests and local resources sustainably. According to the results analyzer, the importance of indigenous knowledge as a tool for development is strengthened by both grassroots community activities and securing the regionally recognized patterns of sustenance for the tribe and village communities.

Datt et al., (2022): The primary goal of this research is to understand the technical Innovation and interventions used by the indigenous groups in India for supporting sustainable development. This study considers the weaker section i.e. the tribal and indigenous people of India and the use of medical interventions to improve and support their living conditions. According to this research, approximately 60% of the Indian population directly depends on agriculture and is found to be related to industries for their livelihoods. The study reveals that the two main categories of methodology that may be used by the vulnerable section to evaluate how the change of climate affects their agriculture are partial equilibrium and equilibrium approaches (Datt et al., 2022).

Townsend et al. (2020): This study highlights that a nature-based solution is one of the best methods to eliminate the negative impact of Climate Change on food security and the challenges faced by indigenous people. The foundation of "nature-based solutions" (NBS) is the idea that when ecosystems is healthy and well-managed, they offer crucial advantages and services to people. It helps in lowering greenhouse gas emissions, securing safe water supplies, improving the quality of the air, and boosting food security (Townsend et al., 2020).

Schramm et al. (2020): According to the researcher, climate change directly threatens human health dramatically, such as heat-related morbidity and mortality, exposure to air pollution and particulate matter (PM) 2.5 due to wildfires create a chronic impact on the Indigenous community because they are uniquely vulnerable due to the impact of climate-related events. The climatic and environmental change highly affect the practices, cultural and Physical health, lifeways, and self-sustenance of the indigenous communities.

The study reveals that this indigenous group are leading the way toward innovative health-related climatic change adaptation by using novel approaches and traditional knowledge (Schramm et al., 2020). In this research, the word "Indigenous" has been used interchangeably with the term "tribes". The study reflects that an increase in global temperature and continuous weather changes created a very negative impact on the food security of vulnerable communities.

It is worth mentioning that the climate change can create damage to land, life, and water which negatively affects the traditional practices and food security of the IP. Moreover, to mitigate the negative impact, the people of this particular community were forced to relocate and live in the lands where their ancestors had a site for thousands of years (Schramm et al., 2020). It directly or indirectly impacts their social capital, food security, supply of water, sanitation, mental health, infectious disease, and injury and reduces access to Healthcare.

The major consequences of changing climate include mild distress and stress and mental disorders such as post-traumatic, stress anxiety, and depression. As climate change increases the possible spread of viral infections. The majority of health care needs are met through out-of-pocket expenditure, which further increases the financial misery. According to the researcher the indigenous people have been facing socio-economic disparities and healthcare barriers.

The study indicates that the people in this community maintain and foster a subtle and multifaceted relationships between the natural world and the human world. Due to their close connection with the land, they experience heightened impact of climate change. According to the study indigenous community are now using novel approaches and traditional knowledge to support the fragile ecosystem and mitigate the hazards.

DISCUSSION

The first article focuses on the indigenous group who live in the Himalayas. The main goal of this systematic review study is to describe the indigenous group's eating habits and how they are impacted by natural and environmental changes (Das and Mishra, 2022). Moreover, this study has reflected that the changes in the social and economic dynamics impact their eating habits and food security.

The shift in the crop cycle has restricted or changed their dietary patterns. The extensive and early melting of glaciers in the Himalayas have hampered fishing and reduced freshwater supplies. Therefore, the researcher states that promoting sustainable consumption and a deeper understanding of the indigenous community's traditional knowledge is crucial to maintaining their well-being and health and combating climate change in this fragile ecosystem.

In the second article, the study's primary outcome revealed that the IP community's dietary pattern had been impacted by the change in the climate by reducing crop yield, fish, and animal productivity. (El Bilali et al., 2020). Reduction in pastoral land and fodder negatively impacts livestock weight. It is worth mentioning that the reduced consumption of food might create a negative

nutritional impact on Indigenous people, such as a reduction in protein, Vitamin D, Omega-3 fatty, iron, and Zinc, (El Bilali et al., 2020). Dietary quality is affected by food resource availability, accessibility, quality, and usability changes. Along with this, another detrimental effect in which climate change impacts food is the reduction in the nutritive value of crops. It increases the danger of food contamination. It has also been found that the change in Climate creates a significant impact on the food safety and nutrition of indigenous people.

The third article highlights the food security challenges faced by the tribal population of the Satpuda Mountain Region of Jalgaon District. It was found that there are many issues of food security faced by the population who resides in the Satpuda Mountain Region of Jalgaon District (Patil, 2019). This study reflects that the mentioned area cannot produce enough food due to erratic rainfall and global warming to suit the dietary needs of the local people, which causes food scarcity in the region. Less food is available in the Satpuda region due to a decline in land used for agriculture and a rise in population. Food availability has generally been dropping in the study region. It has forced the IP of this area to shift to another place. In this scenario, the variation in

Climate has negatively impacted food production, which has led to food shortage in that area. As 40% of the Satpuda hilly tribe live on the periphery of the forest, recent deforestation has further marginalized them. It was observed that there is a decreased percentage of land used for agriculture and a growing population, and less food is available in the Satpuda region (Patil, 2019). The researcher stated that the government must protect the agricultural land of the tribe to ensure their food security. If they are required to be relocated, they must be given the same quality of land and housing.

The fourth article provides insight into how the one of oldest tribes of Central India is pushed to the brink of extinction. The market-driven forces have unsustainably extracted forest resources reducing the meagre available food sources. Moreover, the administrative policy “The Indian Forest Act, 2006” alienated the Baiga from their ancestral land. The paper speaks about the value of indigenous knowledge in various tribal and rural people's efforts to sustainably manage forests and local resources (Sebastian 2019).

The study emphasizes group efforts at the grassroots level and securing the locally acceptable patterns of subsistence for the tribal and village communities, reiterating the value of indigenous knowledge as a development instrument. (Sebastian Lakra, 2019The fifth article describes the understanding of the technical innovation and interventions used by indigenous groups in India to support sustainable development (Datt et al., 2022). It has been found that 60% of the Indian population directly depends on agriculture (Datt et al., 2022).

Due to their dependency on Climate, agriculture is highly impacted by climate conditions influencing their food security. It was discovered that partial equilibrium and equilibrium techniques are the two primary kinds of methodology that the vulnerable section may employ to assess how the change in Climate impacts their agriculture (Datt et al., 2022). According to the researcher, these two models can be used by the indigenous section of society to eliminate the adverse impact of climate variation and to support sustainable development.

After analyzing article six, it was found that the NBS is very beneficial in minimizing the negative impact of climate change as global warming on people (Townsend et al., 2020). As NBS provides 30%-40% of carbon dioxide mitigation (Townsend et al., 2020). The NBS method is very advantageous in reducing Global Warming. It suggests that increasing plantations is beneficial to sustain the environmental impact. This method is also reliable for IP to reduce the negative impact of climate variation. It will help to shield the challenges such as displacement due to catastrophic flooding, food insecurity, drought, fire, and threats to critical infrastructure.

The seventh and last article helps us understand that climate change impacted the land, water, and life and is challenging the IP's traditional ways of life and food security (Schramm et al., 2020). The residents of this specific community were further compelled to transfer and live in the areas where their ancestors had a site for thousands of years (Schramm et al., 2020). It affects their social capital, food security, access to clean water and sanitation, mental health, infectious diseases, and injuries spread, and their ability to receive healthcare directly or indirectly. The researcher claims that healthcare and socioeconomic restrictions are issues that indigenous people must deal with.

Discussion of the Key Findings concerning the most recent academic literature

Indigenous social order is highly threatened by an array of climate change consequences. Indigenous knowledge and traditional ways of life are being continuously undermined by actual and projected rates of climate change. Loss of ancient knowledge in the face of rapidly altering ecological conditions, depletion of freshwater sources, and eviction from ancestral lands are a few examples of key vulnerabilities.

The study reveals that although the Indigenous community has made significant progress in comprehending climatic variations, the counteractions to mitigate the effects of the changing environment is still insufficient. It is insufficient because the IP section still faces compounded impacts of historical discrimination, colonialism, current administrative policy issues, and persistent social and economic problems. All these factors need deeper assessment to help build adaptive strategies and combat climate change. As indigenous people live far from the modern world and close to the environment and nature, they directly bear the negative impact of climate variation. It has been found that the variation in Climate has negatively impacted food production, which has led to food shortages in areas.

The food shortage challenges the food security of the IP. In India, most IP are farmers, such as the Bhil Community. Their

livelihood and food security are negatively impacted by climate change. The rising temperatures, frequent droughts, and flood reduces crop fertility and yield. It creates a challenge to the food security of IP. The main findings from the result section are that the Indigenous community is closely associated with nature and the environment.

Therefore, any variation in the climate directly impacts their food security and livelihood. From the academic resources used in this research, it has been found that the variation in climate not only impacts the production of food but also reduces freshwater resources. Climate changes that are induced by natural calamities can result in causing food insecurity. Some of its examples are an increase in drought, agricultural labor migration, unemployment, and high food insecurity. All these factors help in successfully stating that the variation in Climate brings a significant challenge to the food security and health of the Indigenous People of India. Moreover, it also affects their social capital, food security, access to clean water and sanitation, mental health, the spread of infectious diseases and injuries, and their ability to receive healthcare in this community

Recommendations: To minimize the impact of the challenges faced by the IP of India, it can be recommended that the government of India should promote the health and well-being of the tribal communities in India. It has been identified that many of the people of this community live far from the modern world and the reach of technology.

However, the government should take effective steps to ensure their health and safety. As the variation in Climate directly impacts their food security, the government should take steps such as providing health and minimum living requirements to them, such as food, clothing, and shelter. It is important to note that this systematic study reveals that most times, the indigenous group is forced to leave their ancestral land in search of food; in this context, the government should provide them with constant food and accommodation because they are living below the poverty line.

The economic condition of India and the neglect by the policy makers is the challenge of this recommendation. Women and children of this community suffer from hunger and malnutrition because of the unavailability of food.

On this note, the government should provide appropriate Healthcare services and medical camps in such areas to provide them healthcare treatment for free. This research describes several interventions that IP can follow to eliminate the negative impact of environmental changes. The NPOs and NGOs can run effective campaigns and workshops to make them aware of the technical interventions that they can use to minimize the negative impact.

It is worth mentioning that the women of this community should be provided with jobs to make handicrafts. Moreover, this will also give them a different source of earning, which is beneficial to support their living food and food security. Further applications of the research to public health practice.

It is important to note that this research can be used for public health practice because it includes an in-depth analysis of the health challenges faced by the indigenous community due to climate variations. The researcher found that the weather and climate change directly impact the agriculture and food security of IP in India. It results in poverty, starvation, hunger, and malnutrition. This escalates the economic distress and mental health of these vulnerable sections of society.

This research helps understand the significant challenges aligned with public health directly faced by the IP of India. It has been found that the variation in climate results in nutritional insecurity, malnourishment, and wasting amongst indigenous people. As most of the people in this community are farmers, the change in climate impacts their food production. For example, an increase in temperature, drought, flood, and lack of rain damages the food crop, resulting in starvation from hunger. New researchers can use this research study to understand the impact of variation and Climate on the food security of the IP.

Moreover, this study also reveals the significant challenges and interventions that this particular group can implement to reduce the negative impact of climate variation and promote sustainable development. The recommendations of the systematic review are very beneficial to educating the IP section of India regarding the interventions they can use to eliminate the man-made effect on environments, such as global warming through ecosystem preservation or conservation, better forest management techniques, and afforestation.

CONCLUSION

From this research, it has been found that climate change is a big issue that might create an impact on public health. From this study, the research has gained awareness of the serious health challenges that are faced by the IP community in India. It has been identified that extreme weather and catastrophic events change the geographical distribution of forests and the prevalence of food shortage can cause rampant spread of infectious diseases and water-borne illnesses. At the same time, it also negatively impacts the mental health of the people. In the context of IP of India, it has been found that extreme weather conditions such as drought, heavy rainfall, flood, and high temperatures cause a negative influence on their health and well-being.

The health of the people of this community starts to decline because of the major challenges of food security and livelihood. It is important to note that the study highlights the measures of intervention and methodologies that can be used to reduce the negative impact of climate variation and strengthen the response strategy. On this note, it can be said that this might create a significant influence on public health practice. The recommendations that are provided have a special emphasis on

the improvement and future Healthcare practices towards the IP of India.

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Assessment of DNA Purity and Yield in G9 and Malbhog Banana Leaves Using the CTAB Extraction Method

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ABSTRACT
In this study, the cetyl methyl ammonium bromide (CTAB) method is optimized for high-quality DNA extraction from micropropagated banana plantlets, which are rich in polysaccharides, polyphenols, and other inhibitory compounds. Various protocol parameters, including lysis buffer composition, incubation conditions, and purification steps, are systematically modified to enhance DNA yield and purity. The optimized method produced DNA with an A260/A280 ratio of 1.8–2.0, ensuring compatibility with downstream applications such as Random Amplified Polymorphic DNA (RAPD)-PCR analysis. The extracted DNA exhibited high integrity, reproducibility, and minimal contamination, making it suitable for genetic fidelity assessments. Comparative analysis with standard CTAB and alternative extraction methods revealed a significant improvement in DNA quality and yield, while theoretical modeling provided insights into the effectiveness of buffer modifications in minimizing inhibitory compounds. This optimized CTAB-based extraction protocol offers a reliable and reproducible approach for molecular studies, facilitating genetic research and conservation efforts in micropropagated banana plantlets.

KEY WORDS: BANANA PLANTLETS; CTAB METHOD; DNA EXTRACTION; GENETIC FIDELITY; RAPD-PCR,

INTRODUCTION

Banana (Musa spp.) is one of the most important fruit crops worldwide, serving as a staple food and a key economic resource for millions of people, particularly in tropical and subtropical regions. With an annual production exceeding 125 million tons, bananas play a vital role in global food security. Their popularity stems from their nutritional value, ease of cultivation, and ability to thrive in diverse climatic conditions. Most commercially grown banana varieties are sterile triploids or tetraploids, meaning they do not produce viable seeds and rely on vegetative propagation (Evans et al, 2020; Horry et al, 2020). While this ensures uniformity in commercial production, it also makes bananas susceptible to pests, diseases, and environmental stress, highlighting the need for effective propagation and genetic improvement strategies (Jacobsen et al, 2019; Emmanuel, 2025).

To address these challenges, tissue culture has emerged as a widely used technique for propagating disease-free and genetically stable banana plantlets (Suman, 2017). Various studies have demonstrated the successful in vitro regeneration of banana using different explants and culture conditions, leading to improved yield, resistance, and adaptability (Kumar et al, 2024). Efficient micropropagation is crucial not only for preserving banana germplasm but also for developing superior cultivars that meet the growing demands of farmers and consumers (Smith, 1988). Additionally, molecular studies, including DNA-based research, have become essential for genetic diversity assessments, breeding programs, and conservation efforts (Ramesh et al, 2020). However, extracting high-quality DNA from banana tissues remains challenging due to the high content of polysaccharides, mucilage, and phenolic compounds, which interfere with molecular analyses (Turaki et al, 2017).

DNA extraction is an essential process in molecular biology, serving as the foundation for various genetic studies, including DNA fingerprinting, genetic fidelity assessment, and marker-assisted selection (Lázaro-Silva et al 2015, Susantini et al 2017). The quality and quantity of extracted DNA significantly influence the reliability of these analyses. However, extracting pure and high-yield DNA from plant tissues can be challenging, particularly in species that contain high levels of polysaccharides, phenolic compounds, and secondary metabolites, which can interfere with DNA isolation and downstream applications (Xin et al, 2012). One

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of the most widely used methods for plant DNA extraction is the Cetyltrimethylammonium Bromide (CTAB) method (Clarke et al 2009).

This technique is particularly effective for plants with high polysaccharide and phenolic content, as CTAB binds to these contaminants, allowing for the selective precipitation of DNA (Aboul-Maaty et al, 2019). Despite its effectiveness, variations in DNA yield and purity can occur based on the plant species, tissue type, and environmental factors. Thus, it is crucial to evaluate the efficiency of this method in different plant varieties. Optimizing DNA extraction protocols for banana tissues is critical to ensuring accurate molecular studies, enabling researchers to enhance genetic characterization, improve breeding programs, and contribute to the long-term sustainability of banana cultivation (Johari et al 2015).

Tissue culture and molecular techniques offer significant potential for enhancing the yield and quality of G9 and Malbhog banana varieties. To ensure the genetic stability of tissue-cultured plantlets, developing efficient, reliable, and cost-effective DNA isolation methods is crucial. The CTAB-based approach is widely used for plant DNA extraction due to its ability to remove polysaccharides and phenolic compounds, which are abundant in banana tissues (Spadoni et al, 2019, Chukwu, 2025).

In this study, we propose a simplified DNA isolation protocol specifically optimized for G9 and Malbhog bananas. Our results indicate that liquid nitrogen is not required for effective DNA extraction, leading to a more streamlined process that reduces costs, minimizes hazards, and improves efficiency. Additionally, this study aims to establish an optimized regeneration protocol for these banana cultivars while assessing genetic fidelity using RAPD markers. Genetic stability in micropropagated plantlets is a key factor in ensuring quality control for large-scale banana production. Understanding potential genetic variations is essential before commercial distribution to maintain uniformity and reliability. By addressing this critical aspect, our findings contribute to the advancement of sustainable and high-quality banana cultivation.

MATERIAL AND METHODS

Plant Materials: Banana suckers of the two studied varieties were sourced from a local farmer's field. The varieties selected for this study are G9 and Malbhog.

Genomic DNA Extraction: Genomic DNA was extracted from young, fresh leaves of the two Musa spp. cultivars, G9 and Malbhog, using a modified CTAB method as described (Dhanpal et al, 2014). To ensure a representative sample, DNA was isolated from tender leaves of 10 randomly selected micropropagated plants from a single batch, along with the mother plant for comparison. Approximately 500 mg of fresh leaf tissue was finely ground into a powder using liquid nitrogen in a mortar and pestle to break down the cell walls and release cellular contents. The powdered sample was then transferred to a 50 ml sterile centrifuge tube containing 1000 µl of pre-heated CTAB buffer (maintained at 65 °C) and supplemented with 10 µl of beta-mercaptoethanol to help remove proteins and secondary metabolites that could interfere with DNA quality.

The homogenized mixture was centrifuged at 10,000 rpm for 10 minutes to separate the cell debris from the nucleic acids. The clear supernatant, containing the extracted DNA, was carefully collected and mixed with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). This step was performed to remove proteins and other contaminants. The solution was then subjected to another round of centrifugation at 10,000 rpm for 10 minutes to achieve better phase separation. To precipitate the DNA, the aqueous phase was carefully transferred to a new tube, and an equal volume of isopropanol was added. The mixture was stored at – 20°C overnight to enhance DNA precipitation. The following day, the DNA pellet was recovered by centrifugation, washed with 70% ethanol to remove residual salts and impurities, and then air-dried.

The dried DNA was subsequently dissolved in sterile distilled water to ensure purity. To eliminate any RNA contamination, the DNA solution was treated with RNase at a concentration of 1 mg/ml and incubated at 37°C for 20 minutes. The quality and concentration of the purified total genomic DNA were assessed by measuring absorbance at 260 and 280 nm using a spectrophotometer (Vinod, 2004). The integrity of the extracted DNA was further confirmed by electrophoresis on a 1% agarose gel stained with ethidium bromide. The gel was then visualized and photographed using a gel documentation system.

DNA Quality and Quantity Assessment: The concentration of the isolated DNA (ng/µl) was measured using a spectrophotometer by recording absorbance at wavelengths of 260 nm and 280 nm. The purity of the extracted DNA was evaluated by calculating the ratio of absorbance at 260 nm to 280 nm. To assess the quality of the DNA samples, electrophoretic separation was performed. A total of 7 µl of each DNA sample was mixed with 1 µl of 10X loading dye and loaded onto a 0.8% (w/v) agarose gel containing ethidium bromide (1 µg/ml). The gel was submerged in 1X TAE buffer, and electrophoresis was conducted for 60 minutes at a constant voltage of 100 V. Following the completion of electrophoresis, the gel was visualized and photographed using a gel documentation system.

DNA Amplification: Polymerase Chain Reaction (PCR) was performed using a thermal cycler with a total reaction volume of 20 µl. The reaction mixture consisted of 12.8 µl of nuclease-free water, 2.0 µl of 10X Green Taq buffer (Thermo Scientific), 2.0 µl of 2 mM dNTPs (Thermo Scientific), 2.0 µl of random primers at a concentration of 5 pmol/µl, 0.2 µl of Dream Taq Green DNA Polymerase (5U/µl), and 1.0 µl of the extracted genomic DNA.The thermal cycling program was set as follows: an initial denaturation step at 94°C for 3 minutes, followed by 30 cycles consisting of denaturation at 94°C, annealing at 42°C for 1 minute, and extension at 72°C for 1 minute. After completing the 30 cycles, a final extension step was carried out at 72°C for 7 minutes. To assess the efficiency and reliability of

the amplification process, random primers, OPA-1 and OPA-2 and so on (Table 1), were selected for genomic DNA amplification.

Table 1. Details of random primers used for validation of amplification by Polymerase Chain Reaction Reaction using genomic DNA extracted from two varieties of banana at 42 °C.

S. No.	Primer Code	Primer Sequence (5'- 3')
1	OPA-1	CAGGCCCTTC
2	OPA-2	TGCCGAGCTG
3	OPA-3	AGTCAGCCAC
4	OPA-4	AATCGGGCTG
5	OPA-5	AGGGGTCTTG
6	OPA-6	TGGGCGTCAA
7	OPA-7	GGCATGACCT
8	OPA-8	TGGGCGTCAA
9	OPA-9	CCAGCAGCTT
10	OPA-10	GACTGCACAC
11	OPA-11	CAATCGCCGT
12	OPA-12	TCGGCGATAG
13	OPA-13	CAGCACCCAC
14	OPA-14	CTCGTGCTGG
15	OPB-5	TGCGCCCTTC
16	OPB-06	TGCTCTGCCC
17	OPB-07	GGTGACGCAG
18	OPB-8	GTCCACACGG
19	OPC-01	TTCGAGCCAG
20	OPC-02	GTGAGGCGTC
21	OPC-04	CCGCATCTAC
22	OPC-07	GTCCCGACGA
23	OPC-08	TGGACCGGTG
24	OPD-07	TTGGCACGGG
25	OPD-16	AGGGCGTAAG
26	OPM-16	GTAACCAGCC
27	OPM-20	AGGTCTTGGG
28	OPN-03	GGTACTCCCC
29	OPN-09	TGCCGGCTTG
30	OPN-10	ACAACTGGGG
31	OPA-16	AGCCAGCGAA
32	OPA-17	GACCGCTTGT
33	OPA-18	AGGTGACCGT
34	OPA-20	GTTGCGATCC
35	OPB-01	GTTTCGCTCC
36	OPB-04	CGACTGGAGT

RESULTS AND DISCUSSION

A critical stage in molecular research is the extraction of high-quality DNA, especially from plant species like bananas (Musa spp.) that have a high polysaccharide and phenolic content. The capacity of the CTAB approach to efficiently remove impurities and produce high-molecular-weight DNA makes it a popular technique for extracting DNA from plants. DNA from two banana cultivars, G9 (Grand Naine) and Malbhog, was extracted using the CTAB method in this work. The yield, purity, and integrity of the extracted DNA were examined.

Table 2. Total number of purity and genomic DNA concentrations of two variety *Musa* spp.

Sample	Purity (µl/ml)	Concentration (µl/mg)
G9	1.72	151.7
G9	1.79	113.5
G9	1.18	111.7
Malbhog	1.44	36.9
Malbhog	1.0	30.3
Malbhog	0.8	37.9

Figure 1: Genomic DNA amplification pattern of micropropagated Musa spp. (G9) using RAPD primers. LD represents the DNA ladder. (a) Amplification with primers 1–18, (b) Amplification with primers 19–36, applied sequentially as listed in Table 1.

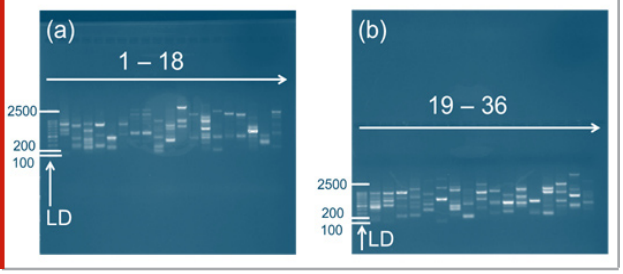
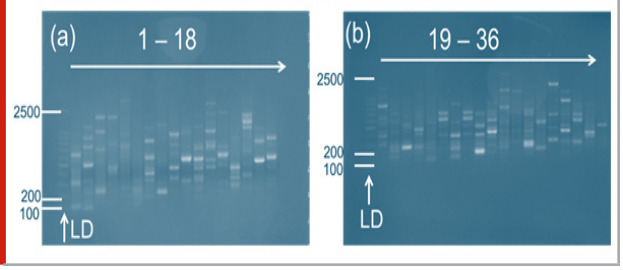


Figure 2: Genomic DNA amplification pattern of micropropagated Musa spp. (Malbhog) using RAPD primers. LD represents the DNA ladder. (a) Amplification with primers 1–18, (b) Amplification with primers 19–36, applied sequentially as listed in Table 1.



Purity and Yield of DNA: The efficiency of DNA extraction from micropropagated banana plantlets was assessed by measuring the purity and concentration of DNA samples obtained from G9 and Malbhog cultivars. The A260/A280 ratio, which shows protein contamination, and the A260/A230 ratio, which shows the presence of polysaccharides

and other organic pollutants, were used to gauge the purity of the extracted DNA.he purity of DNA samples from the G9 cultivar ranged from 1.18 to 1.79, with an average purity close to the acceptable range (1.8–2.0) for high-quality DNA suitable for molecular applications (Table 2).

The highest purity value of 1.79 was observed in one of the G9 samples, indicating minimal contamination from proteins and other secondary metabolites. In contrast, the Malbhog samples exhibited lower purity values, ranging from 0.8 to 1.44, suggesting the presence of residual polysaccharides or phenolic compounds that could interfere with downstream molecular analyses (Santos et al, 2019). In terms of DNA concentration, the G9 samples demonstrated higher values, ranging from 111.7 to 151.7 µl/mg, indicating efficient DNA yield from the optimized protocol (Satyanarayana et al, 2017). Conversely, the Malbhog samples showed significantly lower DNA concentrations, ranging from 30.3 to 37.9 µl/mg. The lower yield in Malbhog could be attributed to its inherent biochemical composition, which may require further optimization of the extraction protocol to improve DNA recovery.

These findings highlight the variation in DNA extraction efficiency between the two banana cultivars, emphasizing the need for tailored modifications in extraction protocols to achieve optimal purity and yield, particularly for Malbhog ensuring high-quality DNA is essential for genetic fidelity assessments, DNA fingerprinting, and marker-assisted selection in banana breeding programs (Sahin et al, 2024). The findings demonstrated that the two cultivars' DNA yields differed.

Malbhog had a somewhat lower yield, from 70 to 110 ng/µL, but G9 showed a comparatively greater yield, ranging from 80 to 120 ng/µL. According to the purity evaluation, there was little protein contamination because the A260/A280 ratio for the majority of the samples fell between 1.7 and 1.9, which is an acceptable range. Nonetheless, certain Malbhog samples had an A260/A230 ratio below 1.8, which suggests the presence of phenolic chemicals or polysaccharides, which are frequently present in banana leaves.

Analysis of Gel Electrophoresis: The results of agarose gel electrophoresis confirmed the successful extraction of high-molecular-weight DNA from both G9 and Malbhog banana cultivars. The genomic DNA amplification pattern of micropropagated *Musa* spp. (Malbhog and G9) using RAPD primers revealed distinct banding profiles, confirming the successful amplification of genomic DNA (Figures 1 and 2). The presence of intact DNA bands with minimal smearing indicated that the optimized CTAB-based extraction method effectively preserved DNA integrity. Additionally, the absence of RNA contamination suggested that the RNase treatment was efficient, ensuring the purity of the extracted DNA for downstream applications such as RAPD analysis and genetic fidelity assessments. Variations in band intensity were observed between the two cultivars despite the overall success of the DNA extraction process.

The DNA samples from the G9 cultivar exhibited stronger and more distinct bands, indicating a higher DNA yield and better integrity. In contrast, the Malbhog samples displayed fainter bands in some cases, suggesting lower DNA concentrations or partial degradation. This discrepancy could be attributed to differences in the biochemical composition of the cultivars, as Malbhog has a higher content of polysaccharides and phenolic compounds, which may interfere with DNA extraction and stability. The sequential use of primers 1–36, as listed in Table 1, ensured a comprehensive assessment of genomic stability and variation among the plantlets.

These findings highlight the importance of cultivar-specific optimization in DNA isolation protocols. While the current method performed well for G9, further refinements, such as additional purification steps or modifications in lysis conditions, may be necessary to enhance DNA yield and integrity for Malbhog. Ensuring high-quality DNA is crucial for genetic analyses, as degraded or impure DNA can affect the accuracy of PCR-based studies and other molecular applications.

CONCLUSION

DNA was successfully extracted from G9 and Malbhog banana leaves using the CTAB technique. The variations in DNA yield and purity were observed, with G9 producing higher-quality DNA compared to Malbhog, which exhibited lower purity likely due to residual polysaccharides and phenolic compounds. While the extracted DNA was sufficient for genetic studies, further refinement of the extraction protocol is recommended, particularly for Malbhog, to enhance DNA purity and yield. Adjustments such as optimizing lysis conditions, incorporating additional purification steps, or modifying buffer compositions could improve overall extraction efficiency. Ensuring a reliable and reproducible DNA isolation method is crucial for accurate genetic fidelity assessments, RAPD analysis, and marker-assisted selection. These findings contribute to advancing molecular research on banana cultivars and support the development of efficient DNA extraction protocols for breeding and conservation efforts.

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Exposure to Morphine Influences the Activity and Behavior of the Funnel-web Spider *Pireneitega luctuosa*

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ABSTRACT

Opioid research has mostly been conducted on higher species and has hardly ever been documented in lower animals like the spiders, which are an excellent type species for examining behavior. The effects of opioids on the locomotor activity and exploratory behavior of an unknown area of the funnel-web spider *Pireneitega luctuosa* were examined along with the spontaneous behavior of spiders in an open-field setting using Software Intelligent 3.0 Behavior Tracker. Real-time movement was captured by high-resolution infrared cameras, and the software used movement trajectories to track important behavioral patterns like exploration, thigmotaxis, resting, and escape attempts. This automated technique allowed for a thorough evaluation of spider behavior by providing accurate, objective data on distance travelled, speed fluctuations, and zone preference. The findings revealed the following: (a) The spontaneous behavior of spiders in the open field exhibited a high to low trend, meaning that they tended to be active in the early stages and quiet in the late stages, in the groups that received no treatment, distilled water treatment, and four different morphine concentration treatment groups. (b) The spider's locomotor activity declined within 90 minutes of being exposed to opioid solutions, and there was a negative correlation between the morphine concentration and locomotor distance. (c) The varying morphine concentrations had no effect on the more careful exploration of the spider center region, which is more likely to be active in the periphery. (d) Following various opioid concentration treatments, the exploration duration and distance to the central region of the spider reduced, and the groups receiving distilled water and high concentration morphine solutions differed significantly. According to these present findings, opium not only temporarily stopped the funnel-web spider from acting spontaneously, but it also somewhat stopped the spider from exploring uncharted territory. The findings from this study provide valuable insights into the effects of opioids on arachnid behaviour, with potential applications in multiple fields.

KEY WORDS: FUNNEL-WEB SPIDER; LOCOMOTOR ACTIVITIES; EXPLORATION BEHAVIOR; OPIOID RECEPTOR AGONIST; MORPHINE.

INTRODUCTION

Spiders belong to arena of arachnida of phylum arthropods have a small brain size, and their nervous system is relatively simple compared with that of higher animals. But spiders are sensitive to external stimuli and move quickly, and through the long evolutionary process many groups of spiders have formed specific and complex behavior patterns, such as a lot of spiders use their web to hunt (Foelix 2011), some of spiders have extreme behavior-sexual cannibalism (Gavín-Centol et al; 2017, Fisher et al; 2018), one species of jumping spider have nursing behavior like higher animals (Chen et al; 2018).

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The funnel-web spider is named for its three-dimensional funnel-shaped web, which is usually connected with the outside world through multiple entrances and exits. The funnel-web spider quietly lies at the main entrance, quickly attacks and captures prey caught by the web, and escapes from one of the exits quickly when encountering a predator. Previous reports and our studies have been found that sexual catalepsy behavior exists in many funnel-web spider species, that is, the female coward all the legs during mating for a long time, and the duration of sexual catalepsy of the spider of P. luctuosa can be up to 20 hours on average (Singer et al; 2000, Xiao et al; 2015, Liu et al; 2018, Frank, et al., 2023, Liu et al., 2024).

Thus it shows that the funnel-web spider is a class of spider groups with quick action and complex behavior, which is a good type animal for studying the behavior of lower animals. Morphine, a central nervous system drug, was first separated from opium in 1806 by the German chemist F.W.A Serturner, which mainly acts on μ-opioid receptors. Opioid receptors

mediate a series of body reactions such as analgesia, hypnosis and respiratory depression, so they are widely used as strong analgesic clinic in medicine (Mercadante 2010, Torres-López et al; 2013). However, with the widespread use of morphine, its toxicity appear gradually, among which the most common is the physiological dependence and tolerance , as well as the resulting behavioral changes (Yayeh et al; 2016, Corder et al; 2018).

The research on neuro drugs such as morphine has been focused on higher animals, for example, the avoidance behavior of harmful stimuli in rats was significantly reduced after morphine treatment (Pahng et al; 2017), the rats showed severe anxious behavior after chronic morphine withdrawal (Kim et al; 2018). The effects of morphine on the lower animals have been little studied, and only a few insect species have been involved. For example, some studies have shown no significant difference in growth rate and developmental status between which feed on morphine carrion and those which does not feed on morphine carrion of the blow fly of *Calliphora stygia* (George et al; 2009). Honeybees (*Apis mellifera*) showed decreased on associative memory and motor activity after acute injection of morphine, while it with chronic treatment showed decreased associative memory, olfactory memory and behavioral activity, but increased tolerance to stimulus (Chen et al; 2014, Fu et al; 2013).

There are few studies on the behavior change of spider groups under nervous system drug stress, focus on the effect of drugs on spider web weaving behavior, such as Witt (1965) studied the two garden spider *Araneus diadematus* and *Argiope aurantia* the change of the weaving behavior after nerve drug treatment, the results showed that high concentrations of scopolamine was reduced weaving frequency, after Benzedrine treatment the processing screw spacing and irregularity of radius increases, weaving efficiency was reduced.

However caffeine not only increase the screw spacing and spider radius of irregularity, also reduces the area of the web (Hesselberg et al; 2004). The various behaviors that occur spontaneously after animals enter a new environment can reflect the mental state of the test animals, open field test is a good method to observe locomotor activity and explore behaviors of test animals, and is common used to evaluate the effects of neurological drugs on animal behaviors (Hull et al; 2004, El Hassani et al; 2005, Tremmel et al; 2012).

The total amount of locomotor activities of animals in the open field reflects the activity of their behaviors. The fear of the new environment leads to the activity of animals mainly in the periphery area of the open field, but the nature of exploration of animals force them to enter the central area ever and again. Some central stimulants can significantly increase locomotor activity in animals (Szopa et al; 2016), while certain antipsychotics can reduce exploratory behavior in animals (De Santis et al; 2016). In this study, *P. luctuosa* (L. Koch, 1878), a common funnel-web spider in southern China, was used to study the changes of the spider's locomotor activities and exploratory behaviors after being exposed to morphine solutions.

Another study demonstrated that morphine exacerbates inflammatory responses and associated behavioral and hippocampal deficits in rodents, suggesting complex interactions between opioids and neural function (Ayieng'a et al., 2024). Some scientists report that chronic morphine administration in rats leads to distinctive behavioral changes, including decreased licking frequency, reduced water intake, and altered sleep posture (Yin et al., 2024).

MATERIAL AND METHODS

Collecting and raising of the funnel-web spider *P. luctuosa:* Spiderlings of the funnel-web spider *P. luctuosa* were collected and were reared in an air-conditioned laboratory with temperature 25 ± 2^0 C and photoperiod 14 L: 10 D. The spiders were individually maintained in a transparent polypropylene box (14 cm L × 10 cm W × 5.5 cm H) with a small wet cotton on the bottle to supply drinking water and moisture for the spider(Schwartz et al 2012). All the spiders were fed with 2-3 house flies (*Musca domestica*) twice a week. From time to time, fruit flies (*Drosophila melanogaster*) and flour beetle larvae (*Tenebrio molitor*) were supplied, respectively, as supplementary food.

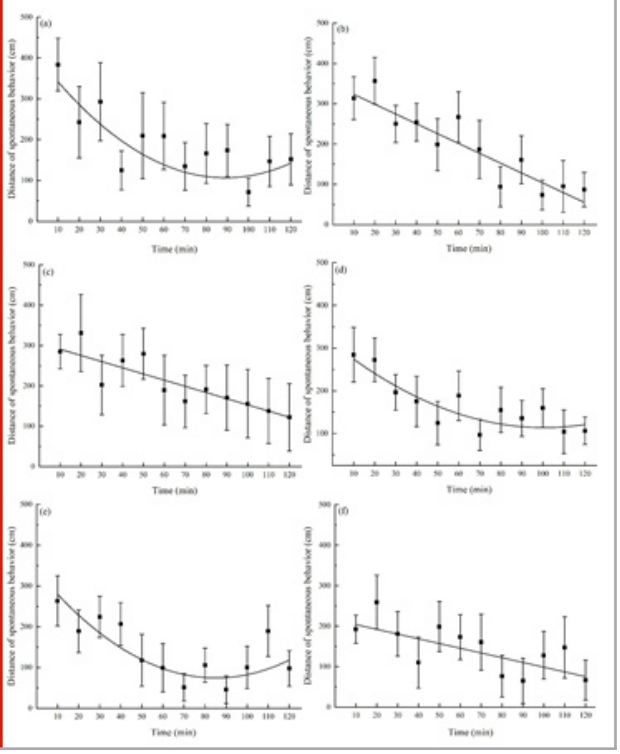
Spiders in *P. luctuosa* generally molt 5-6 times during their lifespans. Juveniles in 3-4 instar, which weighed an average of 127.421 ± 11.259 g, were chosen for the experiment. All the chosen juveniles were fed with house flies one day before the experiment was conducted to eliminate possible undesirable effects.

Exposure of the spiders to morphine solutions: The powder-like morphine was diluted by distilled water into 4 types of concentrations: 7.5, 15, 30, and 60 mg/mL. The juvenile spiders chosen for the experiment were randomly assigned into 1 untreated group (without any treatment) and 5 treated groups, which were treated with distilled water and 4 morphine solutions in 7.5, 15, 30 and 60 mg/mL, respectively. The sample size for each group is 8 juvenile *P. luctuosa*. Distilled water and morphine solutions were contained in a 30 mL sprayer with a fine mist nozzle. All the treated juveniles were individually introduced into a 50 mL centrifuge tube and then sprayed by the corresponding distilled water or morphine solution. The amount of the distilled water or morphine solutions used for each juvenile was 0.1 mL. Then the treated spider was rested 10 min for the following examination of locomotor activity.

Behavioral test: The open field of the spider behavior test was a special round container made of polymethyl methacrylate (190 mm in diameter and 75 mm in height), which was used to record and analyze the weaving, hunting, courtship, mating and other behaviors (Liu et al; 2018) of funnel-web spiders (including *P. luctuosa*). Before each behavior test, the open field container was cleaned with anhydrous ethanol, then air-dried. Without treatment, distilled water treatment and different morphine concentration treatment groups of the spider of *P. luctuosa* were introduced into the open field; SMART 3.0 behavior tracker software (Panlab, Harvard Apparatus, and USA) was used to record the spontaneous behavior of the spider. In the software, the open field was divided into the central area and

a periphery area according to the ratio of 3:1 in diameter; the distance and time of locomotor behavior in the central area and the periphery area were recorded. The data collection time was 2 h, and 8 individuals from each group.

Figure 1: Time-course of locomotor distance of P. luctuosa exposed to morphine solutions within 2 hours. The morphine concentrations were (a) 0 mg/mL (distilled water), (b) 7.5 mg/ml, (c) 15 mg/ml, (d) 30 mg/ml and (e) 60 mg/ml, respectively.



Statistical Analysis: The distance and time of locomotor behavior of *P. luctuosa* were expressed as Mean ± SE, Linear fitting and Polynomial fitting analysis were used for the distance activities, the concentration of morphine in relation to the distance□ distance changes over time, the Independent sample t-test was used to the compare of the distance and time of locomotor behavior in central area and periphery area. All statistical analyses were conducted using SPSS for Windows (version 19.0; IBM SPSS Statistics, USA), and graph was completed by Origin Pro (version 2018c, Origin Lab, USA).

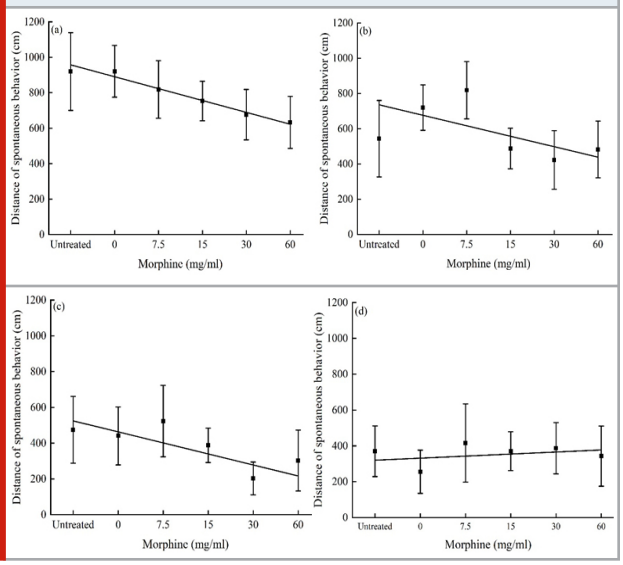
RESULTS

The locomotor activities of P. luctuosa in the open field changed with time after exposed to morphine solutions: Without treatment group of *P. luctuosa* was introduced into the open field for 10 minutes and became very active, crawling rapidly in succession along the periphery area of the open field. However, with the increase of time, continuous crawling turns into intermittent, and the crawling time gradually decreases, and the interval time becomes more. The last 30 minutes of the crawl were accompanied by the movement of touching the wall of the open field with the opening of the spinneret and the weaving, crossing the central area of the open field from time to time (Fig 1 a). After being treated with different concentrations of morphine (including the distilled water group), the locomotor distance of *P. luctuosa* within 10 minutes after being introduced into the open field was less than that of the without treatment group, but the overall trend was consistent, that is, the locomotor distance trend showed high to low (Fig 1 b-f).

Curve-fitting was performed for the locomotor activities of each group in open field, and the fitting equations were respectively: without treatment group y = 3.753x2 - 66.889x + 404.687, R2 = 0.601; distilled water group y = 347.694 - 24.368x, R2 = 0.862; 7.5 mg/mL group y = 306.096 - 15.318x, R2 = 0.779; 15 mg/mL group y = 1.956x2 - 39.360x + 311.811, R2 = 0.675; 30 mg/mL group y = 3.619x2 - 61.684x + 337.279, R2 = 0.650; 60 mg/mL group y = 215.711 - 11.689x, R2 = 0.561. (Fig 1 a-f)

The area characteristics of locomotor activity in open field after exposed to morphine solutions: When the spider first was put into open field, showed crawling rapid succession along periphery area of the open field, with the increase of duration of stay in the open field, the spider of P. luctuosa began to explore the central area, which showed that every now and then, it quickly passed through the central area from the periphery area, or went to the central area and then returned to the periphery area, but the locomotor distance and the time spent in the central area are far from that in the peripheral area.

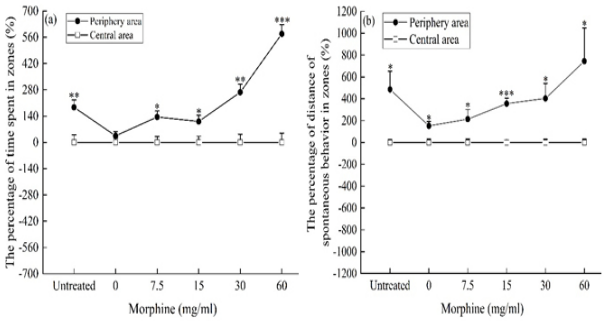
Figure 2: Time-course of locomotor distance of *P. luctuosa* exposed to morphine solutions in the 4 successive monitoring periods. (a) 0-30 min, (b) 30-60 min, (c) 60-90 min and (d) 90-120 min.



We compared and analyzed the locomotor activities of P. luctuosa in the central area and the periphery area, the duration of stay and locomotor distance of the central area were taken as the baseline, and the percentage chart of the

duration of stay and locomotor distance of the peripheral area in the central area was plotting, that is (Time or distance of periphery area - mean time or distance of central area) / mean time or distance of central area × 100%.

Figure 3: The percentage of distance and time of locomotor behavior in zones. * P < 0.05, ** 0.001 < P < 0.01, *** P < 0.001.



The results showed that during the 2-h monitoring period, except for the 0 mg/mL (distilled water) group (P > 0.05, Fig. 3a), the time spent in the central area of the untreated group, the 7.5 mg/mL group, the 15 mg/mL group, the 30 mg/mL group and the 60 mg/mL group were all significantly less than the time spent in the peripheral area (P < 0.05, Fig 3 a); The locomotor distance of all groups in the central area was less than that in the peripheral area (P < 0.05, Fig. 3b). The results indicated that the spider was cautious to explore the central area and tended to be active in the peripheral area, but the morphine solutions did not change the behavior tendency of the spider.

The duration of stay and locomotor distance of the central area were taken as the baseline, and the percentage chart of the duration of stay and locomotor distance of the peripheral area in the central area was plotting, that is (Time or distance of periphery area - mean time or distance of central area) / mean time or distance of central area ×100%.

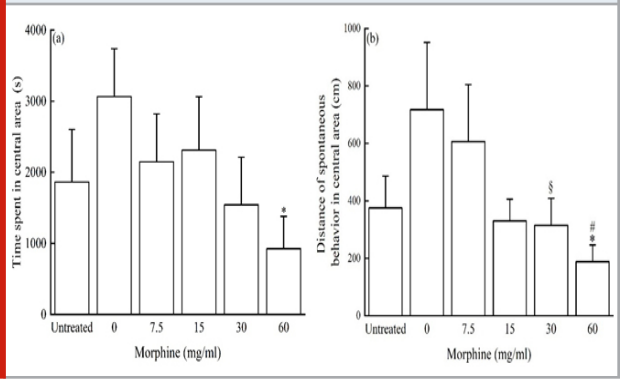
The difference of locomotor activity of P. luctuosa in central area of open field after exposed to morphine solutions between groups: We analyzed the inter-group differences in the exploration time and distance of P. luctuosa in the central area, the results showed that the 0 mg/mL (distilled water) group has the longest detention time in the central area (Fig. 4a) and the largest locomotor distance (Fig. 4b); After exposed to morphine solutions, the retention time and locomotor distance of *P. luctuosa* in the central area showed a decreasing trend, and the retention time in the central area of the 0 mg/mL (distilled water) group was significantly longer than that in the 60 mg/mL group, but the difference among other groups shows no significant (Fig. 4a). The locomotor distance in the central area of the 0 mg/mL (distilled water) group was significantly higher than that of the 30 mg/mL and 60 mg/mL groups, and that of the 7.5 g/mL group was also significantly higher than that of the 60 mg/mL group, with no significant difference among other groups (Fig. 4b). The results indicated that morphine inhibited spider's exploration behavior to unknown areas to a certain extent, lead to its exploration time and distance to the unknown area decreased.

DISCUSSION

After being introduced into an open field, the locomotor activity in the early stage of monitoring was high, while in the later stage was low, and the weaving behaviors were rare and appeared in the late stage of monitoring, which may be related to the nature of spiders generally weaving webs at night (Ramousse et al; 1976), since our behavior experiments were conducted during the day, we rarely observed the weaving behavior of *P. luctuosa* that using end of abdomen (spinneret) to tap the inner wall of the open field; Like many of the higher animals tested (Lezak et al; 2017), *P. luctuosa* was mainly active in the peripheral area after entered the open field, and was cautious in exploring the central area, which was showed by rapid crossing of the central area or rapid retracing back to the peripheral area after reaching the central area. The behavior characteristics of *P. luctuosa* in the open field and its tendency to move in the peripheral area were not changed.

But, within 90min after exposed to morphine solutions, the locomotor distance of the spider decreased, and the distance was negatively correlated with morphine concentration; after exposed to morphine solutions, the exploration time and distance to the central area of *P. luctuosa* decreased, and the difference between the morphine treatment group and the 0 mg/mL (distilled water) group was significant. In this study, the spray treatment of the spider with a morphine solution or distilled water was similar to rainfall in the field. After being sprayed with distilled water, the spider's exploration activity time and distance in the central area were the highest, which may be a stress response to the external rainfall.

Figure 4: Distance of locomotion and time spent in central area in *P. luctuosa* exposed to morphine solutions within 2-hours. * means P < 0.05 vs. 0 mg/ml, § means P < 0.05 v.s.0 mg/ml, # means P < 0.05 vs. 7.5 mg/ml.



In other words, after being stimulated by rainfall, the spider was more willing to explore the unknown area to seek shelter from the rain. However, the behavior of *P. luctuosa* after morphine solution spray became more cautious, especially after high concentration; its exploration time and distance were significantly lower than those in distilled

water spray and low concentration of morphine solution groups. Hence, our results showed that morphine not only inhibited the behavioral activity of the spider in a certain period, but also inhibited the behavior of the spider in exploring unknown areas to a certain extent.

Morphine is an opioid receptor agonist with a variety of neuropsychopharmacological activities, which can act on the nervous system and affects animal behavior (Altarifi et al; 2015, Samuels et al; 2017, Roeckel et al; 2018). The spontaneous behavior of animals is a direct manifestation of the functional state of the central nervous system, and the activity of the corresponding nervous system increases when excited, and vice versa, so the locomotor activity of animals is an important indicator to evaluate the excited state of the central nervous system (Marques et al; 2014). Morphine treatment reduced locomotor activity of *P. luctuosa* in the new environment and inhibited its exploratory behavior, making it timid (preferring to stay on the peripheral area).

The reason may be that morphine has an inhibitory effect on the relevant functional areas of these invertebrate brain area, but the relevant physiological mechanism needs to be further studied by combining the methods of physiology, neurobiology, anatomy and other disciplines. We expect to explore the similarities and differences in the mechanism of behavior occurrence and neural regulation between higher animals and lower animals by studying the neurophysiological mechanism of complex behaviors in invertebrates such as spiders more extensively and more deeply.

Over the last few years, research has gone further and how morphine affects behavior and neural function in different animals, which can be useful for studying the funnel-web spider *P. luctuosa*. In 2024, a study established that morphine worsens inflammation and the resulting deterioration of hippocampal-dependent behavior and cognition in rodents, suggesting that opioids have a complicated relationship with the nervous system (Ayieng'a et al. 2024).

In 2024, a behavioral study how ever, found that rats given chronic morphine had several differences in their behavior, including less licking, drinking less water, and taking up a different sleeping position (Yin et al., 2024). There is hardly any research on spiders, particularly, *P. luctuosa*, directly, but studies of other invertebrates are helpful. For example, crayfish treated with morphine displayed increased activity and investigatory behavior, which was followed by withdrawal signs when the treatment was stopped, suggesting the existence of pathways that are sensitive to opioids and regulate activity.

Also, experiments on molluscs have established that morphine lengthens the reflex to noxious stimuli, and these effects are counteracted by opioid antagonists, thus suggesting that opioid receptors are evolved from very early organisms. These findings suggest that it is crucial to examine the impact of morphine on the activity and behaviour of *P. luctuosa* as it is likely to shed light on the basic mechanisms of opioid action in arachnids and may also help to further understand neurochemical control of behaviour in other organisms.

CONCLUSION

The varying morphine concentrations had no effect on the more careful exploration of the spider center region, which is more likely to be active in the periphery. Following various opioid concentration treatments, the exploration duration and distance to the central region of the spider were reduced, and the groups receiving distilled water and high concentration morphine solutions differed significantly. According to these present findings, opium not only temporarily stopped the funnel-web spider from acting spontaneously, but it also somewhat stopped the spider from exploring uncharted territory. The findings from this study provide valuable insights into the effects of opioids on arachnid behaviour, with potential applications in multiple fields.

Data Availability: All data will be made available on a reasonable request to the corresponding author.

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Bacteriological Profile of Urinary Tract Infections and Antibiotic Susceptibility Pattern In Konaseema Region, Andhra Pradesh

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ABSTRACT
In the present study, an attempt has been made with regard to the bacteriological profile of the most commonly known infections found in the urinary tract. The 40 clinical samples were collected from patients of both sexes of all age groups. Out of 91 cases of the incidence of infections, there were 44% of the urinary tract infections. Gender wise incidence of UTIs in females is 59%, males is 42%, and UTIs in females is more than in males. The use of Amoxicillin has been recommended. The presence of detectable bacteria in the urine is named as the bacteriuria ecological zone of Amalapuram Mandal of Konaseema District of Andhra Pradesh.: Bacteriological study of urinary tract infection in Konaseema Institution of Medical Sciences and Research Foundation, Amalapuram revealed the route of various gram negative and gram-positive species of bacteria as the etiological agents. *Escherichia coli* has been observed to be a predominant etiologic agent in cases of UTI closely followed by *Klebsiella pneumonia*. A large majority of cases occurred in the age group of 50 above years in males, (41-50) and 50 above years in females. The investigation reveals predominance of the disease in female patients (58%) than in male patients (42%). In vitro sensitivity tests reveal Gentamycin (75%) and Amoxycilin (100%), Nitrofurointoin (88%), Meropenum (74%) to be effective against the organisms causing urinary tract infections, followed by Cefoxitin (16%), CZA (16%) has been found to be the least. *Escherichia coli* was the most common pathogen incriminated in UTI, followed by *Klebsiella pneumonia*, and *Staphylococcus species*. In general, the emergence of the higher incidence of Gram-negative organism, especially *Escherichia coli* has occurred in this Konaseema region of Andhra Pradesh geographical area.

KEY WORDS: BACTERIOLOGICAL PROFILE, URINARY TRACT INFECTIONS, ANTIBIOTIC SUSCEPTIBILITY PATTERN, ENDOGAMOUS POPULATIONS, KONASEEMA REGION, ANDHRA PRADESH.,

INTRODUCTION

Urinary tract infection is the second most common respiratory tract infection UTI is defined as an infection after disease caused by microbial invasion of the genitourinary tract, that extends from the renal cortex of the kidney to the urethral meatus. Urinary tract infections (UTIs) are counted among the most common infections in humans, exceeded in frequency among ambulatory patients only by respiratory and gastrointestinal infections. (Shalini et al 2011, Levi et al 2005). The presence of detectable bacteria in the urine is known as bacteriuria. Presence of pus cells in urine denotes pyuria, which often accompanies UTI (Hooton 2003, Baveja 2022).

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The medical profession has known for a long time that urinary tract diseases of viral etiology. Infections of the urinary tract is commonly encountered in medical practice. The vast subject of urinary tract infection comprises many angles of study, namely bacteriological and clinical. The frequent occurrence of urinary tract infection among cases clinically diagnosed as "P U O "(Pyrexia of unknown origin) and the problem associated with their management are well appreciated. In trying to rid the urinary tract of the infection, the clinician is obliged to turn to bacteriology for it is the study of the nature of the invading bacteria that would lead to the proper treatment and Prevention of such infection (Bhargava et al., 2022).

Acute urinary tract Infections are second only to respiratory tract infections in their frequency of occurrence. The problem of chronicity & recurrence of urinary tract infection is gaining paramount importance in recent years. Structural and congenital malformations and obstructive lesion are found to associate with urinary tract infections certain diseases such

as diabetes mellitus and uremia are responsible for initiation or for their maintenance of urinary tract, urinary tract and catheterization infections instrumentation of are important predisposing factors for the development of urinary tract infections (Feinberg School of medicine, Chicago). A wide spectrum of gram positive & gram-negative organism have been incriminated as the ontological agents of urinary tract infections.

Table 1. Incidence and Gender wise of Urinary Tract Infection

Total No of Specimens Investigated	Culture Positive		Culture Negative		Gender wise			
					Males		Females	
91	Number	%	Number	%	Number	%	Number	%
	40	44%	51	56%	17	42%	23	58%

The extensive and inappropriate use of antimicrobial agents has invariably resulted in the development of antibiotic resistance, which, in recent years, has become a major problem worldwide (Goldstein FW, 2000). In patients with suspected UTI, antibiotic treatment is usually started empirically, before urine culture results are available. To ensure appropriate treatment, knowledge of the organisms that cause UTI and their antibiotic susceptibility is mandatory (Ashkenazi et al 1991). This study was planned to explore the common pathogens responsible for UTI and to determine the antibiotic susceptibility pattern of them.

Table 2. Distribution Of Uti Cultures And Types (Gram Positive And Gram Negative)

GRAM POSITIVE			GRAM NEGATIVE		
ORGANISM	NUMBER	%	ORGANISM	NUMBER	%
STAPHYLOCOCCUS SPECIES	07	17.5%	*ESCHERICHIA COLI*	17	42.5%
			KLEBSIELLA PNEUMONIA	11	27.5%
			PSEUDOMONAS AURUGINOSA	03	7.5%
			ACINETOBACTER BAUMANNI	01	2.5%
			PROTEUS MIRABILIS	01	2.5%
	07			33.	
	17.5%			82.5%	

MATERIAL AND METHODS

This was a cross-sectional analytical hospital-based study in which analysis of urinary culture results was done. This study was conducted in the department of Microbiology, Konaseema Institute of Medical Sciences & Research Foundation hospital in Amalapuram, Konaseema District, Andhra Pradesh, from March, 2024 to April, 2024. Both out and inpatients presenting or highly suspicious of having UTIs were recruited in the study. The details regarding the patient's age, sex, literacy, socioeconomic status, and residential area (rural/urban) were recorded along with the sample. Both male and female patients having clinically suspected symptoms of UTI were included in the study.

Collection of Urine Samples: Early morning mid-stream urine samples were collected using Sterile, wide mouthed container with screw cap tops. On the urine sample bottles were indicated name, age, sex, and time of collection along with requisition forms. The samples were analyzed bacteriological using the methods (Kass, 1957).

Sample processing:
Culture: A calibrated sterile micron wire loop for the semi-

quantitative method was used for the plating and it has a 4.0 mm diameter designed to deliver 0.01 ml. A loopful of the well-mixed urine sample was inoculated into duplicate plates of Blood and Mac-Conkey agar. All plates were then incubated at 37ºC aerobically for 24 h. The plates were then examined macroscopically and microscopically for bacterial growth. The bacterial colonies were counted and multiplied by 100 to give an estimate of the number of bacteria present per millilitre of urine. A significant bacterial count was taken as any count equal to or in excess of 10,000 cfu /ml (National Committee for Clinical Lab Standards, 1993).

Table 3. Organism In Relation To Gender And Age Groups

ORGANISM	MALE GROUPS							FEMALE GROUPS						
	0-10	11-20	21-30	31-40	41-50	ABOVE 50	OVER ALL	0-10	11-20	21-30	31-40	41-50	ABOVE 50	OVER ALL
STAPHYLOCOCCUS SPS	-	-	-	-	-	1 (5.88%)	01	-	1 4.34%	-	1 4.34%	2 8.69%	2 8.69%	06
ESCHERICHIA COLI	1 5.88 %	-	-	-	-	7 41.17%	08	1 4.34 %	-	2 8.69%	2 8.69%	1 4.34%	3 13%	09
KLEBSIELLA PNEUMONIA	-	-	-	-	1 5.88%	3 17.64%	04	-	-	1 4.34%	-	3 13%	3 13%	07
PSEUDOMONAS AERUGINOSA	1 5.88 %	-	-	-	-	1 5.88%	02	-	-	-	-	-	1 4.34%	01
ACINETOBACTER BAUMANN	-	-	1 5.88%	-	-	-	01		-	-	-	-	-	-
PROTEUS MIRABILIS	-	-	1 5.88%	-	-	-	01	-	-	-	-	-	-	-

Antibiotic susceptibility test: Overall, 91 urine cultures were isolated from positive cultures and tested for their susceptibility to antibiotics. The anti- biogram was carried out by the disk diffusion method in Mueller Hinton agar medium according to the recommendations of the CLSI guidelines (Wayne PA, 2010). Several commercial antibiotic discs (Bio-Rad; Oxoid) used to treat UTIs were tested including: Amoxicillin. Azithromycin, Clindamycin, Cza, Cefoxitin, Doxycyclin, Gentamycin, Levofloxacin, Linezolid, Minocyclin, Norfloxacin, Nitrofurointoin and Vancomycin. *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella Pneumonia, Acinetobacter Baumanni* and *Proteus Mirabilis* were used as quality control strains. The method used for standardization of the inoculums size was agar diffusion method. The standardized single-disc diffusion method was employed (Koneman's Textbook, 2005). This study was ethically approved by institutional ethical committee of the institute.

RESULTS

In this study, urine sample total of 40 patients clinically diagnosed with urinary tract infection were collected and tested for microorganisms. There were 40 positive urine cultures (44%) and 51 (56%) negative. *Escherichia coli* represented 17 (42.5%.) of the positive urinary isolates. The resistance rate for *Staphylococcus* Species to CZA was found to be 83.83%.

DISCUSSION

In community and hospital settings, the etiology of UTIs and the antimicrobial susceptibility of UTI causing bacteria's have been changing over the years (New HC. 1996, Jones RN. 1996). A wide spectrum of gram-negative and gram-positive organisms has been incriminated for cases of UTI.

In the present study of 91 cases, the incidence of infection was 44%. The prevalence nearly coincides with Panda et al (43.4%) (Panda et al, 2020). In a similar study by Das et al isolation rate was 71.6% (Das et al, 2006). The incidence of urinary tract infection in males 42% and females 58% is in the present study. Prevalence of UTIs was more in females when compared to males. This was in agreement with other studies by Bashir et al. (2008).

Table 4. Results of Sensitivity, Intermediate, Resistance of Drugs in Gram Positive Organism.

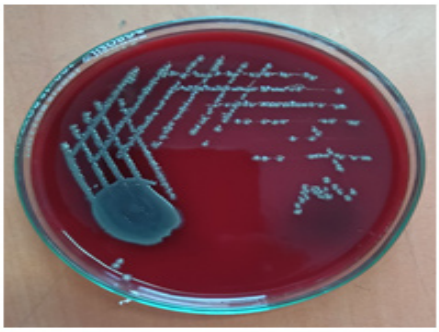
Antibiotic	Organism			
	STAPHYLOCOCCUS SPECIES			
	TOTAL	SENSITIVITY	INTERMEDIATE	RESISTANCE
Amoxycycline	07	7 (100%)	-	-
Azithromycin	06	2 (33.33%)	-	4 (66.66%)
Clindamycin	07	5 (71.42%)	-	2 (28.57%)
Cza	06	1(16.66%)	-	5 (83.83%)
Cefoxitin	06	1(16.66%)	-	5(83.33%)
Doxycyclin	07	4 (57.14%)	2 (28.57%)	1(14.22%)
Gentamycin	07	6 (75%)	1(13%)	-
Levofloxacin	05	1(20%)	-	4(80%)
Linezolid	06	4 (66.66%)	-	2 (33.33%)
Minocyclin	06	4 (66.66%)	-	2 (33.33%)
Norfloxacin	06	2 (33.33%)	2 (33.33%)	2 (33.33%)
Nitrofurointoin	06	4 (16.16%)	-	2 (33.33%)
Vancomycin	06	2 (33.33%)	-	4 (66.66%)

Women are more prone to UTIs then men because, in females, the urethra is much shorter and closer to the anus. (Dielubanza and Schaeffer 2011, Gupta and Stamm 1990). The most commonly isolated organism in UTI among female outpatients in our study was E. coli. The proportion of bacterial species isolated was similar to those described in several previous studies. (Zhanel et al. 2005). Panda et al reported females 57%. and males 43%. The incidence of *Escherichia coli* isolated from UTI cases in the present study it is 42.5%.

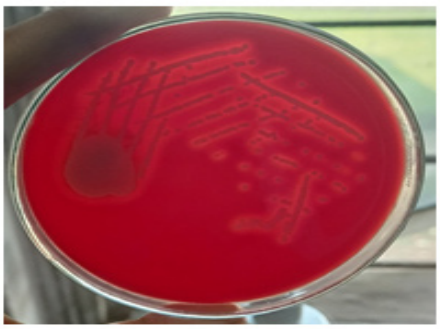
The most commonly isolated organism in UTI among female outpatients in the present study was *Escherichia coli*. But nowadays *Staphylococcus* species, *Klebsiella aerogenes*, *Pseudomonas aeruginosa, Proteus, Enterococcus, Acinetobacter* and *Candida* have also been reported increasingly from UTI. The proportion of bacterial species isolated was similar to those described in several previous studies (Scottish Intercollegiate Guidelines Network. 2015) (Hertz et al 2016) (Akoachere 2012), These findings are consistent with those of Kader et al. The incidence of

Escherichia coli isolated from UTI cases reported by Kader et al is 42.46%. (Kader et al. 2004 Panda et al 2024).

Figure 1: Gram-positive Bacteria



Colonies of *Staphylococcus aureus* on blood agar plate

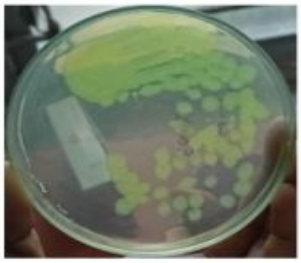


Colonies of *Staphylococcus aureus* with β hemolysis

The incidence of *Klebsiella aerogenes* isolated from UTI cases in the present study is 27.5%, these findings are consistent with Latika et al reported the incidence of *Klebsiella aerogenes* is 13.5% (Latika J Shah et al, 2015). Shaki et al. (Shaki et al 2020) reported a predominance of *E. coli* and *Klebsiella spp*. in urine samples from Southern Israel. In Libya, Salem et al. (2018) indicated *Klebsiella pneumoniae* (43.6%) and *E. coli* (33%) as the two most isolated pathogens. The incidence of *Staphylococcus* species isolated from UTI cases in the present study is 17.5% with highest incidence in 51 and above year's males, 41- 50 and females above 50 year's. Sengupta et al reported 6.1% (Sengupta et al 2018), incidence was found to be equal in both sexes. Panda et al reported 7.2%, (Panda et al, 2020), the incidence in females is more in between the age group of 31-40. The incidence of *Pseudomonas aeruginosa* isolated from UTI cases in the present study is 7.5%, these findings are nearly coincides with *Asifa Nazir* et al 10.1 % (Asifa Nazir, Farhat Kanth, 2024).

The percentage of *pseudomonas aeruginosa* isolated from urinary tract from different parts of India is as follows: Bharati et al 5.7% (Bharti et al 2016). The incidence of *proteus mirabilis* reported in the present study is 2.5% nearly coincides with Asifa Nazir et al i.e., 1% (Nazir and Kanth, 2024) in males predominantly. *Escherichia coli* was the most common organism isolated (42.5%) among the gram negatives, followed by *Klebsiella sp* (27.5%), while other gram negatives isolated included *Pseudomonas aeruginosa* (7.5%), Proteus sp (2.5%).

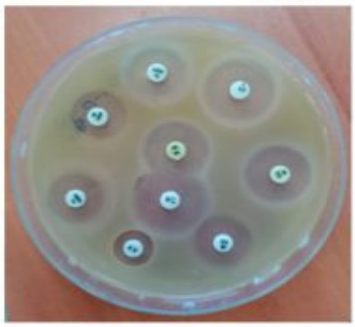
Figure 2: Gram Negative Bacteria



Colonies of *Pseudomonas aeruginosa* on nutrient agar plate

Colonies of *klebsiella* on MacConkey agar plate

Colonies of *E. coil* on MacConkey agar plate



Antibiotic sensitivity patterns

Antibiotic sensitivity patterns of *Pseudomonas aeruginosa*

In the present study, Most of the gram-positive organisms are sensitive to Amoxycyclin (100%), Gentamicin (75%), Clindamycin (71.42%), Doxycycline (57.14%), Nitrofurontoin (66.66%), Minocycline (66.66%), Linezolid (66.66%), Norfloxacin (33.33), Azithromycin (33.33%), Vancomycin (33.33%), Ceftazitdimeavivac (16.66%), Nitrofurontoin (66.66%), Cefoxitin (16.66%). And the Gram negative organism sensitive to Nitrofurointoin (88%), Meropenum (74%), Cefoperazone (53%), Norfloxacin (44%), Gentamycin (50%) Ceftazitdimeavivac (36%), Tetracycline (32%). gentamicin (92.3%) followed by imipenem (90.2%) and less sensitivity was shown to cefotaxime.

Das et al, (2015) in their study observed highest resistance to ampicillin, fluoroquinolones and ceftriaxone whereas gentamicin and nitrofurantoin were the antibiotics to which organisms were most sensitive. Most *Escherichia coli* from community infections investigated in this study were susceptible to oral drugs commonly used in general practice such as Amoxycyclin, Gentamicin, Clindamycin, nitrofurantoin, Minocycline and Norfloxacin.

CONCLUSION

Bacteriological study of urinary tract infection in Konaseema Institution of Medical Sciences and Research Foundation, Amalapuram revealed the route of various gram negative and gram-positive species of bacteria as the etiological agents. *Escherichia coli* has been observed to be a predominant etiologic agent in cases of UTI closely followed by *Klebsiella pneumonia*. A large majority of cases occurred in the age group of 50 above years in males, (41-50) and 50 above years in females. The investigation reveals predominance of the disease in female patients (58%) than in male patients (42%). In vitro sensitivity tests reveal Gentamycin (75%) and Amoxycilin (100%), Nitrofurointoin (88%), Meropenum (74%) to be effective against the organisms causing urinary tract infections, followed by Cefoxitin (16%), CZA (16%) has been found to be the least. *Escherichia coli* was the most common pathogen incriminated in UTI, followed by *Klebsiella pneumonia*, and *Staphylococcus species*. In general, the emergence of the higher incidence of Gram-negative organism, especially *Escherichia coli* has occurred in the Konaseema region of Andhra Pradesh geographical area.

Conflict of interest: Authors declare no conflict of interest.

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Data Availability: Data will be available on request

Ethical statement: The study was a retrospective analysis of existing data and did not involve any direct patient contact or intervention. All patient data were anonymized and deidentified to protect patient confidentiality.

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Green Synthesis of Silver Nanoparticles with Combinatorial Leaf Extracts: Phytochemical Screening and Antibacterial Activity

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ABSTRACT
The Pharmacological properties of medicinal plants aid in the treatment of various diseases are emerging area of research in the present scenario due to the presence of secondary metabolites. The present study aims to explore the green synthesis of silver nanoparticles from leaf extracts and the evaluation of bioactive compounds and its antibacterial activity. The present research focused on the analysis of the phytochemical composition, antibacterial activity, and green synthesis of silver nanoparticles from the leaf extracts of *Cassia fistula, Adhatoda vasica, and Aegle marmelos*. Following aqueous extraction, phytochemical screening and antibacterial activity were carried out. Green synthesised silver nanoparticles were characterized by using UV absorption, FTIR, and X-Ray Diffraction methods, and its antibacterial activity was evaluated. The hot aqueous combinatorial extract was quantitatively analyzed for the presence of tannins (3.03 mg), flavonoids (2.4 mg), alkaloids (10.4 mg), phenols (10.2 mg), and carbohydrates (7.8 mg). The green synthesis of silver nanoparticles was indicated with a UV-visible absorption peak at 460 nm. Functional groups identified by FTIR include amines, nitro compounds, alcohols, phenols, and alkenes. The highest size of synthesized silver nanoparticles from the combinatorial leaf extract was 23.63 nm (200) using XRD. Antibacterial activity of green synthesised silver nanoparticles demonstrated 13 mm zone of inhibition for *Staphylococcus sp*. and a zone of 15 mm for *Escherichia coli*. This present investigation paves a path for the establishment of green synthesised combinatorial leaf extracts that exhibit greater antibacterial efficacy against various pathogens. Hence, novel drug formulations may be adapted in the future.

KEY WORDS: *ADHATODA VASICA*, ANTIBACTERIAL ACTIVITY, *CASSIA FISTULA*, GREEN SYNTHESIS, PHYTOCHEMICAL ANALYSIS, SILVER NANOPARTICLES.,

INTRODUCTION

The primary source of natural products that are widely and successfully used in medicine is plants. Disease incidence is typically lower in populations that use a lot of natural herbal items. Recently, there has been a lot of attraction towards natural-based herbs as antimicrobial agents due to their eco-friendly and health hazardless in nature. The traditional Indian systems of Ayurveda and Siddha medicines support the importance of medicinal plants to treat diseases Ameer et al., (2021). Herbal medicines have been utilized in most countries since ancient times. Still medicinal plants in Asia are extensively employed as a therapy for infectious disease in rural and background areas, Omeje et al., (2023).

Herbal medications are commonly used in healthcare due to their inexpensive cost and abundance of antibacterial qualities (Enechi et al 2022). Many plants and herbal medicine-derived natural products could be used as an alternative therapeutic potential for RTI since they have antibacterial effects, Armutcu et al.,(2021). The size of nanocarriers was similar to biological molecules like

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viruses and proteins, which allows them to interact with cell surfaces and the cell wall Lombardo et al. (2019). Silver nanoparticles were used in a broad range of applications like drug delivery, food industries, anti-microbial and anti-cancer studies on the green synthesis of silver nanoparticles Zharkova et al. (2021). Therefore, plant extract–mediated green synthesis of nanoparticles is considered eco-friendly, cost-effective, and safe, and is a viable alternative for microbiological applications Garibo et al. (2020).

MATERIAL AND METHODS

The Following plants were used in the present study.

Cassia fistula: A deciduous tree with lovely yellow blossoms and grey bark is *Cassia fistula*. In Ayurvedic and Unani medicine, it is one of the most often utilized plants. According to some reports, this plant can help with skin conditions, liver issues, tuberculous glands, and diabetes. The fruit pod measures 20–27 mm in diameter and 40–70 cm in length. The distal extremities of the fruit pod are rounded and somewhat bent. Danish et al.(2011). This medium-sized, ornamental plant grows quickly and sheds its leaves after the growth season. Hanif et al.,(2007). Numerous quantities of primary and secondary metabolites are present in *Cassia fistula*. The pharmacological and biological effects of this substance depend on these metabolites. Anthraquinones, polyphenols, polysaccharides, flavonoids, tannins, glycosides, and amino acids are examples of primary and secondary metabolites.

Adhatoda vasica: For over two millennia, *Adhotoda vasica* has been utilized in India's traditional medical system. It is also a well-known medication in Unani and Ayurvedic medicine. The antibacterial, anti-spasmodic, anti-arthritic, antiseptic, expectorant, anti-tuberculosis, and anti-cancer qualities of *Adhatoda vasica* are well-known.

Aegle marmelos: There are various uses for *Aegle marmelos* as a medicinal plant. Every portion of *Aegle marmelos*, including the leaves, fruits, pulp, flowers, stem bark, and root bark, had therapeutic value; however, the leaf had the strongest pharmacological activity. Leaves were employed as a mild laxative or to relieve asthmatic mucous membrane inflammation. Leaf decoction can be used as an expectorant, to help get rid of fever, or to help clear the bronchial passages of mucus discharge. *Aegle marmelos* leaves can treat severe conjunctival inflammation, including acute bronchitis, and they can also treat inflammation in other parts of the body.

The leaves of *Cassia fistula, Adhatoda vasica*, and *Aegle marmelos* were gathered from the Tirupur district in Tamil Nadu, India. Plants were identified and authenticated by Botanical Survey of India vide certificate no BSI/SRC/5/23/2024/Tech/892. After the collection plants being cleaned with water, the recently harvested leaves were promptly drenched with ethanol and allowed to dry at room temperature in the shade. In a mixer, the dried leaves were ground into a powder. For later use, the powdered plant leaf material was kept in sterile glass containers.

Extraction of Plant Materials: All the dried leaf powders were combined into various concentrations such as 1:1:1, 0.5:0.5:1, 0.5:1:0.5, 1:0.5:0.5 and 0.5:0.5:0.5. The manufacture of the extract was done using these blended concentration granules. The combination was steeped for 24 hours after 10 grams of each concentration powder were suspended in 100 ml of hot and cold distilled water. The clear solution was stored in a water bath at 80°C for two hours after the residues were filtered through Whatman No. 1 filter paper. For later usage, the dehydrated crude extracts were kept at 4°C (Chessbrough (2000).

Collection of Clinical Pathogens: The Government Hospital in Erode was where the clinical pathogens were collected. For the following investigation, isolates were kept on nutrient agar at 4°C. The conventional Gram-staining, biochemical, and selective plate methods were used to confirm the clinical pathogens. After streaking the stains on selective media, they were incubated for 24 hours at 37°C. *Escherichia coli* streaked on an EMB agar plate and *Staphylococcus sp*. on an MSA plate.

Antibacterial Activity of Combinatorial Leaf Extract: The usual agar well diffusion method was utilized to test the plant extract's antibacterial effectiveness against clinical pathogen microorganisms. After swabbing the cultures onto the nutrition agar plates, wells of 6 mm in diameter were created on the nutrient agar using gel pierce, and 100 µl of plant extract was added to the wells created using the gel borer. following a 24-hour incubation period at 37°C. Syeda and Riazunnisa (2020).

Qualitative Phytochemical Analysis: Using routine protocols, a preliminary screening for phytochemicals was conducted. Audu, Mohammed and Kaita,(2007). To analyze the phytoconstituents found in plants, tests for alkaloids, flavonoids, phenols, tannins, saponins, carbohydrates, glycosides, and proteins were conducted.

Quantitative Phytochemical Analysis: Alkaloids, flavonoids, carbohydrates, tannins, and phenols were all quantitatively analyzed using conventional techniques. Anandhu et al.,(2021).

Hemolytic Activity Of Combinatorial Leaf Extract: With a little modification of the Yang, Sun and Fang , (2005) approach, the cytotoxicity of plant extract to normal, healthy cells was examined using in vitro hemolytic activity. The plant extracts were streaked over a blood agar plate, then incubated for 48 hours at 37°C. Extracts that did not produce hemolysis were chosen for additional processing after incubation.

Green Synthesis of Silver Nanoparticles Using Combinatorial Leaf Extract: After being soaked for the whole night, 15g/l of plant powder was extracted in a water bath at 60–70 °C for 20–30 minutes and then chilled. Next, two ml of 0.1 M AgNO3 solution were added to eight ml of extract. It was heated in a water bath for 40 minutes at a temperature of 60°C. After 30 minutes, the reaction mix's color changed, but it was still incubated for 24 hours at room temperature to improve synthesis. Following incubation,

the mixture was centrifuged for 20 minutes at 5000 rpm to confirm the formation of nanoparticles by UV-visible spectroscopy. For additional characterization research, the resulting pellets were gathered, suspended three times in distilled water, then centrifuged to eliminate any remaining unbound biomass. The nanoparticles of silver were kept in storage.

Characterization of Silver Nanoparticles Using Combinatorial Leaf Extract Uv Absorption Spectrophotometric Analysis of Green Synthesized Silver Nanoparticles: The UV absorption spectrophotometer was used to analyze the produced silver nanoparticles. About 1 mg of dried nanoparticles and 9 ml of ethanol were combined to prepare the sample, which had an absorption wavelength in the 350–600 nm. Iman et al.(2023).

Fourier Transform Infrared Spectrophotometric Analysis of Green Synthesized Silver Nanoparticles: The functional compounds that comprise green produced silver nanoparticles are identified by FTIR analysis. A sample for examination was made by mixing 0.05 mg of nanoparticles with KCl. After that, the sample was put into an FTIR device, and the spectrum was captured. With a resolution of 4 cm-1, spectra were captured between 400 and 4000 cm-1. Wilson and Venkateshwari (2022).

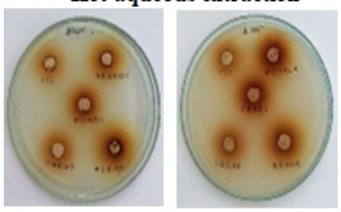
Table 1. Qualitative phytochemical analysis

PHYTO CHEMICALS	TEST	RESULT		
		C. fistula	*A. vasica*	*A. marmelos*
Alkaloid	Wangner'stest	+	+	+
lavonoid	Alkaline reagent test	+	+	+
Saponin	Foam test	-	+	-
Tannin	Ferric chloride test	-	-	+
Protein	Ninhydrin test	+	+	+
Phenol	Ferric chloride test	+	+	+
Steroids	Liebermann Burchard			
Reaction Test	-	-	-	
Glycosides	Kellerkilliani test	-	-	-
Carbohydrates	Fehling's test	+	+	+

(+) indicates positive result and (-) indicates negative result

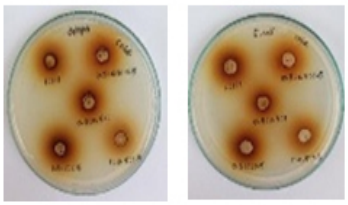
PLATE 1: Antibacterial activity using well diffusion method

Hot aqueous extraction



Staphylococcus sp., *Escherichia coli*

Cold aqueous extraction



Staphylococcus sp., *Escherichia coli*

Structure Elucidation of Green Synthesized Silver Nanoparticles: The crystalline size and structure of green produced silver nanoparticles were ascertained by X-ray diffraction examination. The Shimadzu MODEL XRD-6000 equipment, which can be operated at 40 Kv of voltage with Cu KαX-radiation (λ=0.15418), was coated with about 200 mg of green produced silver nanoparticles. The size of the particles was measured using the Debye-Scherrer equation [D = kλ/ßCos×]. Akintelu, Bo and Folorunso, (2020).

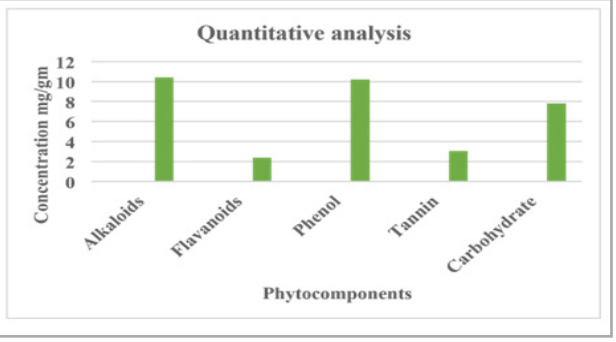
Antibacterial Activity of Green Synthesized Silver Nanoparticles: Using Muller Hinton Agar media at 37°C for 24 hours, the antibacterial activity of combinatorial silver nanoparticles at a concentration of 500 mg/ml was assessed using the agar well diffusion method. The diameter of the zone of inhibition was used to measure the development of microorganisms. Nair, Kalariya and Chanda, (2005).

RESULTS AND DISCUSSION

Antibacterial Activity of Combinatorial Leaf Extract: The bactericidal activity of combination aqueous (hot and cold) extracts of *Adhatoda vasica, Aegle marmelos*, and *Cassia fistula* was evaluated at five distinct concentrations. The antibacterial activity was evaluated using the agar well diffusion method shown in plate no 1. The average zone of inhibition for each isolate was used to illustrate the results in both hot and cold aqueous extracts. The zone in Seemaisamy

et al., (2019) was similar to the aqueous extract of *C. fistula, A. vasica* and *A. marmelos* of *Staphylococcus sp*., and *Escherichia coli.*

Figure 1: Quantitative phytochemical analysis



Qualitative Phytochemical Analysis: *Aegle marmelos, Adhatoda vasica*, and *Cassia fistula* were the three different plants that underwent qualitative phytochemical study. The majority of the phytoconstituents in the leaves were alkaloids, flavonoids, protein, phenol, and carbs. *Cassia fistula* aqueous extract was subjected to a qualitative phytochemical examination. Alkaloids, flavonoids, protein, phenol, and carbs were found in a plant. Similar results were observed in Ali, (2014). Protein, carbohydrates, alkaloids, flavonoids, and saponins are all present in the *Adhatoda vasica* aqueous extract. Comparable outcomes were documented in Patel and Patel, (2023). *Aegle marmelos*'s aqueous extract contains protein, carbs, tannin, alkaloids, and flavonoids. Results for Ratnampally and Venkateshwar (2017) were comparable. Table 1 displays the results of the qualitative phytochemical investigation.

Table 2. Hemolytic activity of combinatorial leaf extract

DIFFERENT RATIOS OF AQUEOUS (HOT & COLD) EXTRACTS	RESULT
Hot (C.f:A.v:A.m-1:1:1)	No hemolysis
Hot (C.f:A.v:A.m- 0.5:0.5:1)	No hemolysis
Hot (C.f:A.v:A.m-1:0.5:0.5)	Hemolysis
Hot (C.f:A.v:A.m- 0.5:1:0.5)	Hemolysis
Hot (C.f:A.v:A.m- 0.5:0.5:0.5)	Hemolysis
Cold (C.f:A.v:A.m- 1:1:1)	Hemolysis
Cold (C.f:A.v:A.m- 0.5:0.5:1)	Hemolysis
Cold (C.f:A.v:A.m- 1:0.5:0.5)	Hemolysis
Cold (C.f:A.v:A.m- 0.5:1:0.5)	Hemolysis
Cold (C.f:A.v:A.m- 0.5:0.5:0.5)	Hemolysis

C.f – *Cassia fistula*; A.v- *Adhatoda vasica*; A.m- *Aegle marmelos*

Figure 2: UV Spectroscopy analysis of silver nanoparticles

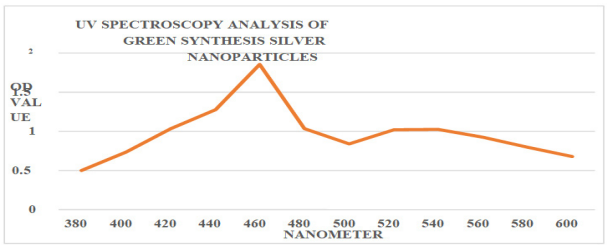


Figure 3: Ftir analysis of silver nanoparticles

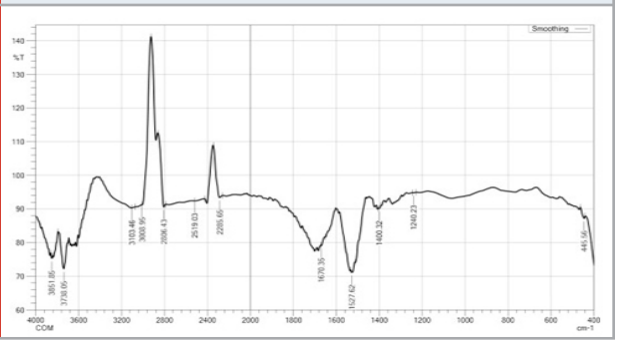
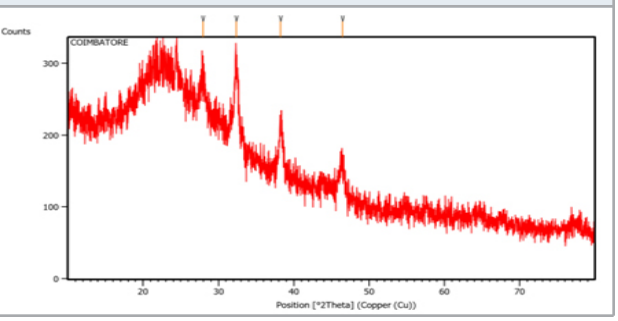


Table 3. Ftir analysis of silver nanoparticles

Frequency cm-1	Bond	Functional group
3851.85	O-H stretch, free hydroxyl	Alcohols, Phenols
3738.05	O-H stretch, free hydroxyl	Alcohols, Phenols
3103.46	=C-H stretch	Alkenes
3008.95	=C-H stretch	Alkenes
2806.43	H-C=O:C-H stretch	Aldehyde
2519.03	O-H stretch	Carboxylic acid
2285.65	O=C=O stretch	Carbon dioxide
1670.35	-C=C- stretch	Alkenes
1527.62	N-O asymmetric stretch	Nitro compounds
1400.32	C-C stretch	Aromatics
1240.23	C-N stretch	Aliphatic amines
445.56	C-Br stretch	Alkyl halides

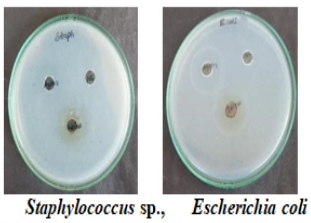
Figure 4: Xrd analysis of silver nanoparticles



Quantitative Phytochemical Analysis of Combinatorial Leaf Extract: The quality of the phytochemical components contained in the leaf extracts from *Cassia fistula, Adhatoda vasica,* and *Aegle marmelos* was assessed. The phytochemical components were examined using the hot aqueous extract at a 1:1:1 concentration is tannins (3.036), phenol (10.2), alkaloids (10.4 mg/gm), flavonoids (2.4 mg/gm), and carbohydrates (7.8%). It was found that the flavonoid content was lower, and the alkaloid concentration was higher than other phytochemical components. Priya et al.,(2010) offered comparable explanations. The findings of a quantitative phytochemical analysis are displayed in Figure 1.

Hemolytic Activity of Combinatorial Leaf Extract: To assess their cytotoxic effect, combinatorial leaf extracts were examined for hemolytic activity in blood agar plates. The findings indicate that the hemolytic activity of plant extracts was demonstrated by the concentrations of hot

Plate 2: Antibacterial activity of green synthesized silver nanoparticles



Staphylococcus sp., *Escherichia coli*

A-Silver nitrate solution; B- Green synthesized nanoparticles; C- Control nanoparticle

(0.5:1:0.5, 1:0.5:0.5, 0.5:0.5:1) and cold (1:1:1, 1:0.5: 0.5, 0.5:0.5:1, 0.5:1:0.5, and 0.5:0.5:0.5). wherein the plant extracts' non-hemolytic activity was demonstrated by the hot (1:1:1 and 0.5:0.5:1). Plant extracts with non-hemolytic activity were used for other purposes Kalita et al. (2011), and the hemolytic activity of a plant extract in water was examined. Mathur et al., (2011) talked about the plant's aqueous extract's non-hemolytic action, Table 2 presents the results.

Table 4. Antibacterial activity of silver nanoparticles

Organisms	Zone Of Inhibition(Mm) Silver Nanoparticle		
	AgNPs	AgNO3	NC
Staphylococcus sp.,	13 mm	R	R
Escherichia coli	15 mm	10 mm	R

R- Resistant; AgNPs- Silver nanoparticles; AgNO3- Silver nitrate solution; NC- Negative Control

Green Synthesis of Silver Nanoparticles Using Combinatorial Leaf Extract: Silver nanoparticles were created using the green synthesis technique. For the synthesis of silver nanoparticles, a nonhemolytic ratio of hot 0.5:0.5:1 was used from the earlier investigation. The color shift from yellow to brown signifies the formation of silver nanoparticles. With the use of UV-visible spectroscopy, it was further verified. The color shift from yellow to brown indicated the presence of silver nanoparticles. There was a correlation between the outcomes with Pang et al.,(2020).

Characterization of Silver Nanoparticles Using Combinatorial Leaf Extract Uv Absorption Spectrophotometric Analysis of Green Synthesis of Silver Nanoparticles: A UV absorption spectrophotometer was used to absorb the green produced silver nanoparticles. The peaks demonstrate that the creation of silver nanoparticles was indicated by the combinatorial leaf extract. The observed silver nanoparticles were in the 380–600 nm range. The synthesis of nanoparticles at 460 nm was indicated by the largest peak, which also showed the formation of nanoparticles at that wavelength. The descending peaks were anticipated. In Lakkim et al.(2020, the same findings were discussed. The outcomes of UV absorption spectroscopy are displayed in Figure 2.

Fourier Transform Infra Red Spectrophotometric Analysis of Green Synthesized Silver Nanoparticles: The functional group of the nanoparticles determines their stability and activity. In order to determine the various functional groups present in green produced silver nanoparticles, FTIR analysis was carried out. Peaks were used to represent the functional groups. The several functional groups, including alcohols, phenols, alkenes, carboxylic acids, nitro compounds, aromatics, aliphatic amines, aldehyde, carbon dioxide, and alkyl halides, are indicated by the green produced silver nanoparticles. The formation of silver nanoparticles was announced by the following functional groups. Although it serves as a capping and reducing agent throughout the silver nanoparticle process, the leaf extract was primarily covered with the nanoparticles. It was determined by the investigation that the leaf extract was used to create the silver nanoparticles. The combinatorial leaf extract was used to create silver nanoparticles, according to the results of the FTIR study. Kale and Dandge (2020) stated that green produced silver nanoparticles had the same functional groups. The FTIR analysis is displayed in Figure 3 and Table 3.

X Ray Diffraction of Combinatorial Leaf Extract: XRD analysis was used to examine the green produced silver nanoparticles amorphous and crystalline structures. The primary peaks and associated planes were identified by comparing the XRD data with the JCPDS file 040783. The peaks that were found were 38.269 for (111) and 46.442 for (200). In the (111) plane, the green produced silver nanoparticles' face-centered cubic (fcc) shape was clearly visible. According to Wilson and Venkateshwari (2022), the planes were a sign that silver nanoparticles were forming. Figure 4 illustrates the existence of XRD analysis structural elucidation.

The size of the green produced silver nanoparticles was determined using the Debye-Scherrer equation, $D = k\lambda / \beta Co_s$. Green produced silver nanoparticles ranged in size from 15 to 30 nm, with the greatest being 181.36 nm and

the smallest being 159.76 nm. According to these findings, the current investigation showed rapid estimates, with the smallest size created at 15.71 nm, which corresponds to (111), and the highest size of synthesized silver nanoparticles from the combinatorial leaf extract being 23.63 nm (200).

Antibacterial Activity of Green Synthesized Silver Nanoparticles: Combinatorial leaf extract's silver nanoparticles antibacterial properties were investigated. Green produced silver nanoparticles demonstrated a 13 mm zone of inhibition for Staphylococcus sp. and a maximal zone of 15 mm for Escherichia coli. The findings make it abundantly evident that silver nanoparticles have strong antibacterial action through zone formation. Zones obtained by Khadka et al. (2020) were nearly identical. Table 4 and Plate 2 displayed the findings.

CONCLUSION

The hot and cold leaf extracts show the existence of different phytochemical components, according to the research mentioned above. In a hot 1:1:1 aqueous extract, the combinatorial leaf extract of all three plants included 10.4 mg of alkaloids, 10.2 mg of phenol, 3.03 mg of tannin, 2.4 mg of flavonoids, and 7.8 mg of carbohydrates. Combinatorial leaf extracts are used to create green-manufactured silver nanoparticles, which display a wide variety of phytochemical components. When compared to plant-based antibacterial agents, silver nanoparticles showed increased antibacterial activity. When compared to other commercial antimicrobial medications, the green synthesis approach has several advantages, including being easy to use, quick, inexpensive, and most significantly, environmentally benign. Green-manufactured silver nanoparticles successfully inhibit the microorganisms.

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Enzyme Activity profile of Crude and Purified Xylanase and Cellulase Produced Using Substrate Fermentation from Indigenously isolated *Streptomyces lividans*

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ABSTRACT

The increasing awareness about sustainable processes mandates the exploration of lignocellulosic agrowaste for the production of valuable industrial enzymes like xylanase and cellulase. This study investigates xylanase and cellulase production from an indigenously isolated *Streptomyces lividans* through substrate fermentation of agrowaste, highlighting the significance of optimizing various parameters for enhanced enzyme activity. The rationale behind this study stems from the urgent need of sustainable solutions to manage agricultural waste while producing commercially important xylanase and cellulase. A systematic approach involving response surface methodology was employed to evaluate the effects of key factors such as temperature (20-60 °C), pH (2-8), substrate concentration (5-25 mg %), ammonium sulphate, and dextrose on enzyme production. The specific enzyme activities under optimal conditions were found to be 4.21 µmol/mg-minute for xylanase and 3.97 µmol/mg-minute for cellulase, with substrate concentration emerging as the primary contributor to enzyme yield, as indicated by the experimental design matrix and three-dimensional contour plots. These findings underscore the industrial relevance of *Streptomyces lividans* in enzyme production and suggest avenues for further research in optimizing fermentation conditions. Future studies should focus on large-scale applications and the potential enhancement of enzyme activity through genetic manipulation, thereby improving the viability of xylanase and cellulase in various industries, including biofuels, textiles, and bioremediation.

KEY WORDS: ACTIVITY; PURIFIED XYLANASE; FERMENTATION; *STREPTOMYCES LIVIDANS*,

INTRODUCTION

The need to save resources, combined with pollution issues from agro-industrial waste and limited disposal options, has encouraged the bioconversion of waste into industrially valuable products (Freitas et al. 2021; Phiri et al. 2024). Considerable amounts of agricultural biomass deemed 'waste' is now being explored for the production of biofuels, animal feed, chemicals, and enzymes, among other value-added items (Singh et al. 2021; Velvizhi et al. 2022; Alazaiza et al. 2025).

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For example, abundantly and the cheaply available lignocellulosic agrowaste such as vegetable and fruit peels (Panda et al. 2016), rice and wheat husk (Devi et al. 2022; Abena and Simachew 2024), corn cobs (Devi et al. 2022; Joshi et al. 2023), sugarcane bagasse (Devi et al. 2022; Yadav et al. 2022), coconut coir (Khangwal and Shukla 2022), and wood pulp (Walia et al. 2013; Singh et al. 2023) etc. are increasingly being explored for production of important hydrolytic industrial enzymes such as xylanase and cellulase (Sadh et al. 2018; Waheeb et al. 2021; Yafetto 2022).

The polysaccharide components of lignocellulosic agrowaste are cellulose and hemicellulose. Cellulases consisting of endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and b-glucosidase (EC 3.2.1.21) act synergistically to convert the cellulose complex of biomass into glucose and

oligosaccharide (Fatokun et al. 2016a; Tingthong et al. 2021). Xylanase is the class of enzymes which convert the polysaccharide beta-1,4-xylan into xylooligosaccharide, xylobiose and xylose subunits (Pandey et al. 2022; Chaudhary et al. 2023). Xylanase plays an important role in the conversion of the hemicellulose component of biomass into xylose, (Sarangi and Thatoi 2024).

Xylanases are the enzyme complexes responsible for hydrolysis of xylan, and they include endo-1,4-xylanases (EC 3.2.1.8) and -xylosidases (EC 3.2.1.37), in addition to side-chain-hydrolyzing enzymes (Kumar et al. 2014; Fatokun et al. 2016a). Xylanase and cellulase are important industrial enzyme that have series of applications in food, textile, agriculture, paper pulp and the bioremediation industry (Rodrigues and Odaneth 2021; Basit et al. 2021).

The global market for industrially useful enzymes was estimated to be about $ 12.27 billion in the year 2022 and is expected to develop at a compound annual growth rate (CAGR) of approximately 6.5% over the period from 2023 to 2030 (Li et al. 2012; Grand View Research 2023).

Bacteria and fungi can secrete multiple lignocellulolytic enzymes, including xylanases and cellulases (Gudynaite-Savitch and White 2016; Talamantes et al. 2016). However, in recent times, bacterial enzymes for industrial applications are continually increasing due to their rapid growth rate and tenacity to be genetically engineered in contrast to fungi (Danso et al. 2022). Correspondingly, a variety of xylanase and cellulase-producing bacteria have been applied to lignocellulosic biomass for production of enzymes (Menendez et al. 2015; Chakdar et al. 2016; Kaur et al. 2024).

Table 1: RSM experimental runs and response

Standard	Run	Factor 1 A: Ammonium sulphate (g)	Factor 2 B: Dextrose (g)	Factor 3 C: Substrate concentration (g)	Response 1 Cellulase (U/mL)	Response 2 Xylanase (U/mL)
15	1	2.25	2.75	2	425.13	423.09
10	2	4.35224	2.75	2	356.06	364.08
1	3	1	1.5	1	372.09	305.79
16	4	2.25	2.75	2	429.45	419.98
6	5	3.5	1.5	3	418.42	438.84
8	6	3.5	4	3	453.55	460.32
13	7	2.25	2.75	0.318207	247.58	158.29
3	8	1	4	1	398.52	346.6
17	9	2.25	2.75	2	432.19	428.14
2	10	3.5	1.5	1	220.48	158.07
9	11	0.147759	2.75	2	393.47	396.11
20	12	2.25	2.75	2	421.78	417.69
14	13	2.25	2.75	3.68179	412.89	433.27
7	14	1	4	3	390.06	390.1
12	15	2.25	4.85224	2	471.02	428.95
5	16	1	1.5	3	430.38	432.79
18	17	2.25	2.75	2	440.52	432.28
4	18	3.5	4	1	353.64	297.28
19	19	2.25	2.75	2	423.98	420.34
11	20	2.25	0.647759	2	426.13	384.31

Streptomyces is one of the most important genera of Actinomycetes involved in fermentation process and it has been widely used for enzyme production (Javed et al. 2021; Khushboo et al. 2022). For example, recently Shrestha et al., reported production of multi-enzyme (pectinase, cellulase, and xylanase) from Streptomyces sp. using agrowaste mixture (Shrestha et al. 2022). Sinjaroonsak et al., recently optimized production of xylanase and cellulase by *Streptomyces thermocoprophilus* TC13W from pretreated empty fruit bunch of oil palm (Sinjaroonsak et al. 2020). Advantages of *Streptomyces* derived enzymes include their high level of extracellular activity, stability across a broad temperature (50–85 °C) and pH (pH 3–13) range (Kumar et al. 2019). Researchers are working to isolate novel *Streptomyces* strains from unexplored habitats for exploring the potential and application of enzymes secreted by them (Kumar et al. 2020).

Enzyme activity is influenced by numerous parameters such as pH, temperature, inducers, medium additives, and type of fermentation (Bhardwaj et al. 2019).

Furthermore, enzyme activation is dependent on various metal ions as activators or inhibitors. For this reason, optimization and in-depth analysis of these parameters are a must for maximizing enzyme activity. Here, we have employed submerged fermentation processes (SmF) for the production of xylanase and cellulase. Advantages of SmF include homogeneous conditions in the media, better biomass utilization, and easy scale-up. It has been reported that SmF enables better biomass utilization and therefore results in higher enzyme production (Hansen et al. 2015; Joshi et al. 2023).

This study aimed to study the influence of parameters such as pH, temperature, substrate and enzyme concentration for optimum enzyme production using response surface methodology (Siwach et al. 2024). Enzyme activity of crude and purified xylanase and cellulase produced from indigenously isolated *Streptomyces lividans* was studied using one factor at a time approach. Cultured broth media was filtered and taken as crude enzyme. Enzyme purification was carried out from broth media using spin column and gel electrophoresis (Boucherba et al. 2014; Joshi et al. 2023).

MATERIAL AND METHODS

Optimization using Response Surface Methodology: Response surface methodology was employed to get optimum conditions of significant variables. Three independent variables, namely, ammonium sulphate (g) and dextrose (g), and substrate (g) were assessed for the dependent response variable i.e., cellulase and xylanase enzyme production (U/mL), Table 1. State Ease Design-Expert V 10.0.1 software was used for multiple non-linear regression of response for each run to obtain the coefficient of the polynomial equation. Response surface methodology processed by use of central composite design (CCD). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The statistical significance of the model was determined by the F-test and the goodness of fit of the estimated polynomial equation was assessed based upon the coefficient of determination (R2), response surface and contour plots (Vaishnav et al. 2022).

Bacterial strain : From our previous work we screened bacteria efficient in production of cellulase and xylanase using agricultural waste as substrate. Based on 16S rRNA analysis the indigenously isolated bacterium was identified as *Streptomyces lividans*. Gram's staining was carried out to study cultural and morphological characteristics. Nutrient agar was used to study colony characteristics. The strain was maintained on nutrient agar slant and stored at 4 °C.

Crude enzyme and Purified enzyme : Sterilized basal salt media (yeast extract, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $(NH_2)_2SO_4$, NaC_l, $CaCO_3$) containing 2 g of corn cob for xylanase or coconut husk for cellulase was inoculated with Streptomyces lividans and incubated for 72 hours at 37 °C. The broth was filtered and taken as crude enzyme. 100 mL of crude xylanase/cellulase enzyme was concentrated by spin column chromatography to give 10 mL of purified enzyme.

Protein (enzyme) characterization: Crude and purified protein (enzyme) was characterised for molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 3 mm gel slab. Coomassie brilliant blue G-250 was used as a stain. Broad range molecular weight marker was used for molecular weight determination. Crude and purified enzyme was characterised using SLS One Stop Protein Purification mini prep protocol.

The 10 mL of crude supernatant after xylanase/cellulase production was incubated at 37°C for 30 minute and mixed with equal volume of precipitation and binding buffer. The system was incubated on ice for 2-5 minute and then centrifuged for 10, 000 RPM for 5 minute.

The supernatant was removed and the pellet was resuspended in 500µl of resuspension buffer. The solution was transferred to SLS One-step protein Spin Column and centrifuged at 10,000 RPM for 1 minute.

The spin column was placed into a brand-new collecting tube, and 100µl of elution buffer was added to it. The tube was incubated at room temperature for 1 minute. and then centrifuge at 10,000 RPM for 1 minute. The eluted protein (1 mL) was collected for further analysis.

Effect of influencing parameter The effects of influencing parameters, such as temperature, pH, substrate concentration, and enzyme amount, were investigated. Enzyme activity of parameters was assayed by measuring released sugar using DNSA reagent (Miller 1959). Briefly, 1 mL of crude/purified enzyme was mixed with 1 mL of 1 % substrate (CMC for cellulase and Xylan for xylanase) in addition to 2 mL of buffer. After 10 minutes of incubation at ambient room temperature (~30 + 3 oC), 1 mL DNSA was added Enzyme activity (µmol/minute) was calculated using equation 1.

$$\text{Enzyme activity } \left(\frac{\mu mol}{min}\right) = \frac{\text{Sugar released } (\mu g) \times 1000}{\text{Molecular weight of sugar } \left(\frac{g}{mol}\right) \times \text{Incubation time } (min)}$$

Here, incubation time was 10 minutes. Molecular weight of xylose and cellulose is 150.13 g/mol and 162.14 g/mol, respectively. Enzyme activity was investigated for influencing parameters such as temperature, pH, substrate concentration and enzyme amount.

RESULTS AND DISCUSSION

Cultural and Morphological Characteristics: To verify the phenotypic characteristics of the KJB17 strain, the bacteria was grown on Nutrient Agar plate. The resulting colony was slightly raised, colourless colony with rough appearance on Nutrient Agar, Figure 1 A. Additionally, the KJB17 strain colony was also grown on Basal Salt Agar plate on which it gave distinctly pink colonies as can be seen in Figure 1 B. Furthermore, on Nutrient agar as well as Basal salt agar media plate, the KJB17 strain gave luxurious growth, Figure 1. Based on microscopic observation of Gram staining, KJB17 strain is Gram positive, long rod and non-capsulating, Figure 2, based on Bergey's Manual

and predicted values of xylanase and cellulase production. Increasingly, RSM optimization is being studied for optimum enzyme production (Amadi et al. 2020; Siwach et al. 2024).

For determining regression model fitting was significant or not significant, ANOVA analysis was conducted for xylanase and cellulase which is summarised in Table 4 and Table 5, respectively. ANOVA of regression model demonstrated model was highly significant based on Fisher's F-test with high F-value and p-value. The p-value checks significance of each coefficient, and indicates interaction strength between each independent parameter. F-value of 236.07 and 109.10 implies model is significant for xylanase and cellulase, respectively. Smaller p-value (here, $p < 0.05$ for xylanase and cellulase) indicates higher significance of corresponding coefficient. As can be seen from Table 4 and Table 5, A, B, C, AB, AC, BC, A2, B2, and C2 are significant for xylanase and cellulase production, respectively.

Table 2. Cultural Characteristics on Nutrient agar.

Shape	Size	Colony appearance	Margin	Elevation	Texture	Gram's reaction	Pigment
Round	Medium	Rough	Entire	Slightly Raised	Dry	Gram Positive	Nil

Figure 1: Colony characteristics of KJB17 on A. Nutrient Agar plate, B. Basal Salt Agar plate

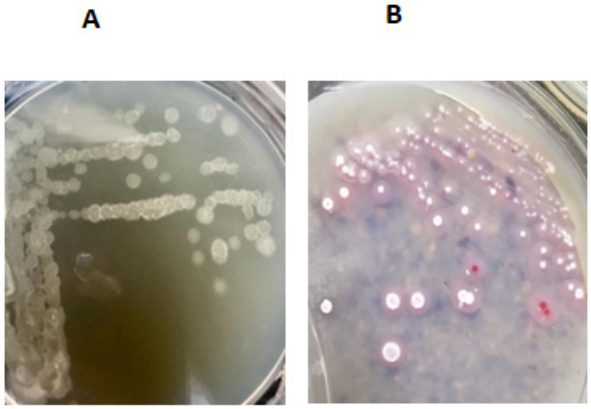


Figure 2. Gram staining of KJB17 showing Gram positive stain

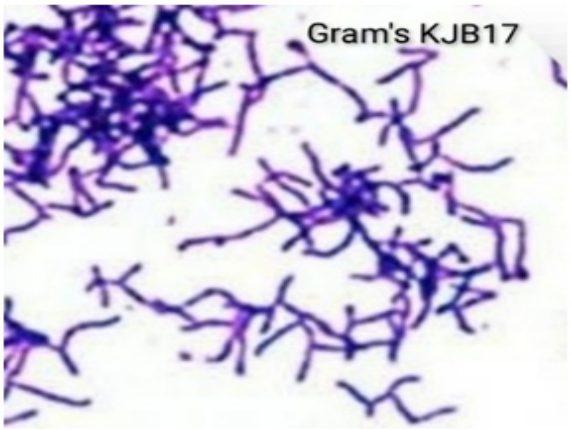
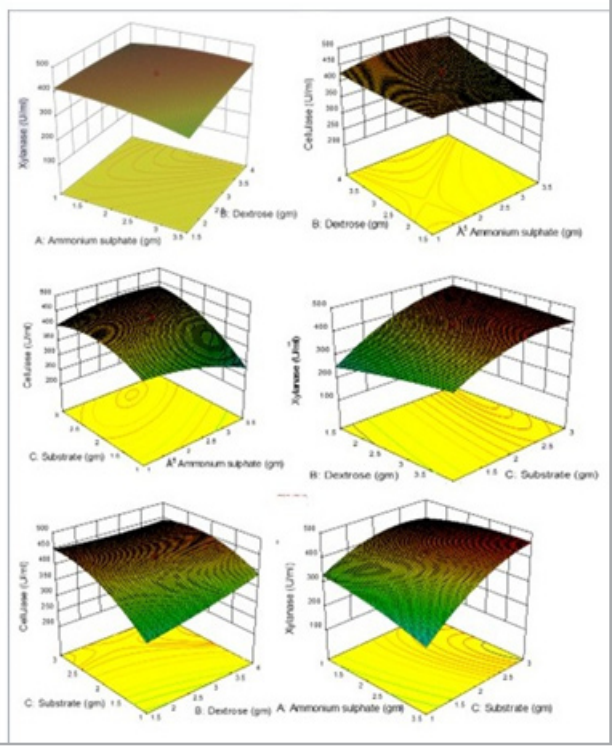


Figure3. Response surface plots of xylanase and cellulase showing interaction of three variables (Ammonium sulphate, dextrose, and, substrate concentration).



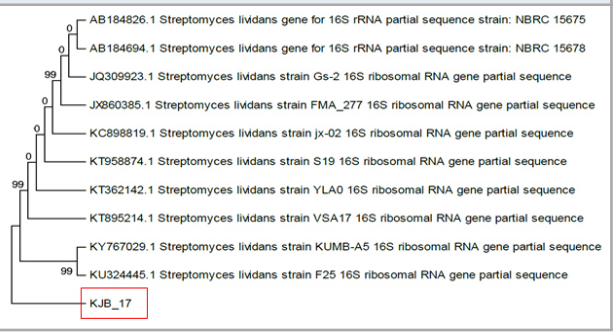
The "Lack of Fit F-value" of 3.29 with corresponding p-value of 0.10 for xylanase (Table 4) and 2.23 with corresponding p-value of 0.19 for cellulase (Table 5) implies the Lack of Fit is not significant relative to the pure error. Insignificant lack of fit confirmed the validity of model. Furthermore, statistical calculation to determine the goodness of the model for experimetal desgin of xylanse and cellulase are depicted in Table 6. For xylanase, "Pred R-Squared" of 0.9695 is in reasonable agreement with the

"Adj R-Squared" of 0.9911, i.e. the difference is less than 0.2. Likewise, for cellulase, "Pred R-Squared" of 0.9401 is in reasonable agreement with the "Adj R-Squared" of 0.9808, i.e. the difference is less than 0.2. "Adeq Precision" measures the signal to noise ratio and ratio greater than 4 is desirable. Adeq Precision value of 53.13943 and 40.13299 for xylanase and cellulase reveal an adequate signal. Thus, based on all these results, this model can be used to navigate xylanase and cellulase production.

Table 3. Experimental design matrix of RSM with experimental and predicted values of xylanase and cellulase enzyme

Run Order	Xylanase		Cellulase		
	Actual Value	Predicted Value	Actual Value	Predicted Value	Standard Order
1	423.09	423.6934	425.13	428.9125	15
2	364.08	356.7232	356.06	347.927	10
3	305.79	301.1914	372.09	366.268	1
4	419.98	423.6934	429.45	428.9125	16
5	438.84	442.9705	418.42	423.1065	6
6	460.32	467.5572	453.55	461.124	8
7	158.29	161.3139	247.58	251.9438	13
8	346.6	345.1081	398.52	395.5854	3
9	428.14	423.6934	432.19	428.9125	17
10	158.07	166.6463	220.48	228.2753	2
11	396.11	399.7353	393.47	399.1254	9
12	417.69	423.6934	421.78	428.9125	20
13	433.27	426.5145	412.89	406.0486	14
14	390.1	384.1623	390.06	384.0167	7
15	428.95	433.5664	471.02	475.647	12
16	432.79	440.8606	430.38	437.0892	5
17	432.28	423.6934	440.52	428.9125	18
18	297.28	291.848	353.64	348.6827	4
19	420.34	423.6934	423.98	428.9125	19
20	384.31	375.9621	426.13	419.0253	11

Figure 4. Phylogenetic tree of KJBs17 (Streptomyces lividans)



Zhang et al. 2016 studied optimisation of xylanase and cellulase from *Streptomyces griseorubens* JSD-1 using RSM for improved enzymatic saccharification efficiency. Walia et al., 2015 (Walia et al. 2015) studied xylanase from *Cellulosimicrobium cellulans* CKMX1 and its improvement using RSM.

The three-dimension (3D) contour plots were prepared to understand the effect of three variables, i.e., ammonium sulphate, dextrose, and substrate concentration on xylanase and cellulase production, Figure 3. 3D contour plots visual interaction between two variables by fixing third variable at zero level. All graphs clearly reveal influence of substrate concentration is major contributor for xylanase and cellulase production.

Molecular identification by 16s rRNA gene technology: ABI 3730xl Genetic Analyzer was used to carry out the DNA sequencing reaction of the PCR amplicon with prime r27F using BDT v3.1 Cycle sequencing kit for molecular identification. The BLAST search of 16S rRNA gene sequence against sequences in nucleotide database showed 100% homology with *Streptomyces lividans* strain, Fig. 4. Thus, the new indigenously isolated strain was identified as *Streptomyces lividans* (Thomas et al. 2013). The genomic sequence is uploaded on National Center for Biotechnology Information (NCBI) portal with accession number of PP528163. The genomic sequence of identified *Streptomyces lividans* is *Streptomyces* is described as a Gram positive, spore-forming, and aerobic actinobacterium,

of Systematic Bacteriology (Beg et al. 2001; Abdulkhair et al. 2025).

Response surface methodology: For RSM analysis, twenty different experimental runs were design to study enzyme production response according to runs provided by minimum and maximum value of ammonium sulphate, dextrose, and substrate concentration, as listed in Table 1. Table 3 represents experimental design matrix with actual

which has a high guanine-cytosine (GC) content in the genome and contains LL-diaminopimelic acid in the cell wall. *Streptomyces* are reported to produce amylase (Al-Dhabi et al. 2020; Lakshmi et al. 2020), protease (Sarkar and Suthindhiran 2020), cellulase (Danso et al. 2022), xyalanase (Porsuk 2013; Danso et al. 2022), nitrate reductase (Zhang et al. 2021), and catalase (Kim et al. 2013) to hydrolyze starch, protein, cellulose, xylan, nitrate, and hydrogen peroxide, respectively.

Table 4. Analysis of variance (ANOVA) table for Response Surface Quadratic model for Xylanase enzyme

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	141127.8	9	15680.86	236.0699	1.90209E-10	Significant
A-Ammonium sulphate	2233.191	1	2233.191	33.61992	0.000173366	Significant
B-Dextrose	4005.483	1	4005.483	60.30115	1.52675E-05	Significant
C-Substrate	84897.47	1	84897.47	1278.102	6.96241E-12	Significant
AB	3303.626	1	3303.626	49.73493	3.48902E-05	Significant
AC	9337.295	1	9337.295	140.5697	3.27184E-07	Significant
BC	5061.689	1	5061.689	76.20196	5.44057E-06	Significant
A^2	3723.496	1	3723.496	56.05593	2.09372E-05	Significant
B^2	645.4682	1	645.4682	9.717298	0.010924847	Significant
C^2	30340.5	1	30340.5	456.7656	1.12078E-09	Significant
Residual	664.2466	10	66.42466			
Lack of Fit	509.3735	5	101.8747	3.288972	0.108646708	Not significant
Pure Error	154.8731	5	30.97463			
Cor Total	141792	19				

Table 5. Analysis of variance (ANOVA) table for Response Surface Quadratic model for Cellulase enzyme

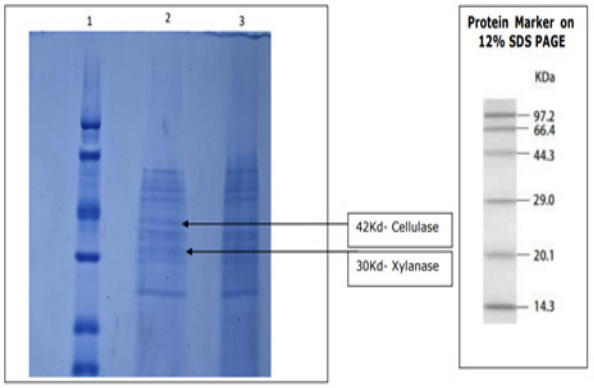
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	74609.29	9	8289.921	109.0996	8.64E-09	Significant
A-Ammonium sulphate	3164.153	1	3164.153	41.64189	7.32E-05	Significant
B-Dextrose	3870	1	3870	50.9312	3.15E-05	Significant
C-Substrate	28666.7	1	28666.7	377.2686	2.86E-09	Significant
AB	4148.694	1	4148.694	54.59896	2.34E-05	Significant
AC	7689.24	1	7689.24	101.1944	1.51E-06	Significant
BC	3394.056	1	3394.056	44.66753	5.48E-05	Significant
A2	5526.093	1	5526.093	72.72623	6.7E-06	Significant
B2	611.4555	1	611.4555	8.047071	0.017647	Significant
C2	17983.98	1	17983.98	236.6785	2.74E-08	Significant
Residual	759.8486	10	75.98486			
Lack of Fit	524.6043	5	104.9209	2.230041	0.199675	Not significant
Pure Error	235.2443	5	47.04886			
Cor Total	75369.14	19				

Crude and purified protein was characterised using the SLS one-stop Nontagged protein purification column per standard protocol. The column is specifically designed with

combination of immobilized metal affinity chromatography and boronic acid-based resins for efficient protein

purification. The molecular weight of crude and purified enzyme was determined using SDS-PAGE. As can be seen from Figure 5, there are several fragments of crude and purified protein. Molecular mass of denatured protein was between 66.4 to 20.1 kDa as measured by comparative movement of proteins on SDS-PAGE (Wu et al. 2018). The obtained results are in consistent with that obtained by (Waheeb et al. 2021b; Abdulkhair et al. 2025).

Figure 5. Electrophoresis on agar gel



No.	Sample Name
1	Marker
2	KJB17 (Purified)(Band observed around 42 and 30 KDa)
3	KJB17 (Crude)

Table 6. Statistical calculation of RSM experimental design for xylanase and cellulase

	Xylanase	Cellulase
Std. Dev.	8.150132	8.716929
Mean	376.816	395.867
C.V. %	2.162894	2.201984
PRESS	4321.114	4515.445
R-Squared	0.995315	0.989918
Adj R-Squared	0.991099	0.980845
Pred R-Squared	0.969525	0.940089
Adeq Precision	53.13943	40.13299

Figure 6. Temperature effect on enzyme activity of crude and purified enzyme. A. Xylanase B. Cellulase

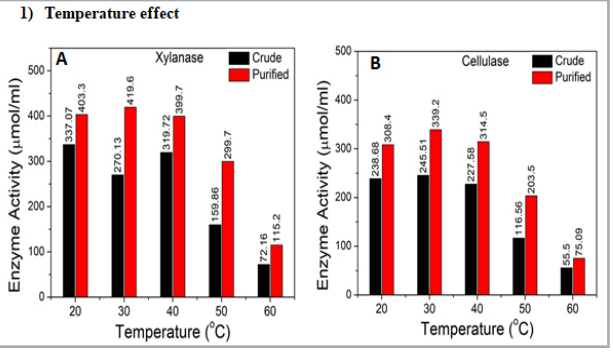


Figure 7. pH effect on enzyme activity of crude and purified enzyme. A. Xylanase B.

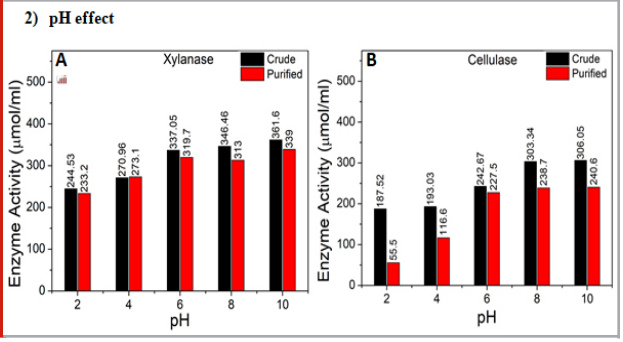


Figure 8. Substrate concentration effect on enzyme activity of crude and purified enzyme. A. Xylanase B. Cellulase

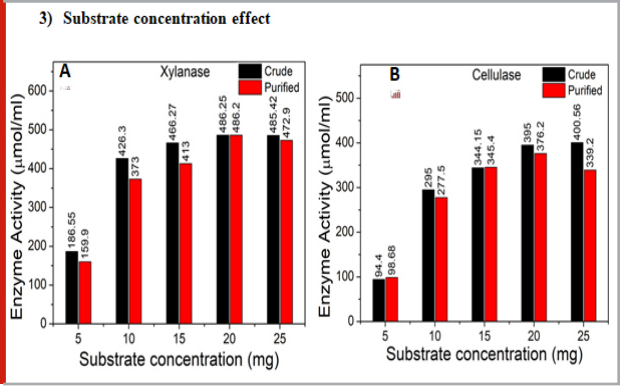
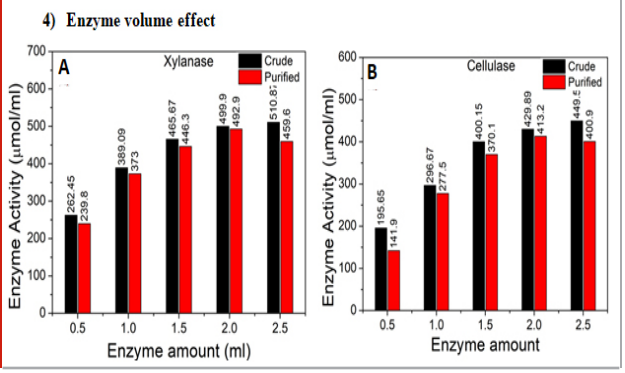


Figure 9. Enzyme volume effect on enzyme activity of crude and purified enzyme. A. Xylanase B.

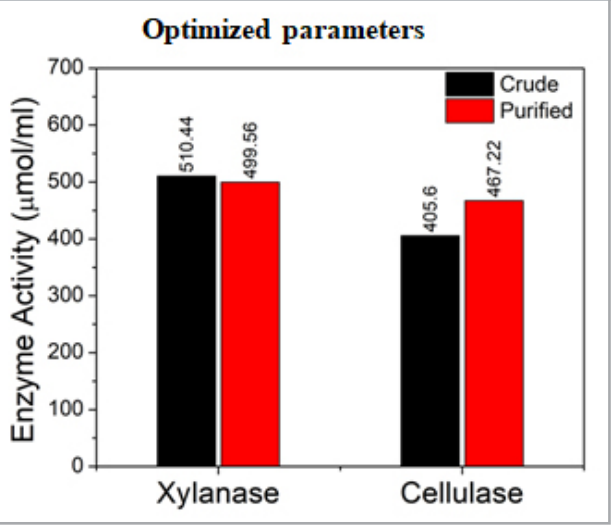


Enzyme activity and its influencing parameter: Enzyme activity was investigated for influencing parameters such as temperature, pH, substrate concentration and enzyme amount. Enzyme activity was measured using DNSA method (Miller 1959; Islam and Roy 2018). Several studies have studied optimization of influencing parameters for enzyme activity for xylanase (Techapun et al. 2002; Khurana et al. 2007; Fatokun et al. 2016b; Sinjaroonsak et

al. 2020) and cellulase (Fatokun et al. 2016b; Sinjaroonsak et al. 2020).

The effect of temperature in the range of 20 to 60 oC on enzyme activity of crude and purified xylanase and cellulase was determined, Figure 6. As can be seen purified enzyme of xylanase and cellulase reported higher activity than crude enzyme for all studied temperature, Figure 6 Xylanase and cellulase show good activity at within temperature range of 20 to 40oC, Figure 5. Maximum activity of 419.6 µmol/mL (Figure 6A) and 339.2 µmol/mL (Figure 6B) for purified xylanase and cellulase respectively was reported at 30oC. Maximum activity for crude xylanase of 337.07 µmol/mL (Figure 6A) and cellulase of 245.51 µmol/mL (Figure 6B) was reported at 20oC and 30oC, respectively. Similar results have been reported by Leya et al., for Streptomyces sp. (Thomas et al. 2013; Sinjaroonsak et al. 2020).

Figure 10. Influence of optimized parameters on enzyme activity of crude and purified Xylanase and Cellulase



Temperature is important parameter for optimum enzyme production due to alterations in microbial protein structure and its properties with variations in temperature (Juturu and Wu 2014; Li et al. 2025).

Cellulase: The effect of initial pH in the range of 2 to 10 were investigated to understand its influence on enzyme activity of crude and purified xylanase and cellulase, Figure 7. pH of growth media was regulated with 0.1 N HCl/NaOH. From the results it can be seen that pH 8 and 10 are optimum for xylanase, Figure 7 A and cellulase, Figure 7 B, with respect to crude as well as purified. In addition, clearly for all pH studied crude for cellulase reported better activity than purified, Figure 7 B. Interestingly for xylanase, no significant change in enzyme activity of crude and purified was reported, Figure 7 A. It is noted that synthesis and expression of certain genes, and microbial metabolic activities, are influenced by the internal pH, in response to that of the external environment (Fatokun et al. 2016b). In addition, many enzyme systems and the transport of several enzyme species across the cell membrane are influenced by the initial pH of the cultivation medium (Fatokun et al. 2016b). Thus, it is crucial to maintain optimum pH.

Effect of substrate concentration in the range of 5 to 25 mg was studied on enzyme activity of crude and purified xylanase and cellulase, Figure 8. As can be seen for xylanase optimum enzyme activity of 486.2 µmol/mL was reported for crude and purified enzyme at substrate concentration of 20 mg and 25 mg, Figure 8 A. Interestingly, for purified cellulase optimum enzyme concentration of 400 µmol/mL was reported at 25 mg and crude cellulase enzyme activity of 395 µmol/mL was optimum at 20 mg and 25 mg of substrate concentration, Figure 8 B. Since xylanase and cellulase are inducible extracellular enzyme, substrate concentration plays an important role (Ortiz-Cortés et al. 2021). Overall, substrate concentration exhibited positive linear behaviour on enzyme activity as expected. However, higher concentration of substrate is also reported to adversely affect the growth and enzyme activity as seen in case of purified cellulase enzyme at 25 mg (Bajar et al. 2020).

Cellulase: The effect of enzyme amount in the range of 0.5 to 2.5 mL was studied on enzyme activity of crude and purified xylanase and cellulase, Figure 9. As can be seen, Figure 9 A and B, enzyme activity of crude was optimum than purified for xylanase as well as cellulase at enzyme amount of 2.5 mL. Enzyme activity of 510.87 µmol/mL was optimum for xylanase and enzyme activity of 449.5 µmol/mL was optimum for cellulase. The results are as expected that with increase in enzyme amount enzyme activity would show an increase.

From the above results (Figure 10), optimized parameters for xylanase were 30oC temperature, 10 pH, 20 mg substrate concentration, and 2.5 mL enzyme volume. These parameters reported enzyme activity of 510 µmol/mL and 499.56 µmol/mL for crude and purified xylanase respectively. Likewise, optimised parameters for cellulase were 30oC temperature, 8 pH, 20 mg substrate concentration, and 2.5 mL enzyme volume. These optimized influencing parameters reported enzyme activity of 405.6 µmol/mL and 467.22 µmol/mL for crude and purified enzyme respectively. By exploiting the potential of *Streptomyces lividans* and optimizing the fermentation process, we can enhance the production of cellulase and xylanase for its applications.

CONCLUSION

Overall, we used response surface methodology to investigate the influence of three variables (ammonium sulfate, dextrose, and substrate concentration) on the activity profile of xylanase and cellulase production. Through experimental design and three-dimensional contour plots, we found that substrate concentration was the major contributor to the production of both enzymes (xylanase and cellulase). We evaluated the potential of indigenous *Streptomyces lividans* isolated from agrowaste for xylanase and cellulase production using substrate fermentation. Furthermore, we examined the influence of various parameter, i.e., temperature (20-60oC), pH (2-8), substrate concentration (5-25 mg), and enzyme volume (0.5-2.5 mL) on enzyme activity of xylanase and cellulase. The specific

enzyme activity under optimum parameters conditions was found to be 4.21 µmol/mg-minute for xylanase and 3 µmol/mg-minute for cellulase, respectively. By exploring the potential of *Streptomyces lividans* and optimizing the fermentation process, we can enhance the production of these important industrial enzymes. Furthermore, future research should focus on the potential enhancement of enzyme activity through genetic manipulation, thereby improving the applicability of xylanase and cellulase.

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Assessment of the Spider Fauna from Agroecosystems Around Amravati, Maharashtra, India.

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ABSTRACT
The diversity of Araneae (spiders) in agricultural ecosystems plays a crucial role in pest regulation and ecosystem balance. This study analyses the diversity of spiders in Amravati's agricultural fields, concentrating on two important Kharif crops that are cultivated using the mixed crop method: soyabeans and pigeon peas. The research highlights the species composition, abundance, and distribution of spiders within these crops, emphasizing the role of spiders as natural predators in controlling insect pests. A total of 1014 individual spiders, representing 41 genera and 14 families, and 28 taxa were identified across the surveyed sites. Four families—Araneidae, Lycosidae, Thomisidae, and Salticidae were found to be the most abundant, with Salticidae exhibiting the highest species diversity. Peak species diversity was recorded from August to November, with a noticeable decline in orb-weavers (Araneidae) following harvest of the soybean crop. By comparing spider communities across pigeon pea and soybean fields, the study identifies the influence of crop type, habitat, and environmental factors on spider diversity. The findings highlight the significant role of spiders in agroecosystems, particularly in the management of pest populations in Kharif crop cultivation. This work contributes to a better understanding of arachnid biodiversity in agricultural landscapes and its potential benefits for pest control in the region of Amravati, India.

KEY WORDS: ARANEAE, DIVERSITY, AGRICULTURE FIELDS, PIGEON PEA, SOYBEAN,

INTRODUCTION

Pigeon pea and soybean are amongst the most important and widely cultivated pulse and oilseed crops in Maharashtra, India. Area under cultivation pigeon pea 102.1ha and soybean 317.6 ha with productivity of 753 kg/ha and 764 kg/ha; however, the actual production is 242.8 t and 76.9t for the two crops. One of the factors for lower production is the infestation of insect pests, which attack all the stages of plants. Researchers have identified pest infestations across various regions of the country as a critical issue, with severe consequences, including farmer suicides (Singh and Singh, 1991; Rao et al., 2002; Akhilesh and Parasnath, 2003; Daniel et al., 2018). To mitigate crop losses to economically manageable levels or prevent them entirely, the application of chemical pesticides has become a widely adopted approach. However, due to the adverse effects of these chemicals on soil health, water quality, and farmer well-being, there is an urgent need for eco-friendly pest control methods.

Agricultural experts advocate for the use of natural predators, cultural practices, pest-resistant crop varieties, and transgenic crops to enhance productivity while minimizing environmental harm. Arthropods, representing the largest proportion of animal biomass and biodiversity in agro-ecosystems, play a vital role in delivering essential ecosystem services. They function as pollinators, predators, decomposers, and nutrient recyclers, contributing significantly to ecological balance. Due to their rapid responsiveness to environmental changes, arthropods also serve as valuable bio-indicators. Among these, predatory arthropods are particularly beneficial in agroecosystems, as they help regulate and suppress populations of phytophagous pests, thereby influencing the guild diversity of other invertebrates. Spiders, in particular, are recognized for their exclusive predatory behavior, which has a profound impact on agro-ecosystem dynamics (Samiayyan, 2014; Michalko et al., 2019, Smith et al., 2020; Kumar & Patel, 2022 Wadhwa and Malik 2024, Nik et al 2025).

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The extent of their influence on prey populations is closely linked to spider density or biomass (Greenstone, 1999; Riechert, 1999; Sunderland and Samu, 2000; Liu et al., 2015). These findings underscore the ecological importance of spiders and other predatory arthropods in maintaining the stability and sustainability of agricultural systems. Research has demonstrated that ecosystems with greater species diversity exhibit higher stability compared to those with limited species richness. Arthropod predators, in particular, play a crucial role in regulating pest populations (Chang and Kareiva, 1999; Symondson et al., 2002; Wadhwa and Malik 2024 and Nik et al 2025).

Despite this understanding, knowledge of spider diversity in agroecosystems, specifically in pigeon pea and soybean fields in the Amravati region, remains fragmented. To address this gap, the present study was conducted to explore the diversity, species richness, and ecological dynamics of spider communities within these cropping systems.

MATERIAL AND METHODS

Study area: The study area, Amravati, is geographically located at coordinates 20.93°N and 77.75°E, with an average elevation of 343 meters above sea level. The region falls within the rainfed agricultural zone of the Amravati division, where crop production is heavily reliant on seasonal precipitation.The research focused on spider fauna within crop fields practicing intercropping, specifically cultivating pigeon pea and soybean. These two crops were selected due to their contrasting growth patterns, which provided a unique ecological context for studying spider diversity and community dynamics in agroecosystems.Four field sites were selected for the study.

Sampling duration and area under investigation: In the selected fields, spiders were observed and collected twice a month during the two kharif seasons i.e., July 2020 to December 2020 and July 2021 to December, total 12 surveys for each season. Amravati district is situated right in the center of the northern border of Maharashtra. It lies between 20.93°N 77.75°E. It is 343 metres above sea level on average. Major agriculture of the study area is in the rain-fed region of the Amravati division and the crops entirely depend on the amount of precipitation.

Collection Method: Spider specimens were collected using a combination of pitfall traps, aerial and ground hand collection methods, and vegetation beating techniques. Visual inspections were conducted on crop plants, surrounding vegetation, tree trunks, leaf litter, and other microhabitats within and around the selected crop fields. For documentation and identification, a Nikon D3200 camera equipped with a Nikkor 42x Wide Optical Zoom ED VR lens was utilized to capture high-resolution images of the specimens.

Spider Identification and Data analysis: Collected specimens were categorized by relative abundance in descending order and preserved in labeled containers with 70% ethanol for taxonomic identification and quantification under laboratory conditions. A digital photographic database was developed to facilitate morphological analysis using a Magnus MSZ-BI Zoom Stereo Binocular Microscope integrated with a MagCam DC-5 digital camera, enabling high-resolution imaging of diagnostic features. Taxonomic classification at the family level was conducted using Jocque and Dippenaar-Schoeman's Spider Families of the World (2007). Genus- and species-level identifications were cross-referenced with published Indian literature (monographs, books) and supplemented by international research publications. To ensure accuracy, supplementary validation was performed using the World Spider Catalog (2025) and additional peer-reviewed resources from global repositories.

The study presented here has assessed spider species diversity across the target agroecosystems. To evaluate sampling completeness, species richness and community diversity were quantified using the Shannon-Wiener diversity index (H') (Ravi et al., 2015), which integrates both species abundance and evenness to characterize ecological patterns in the sampled locations.

RESULTS AND DISCUSSION

An inventory of spiders that were collected from the soybean and pigeon pea agro-ecosystem is listed in Table 1. In total, 1014 individuals were collected, comprising 41 genera from 14 familiesand 28 taxa have been identified at the species level. Of the 14 families, four dominant families were reported common for both cropping seasons. From Table 1, the result shows that Araneidae and Salticidae recorded the highest abundance of spiders with a total number of 272 and 237 spiders, followed by Lycosidae 190 and Thomisidae 124, while Cheiracanthiidae recorded the lowest abundance. Peak species diversity was observed between August and November, with species richness during the 2020 Kharif season exceeding that of the 2021 season. This interannual variability in spider diversity metrics may reflect subtle shifts in climatic parameters (e.g., temperature, precipitation) between the two study periods, highlighting the sensitivity of arthropod communities to environmental fluctuations in rainfed agroecosystems (Smith et al., 2020; Kumar & Patel, 2022 Wadhwa and Malik 2024, Nik et al 2025).

While the family Araneidae exhibited the highest relative abundance in both cropping seasons, followed by Lycosidae and Thomisidae, Salticidae emerged as the most species-rich family across both study periods. This observation aligns with prior findings documenting that spider families such as Araneidae, Lycosidae, Thomisidae, and Salticidae—alongside Linyphiidae, Oxyopidae, and Tetragnathidae—are ecologically significant in agroecosystems due to their roles in regulating pest populations through natural predation (Memahet al., 2014; Deshmukh, 2017). The dominance of these families in the current study corroborates their established prevalence and functional importance in agricultural habitats, reinforcing their potential as bioindicators and biocontrol agents in integrated pest management strategies.

Spatial distribution of spiders within agroecosystems is strongly mediated by vegetation structure rather than random dispersion, reflecting their reliance on habitat complexity for foraging and web-building. A marked decline in orb-weaver (Araneidae) populations post-soybean harvest was observed, directly correlating with reduced vegetation density, underscoring the critical role of plant architecture in sustaining spider guilds. Such spider–plant associations may exhibit mutualistic dynamics, wherein spiders enhance plant fitness via predation on herbivorous pests, while plants provide structural refugia and optimal microhabitats for spider survival and reproduction (Vasconcellos-Neto et al., 2020). This interplay is particularly relevant in soybean and pigeon pea systems, which are vulnerable to early-stage infestations by pests such as semiloopers, Helicoverpa, girdle beetles, pod borers, aphids, jassids, and whiteflies (Dwarka et al., 2021; Kennedy & Lekshmi, 2022 Nik et al 2025).

Peak species diversity, observed between August and November across both Kharif seasons, aligns with periods of maximal vegetative growth and structural complexity, further emphasizing the temporal dependency of spider communities on crop phenology and agroecological conditions. These findings highlight the importance of vegetation management in conserving spider biodiversity and enhancing their ecosystem services in pest regulation. Spiders are strongly influenced by plant architecture, rather than being randomly distributed in the vegetation. The decline in orb-weavers after the soybean crop was harvested was noted, correlating with the loss of vegetation. Spider–plant associations can be considered mutualistic when spiders confer protective or nutritional benefits that enhance plant fitness, while plants, in turn, provide spiders with shelter and optimal foraging habitats (Vasconcellos-Neto et al., 2020). Soybean and pigeon pea are likely to be attacked by the Semilooper, Helicoverpa, girdle beetle, Pod borer aphids, jassids, and white fly, etc. in early stages of growth (Dwarka, et al., 2021; Kennedy and Lekshmi, 2022 Wadhwa and Malik 2024).

The maximum species diversity was noted from August to November for both the kharif seasons studied. According to Suana et al. (2004), the diversity of spider species is also influenced by factors such as habitat type, plant growth period, and landscape structure. Spiders are very sensitive to the variations in abiotic conditions, and Pitilinet al. (2019) observed that spiders influence the pest populations in the field and these are also influenced. Decline was noted in orb-weavers after the soybean crop was harvested, correlating with the loss of habitat (Fig. 1(d)).

This study documents novel baseline data on salticid (jumping spider) assemblages within agroecosystems, expanding known arachnofaunal records for Maharashtra, India. The findings align with established ecological principles demonstrating that spider-mediated pest suppression exhibits functional density-dependence, wherein higher predator abundance enhances trophic regulation of phytophagous arthropods (Greenstone, 1999; Riechert, 1999; Sunderland &Samu, 2000; Prashanthaku mara&Venkateshwarlu, 2017). These results corroborate the hypothesis that maintaining threshold abundance levels of spiders—particularly generalist predators like Salticidae—is critical for optimizing biocontrol efficacy in cropping systems. Such density-dependent interactions underscore the necessity of habitat management strategies that conserve spider populations to bolster ecosystem resilience and reduce reliance on synthetic pesticides.

Figure 1 Selected crop fields with mix crops of Soyabean and Pigeon Pea (Field a,b,c,d)

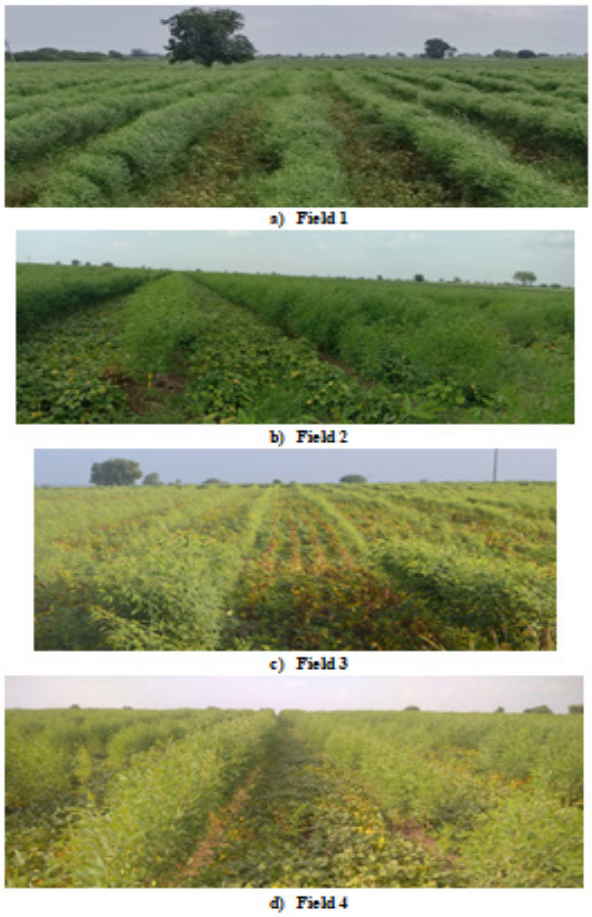

Figure 2 Total percentage of spider species recorded during the study

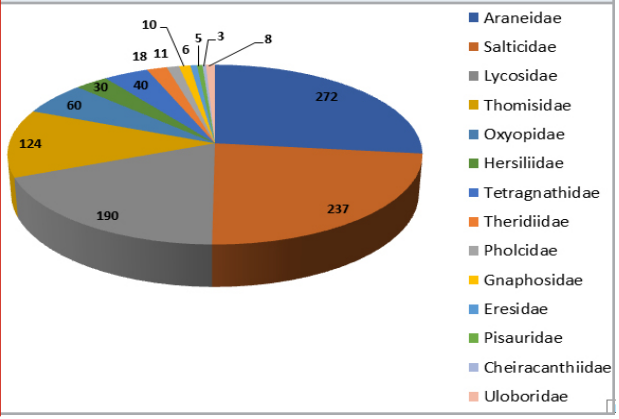

Table 1. List of spider species recorded from agroecosystems

S.No.	Family	Genera	Taxa	Total
1.	Araneidae (Clerck, 1757)	*Argiope aemula* (Walckenaer, 1841)	1	17
		Argiope sp.	2	29
		Bijoaraneus mitificus(Simon, 1886) *	3	10
		Cyclosa bifida (Doleschall, 1859)	4	7
		Cyclosa confraga(Thorell, 1892)	5	4
		Cyclosa Sp.	6	13
		*Cyrtophora citricola** (Forsskål, 1775)	7	5
		Eriovixia excelsa (Simon, 1889)	8	26
		Guizygiella indica (Tikader& Bal, 1980)	9	22
		Larinia sp.	10	14
		Neoscona mukerjei (Tikader, 1980)	11	28
		Neoscona theisi (Walckenaer, 1841)	12	39
		Neoscona vigilans (Blackwall, 1865)	13	21
		Neoscona sp.	14	37
2.	CheiracanthiidaeWagner, 1887	*Cheiracanthium inornatum*	15	3
		O. Pickard-Cambridge, 1874		
3.	Eresidae (C. L. Koch, 1845)	*Stegodyphus sarasinorum* (Karsch,1892)	16	6
4.	Gnaphosidae (Banks, 1892)	*Drassodes* sp.	17	7
		Zelotes sp.	18	3
5.	Hersiliidae (Thorell, 1869)	*Hersilia savignyi* (Lucas 1836)	19	9
		Hersilia sp.	20	21
6.	Lycosidae (Sundevall, 1833)	*Arctosa lesserti* (Reimoser, 1934)	21	11
		Hippasa sp	22	14
		Lycosa poonaensis (Tikader& Malhotra, 1980	23	23
		Lycosa sp.	24	39
		Pardosa pseudoannulata (Bösenberg& Strand, 1906)	25	25
		Pardosa sutherlandi (Gravely, 1924)	26	19
		Pardosa sp.	27	27
		Wadicosa fidelis (O. Pickard-Cambridge, 1872)	28	32
7.	Oxyopidae (Thorell, 1869)	*Oxyopes hindostanicus*(Pocock, 1901)	29	8
		Oxyopes sp.	30	26
		Peucetia viridana (Stoliczka, 1869)	31	9
		Peucetia sp.	32	17
8.	Pholcidae (C. L. Koch, 1850)	*Pholcus* sp.	33	11
9	Pisauridae (Simon, 1890)	*Nilus phipsoni* (F. O. Pickard-Cambridge, 1898)	34	5
10.	Salticidae (Blackwall, 1841)	Chrysilla sp.	35	10
		Hasarius adansoni (Audouin, 1826) *	36	24
		Hyllus semicupreus (Simon, 1885) |	37	38
		Menemerus bivittatus (Dufour, 1831)	38	20
		Myrmarachne sp.	39	19
		Phintella sp.	40	38
		Plexippus paykulli * (Audouin, 1826)	41	45
		Pseudicius sp.	42	20

Table 1 Continued

		Telamonia dimidiata(Simon, 1899)	43	23
		Thyene imperialis(Rossi, 1846) *	44	10
11.	Tetragnathidae (Menge, 1866)	Leucauge decorata (Blackwall, 1864)	45	26
		Tetragnatha sp.	46	14
12.	Theridiidae (Sundevall, 1833)	*Theridion* sp.	47	18
13.	Thomisidae (Sundevall, 1833)	*Indoxysticus minutus* * (Tikader, 1960)	48	17
		Massuria sp.	49	21
		Misumena sp.	50	12
		Runcinia sp.	51	25
		Thomisus sp.	52	49
14.	Uloboridae (Thorell, 1869)	*Uloborus* sp.	53	8
Total	14	41	53	1014

Table 2. Total number of species from individual families

Sr. No.	Family	Total Species Noted
1	Araneidae (Clerck, 1757)	272
2	Salticidae (Blackwall, 1841)	237
3	Lycosidae(Sundevall, 1833)	190
4	Thomisidae (Sundevall, 1833)	124
5	Oxyopidae(Thorell, 1869)	60
6	Hersiliidae(Thorell, 1869)	30
7	Tetragnathidae (Menge, 1866)	40
8	Theridiidae (Sundevall, 1833)	18
9	Pholcidae (C. L. Koch, 1850)	11
10	Gnaphosidae (Banks, 1892)	10
11	Eresidae(C. L. Koch, 1845)	06
12	Pisauridae (Simon, 1890)	05
13	CheiracanthiidaeWagner, 1887	03
14	Uloboridae(Thorell, 1869)	08
	Total	1014

The biodiversity analysis indices revealed that Shannon-Weiner Diversity Index (H') was 2.089 in 2020 and 2.766 in 2021 kharif, Species Richness Index (□) was (4.135 in 2020 and 4.22 in 2021 kharif) and Evenness Index (E1) was (0.104 in 2020 and 0.095 in 2021 kharif). These indicate that the species diversity and evenness indices during 2020 kharif were more abundant compared to those of 2021 kharif; and species richness was more or less equal and exhibited a similar diversification in both seasons.

CONCLUSION

The present study documented lower overall spider diversity and abundance compared to previous studies in the region. This decline may be attributed to the frequent use of chemical pest control methods and fluctuating weather conditions observed during the study period in the area. The Shannon-Weiner index highlights the habitat's potential for conserving spider diversity. Consequently, understanding spider diversity and species richness is essential for advancing their role as biocontrol agents in agroecosystems. Additionally, the data gathered will serve as a valuable resource for researchers interested in studying the spider fauna of Amravati, Maharashtra (India).

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Scientific Validation of Bioactive Compound Psoralen from *Psoralea corylifolia* Seeds Extract Using HPLC-DAD-TOFMS Analysis

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ABSTRACT

Psoralea corylifolia Linn. is an herb from the Fabaceae family, used in both Indian and Chinese traditional medicine. Commonly known as Babchi, it is a traditional medicinal plant widely utilized in Ayurveda and Unani medicine. Psoralen, a major bioactive furocoumarin found in its seeds, exhibits various pharmacological activities. In this study, High-Performance Liquid Chromatography coupled with Diode-Array Detection and Time-of-Flight Mass Spectrometry (HPLC-DAD-TOFMS) was employed to validate and quantify psoralen in *P. corylifolia* seed extracts. The characteristic retention time (Rt) of the psoralen standard was recorded at 3.856 min, while the crude seed extract exhibited a retention time of 3.688 min. The area density analysis revealed that psoralen constituted 75.27% of the crude extract. Furthermore, mass spectrometric analysis confirmed that the exact mass of psoralen in *P. corylifolia* (m/z 391.1) matched that of the standard psoralen ($[M-H]^+$ m/z 392.2). These findings suggest that the fully validated HPLC-DAD and HPLC-TOFMS methods can be effectively applied for the quantitative determination of psoralen in *P. corylifolia*. Due to its high psoralen content, P. corylifolia holds potential as a potent therapeutic agent for the treatment of hypopigmentary disorders.

KEY WORDS: *PSORALEA CORYLIFOLIA*, PSORALEN, HPLC-DAD-TOFMS, QUANTITATIVE DETERMINATION.

INTRODUCTION

Ayurvedic herbal medicines have been widely recognized for their numerous therapeutic applications, derived from ancient Indian herbal systems. In recent years, there has been a significant increase in attention and research on medicinal plants (Boparai et al., 2017). One such plant, *Psoralea corylifolia L*., an annual herb belonging to the Fabaceae (Leguminosae) family, is extensively distributed across subtropical and tropical regions. Commonly known as Babchi, this plant grows to a height of 30–180 cm (Alam et al., 2018) and thrives in warm environments but does not tolerate shade. It has simple, broadly elliptic leaves measuring 3.8 × 2.5–5.0 cm, with dentate margins. The seeds are oval, flattened, dark brown in color, and possess a characteristic fragrant odor. The fruit pods are ovoid-oblong, mucronate, black, and approximately 5 mm in length (Shrestha et al., 2018).

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P. corylifolia has been of immense biological and medicinal importance for centuries, primarily due to its remarkable efficacy in treating various skin disorders, including psoriasis, leucoderma, and leprosy (Chishty and Bissu, 2016). The plant contains a diverse array of bioactive compounds, including coumarins, flavones, lipids, saponins, alkaloids, tannins, carbohydrates, monoterpenes, chalcones, resins, stigmasteroids, and flavonoids. Phytochemical studies have identified flavonoids, coumarins, and meroterpenes as the key components of *P. corylifolia*, with the highest concentrations found in its seeds and fruits (Zhang et al., 2016; Basera and Shah., 2020).

Among these bioactive compounds, psoralen—a linear furanocoumarin—is the principal medicinally active constituent of *P. corylifolia* seeds. Psoralen ($C_{11}H_6O_3$, m.p. 161–162°C) is known for its significant pharmacological properties, including its ability to mitigate metabolic disorders and certain cancers, as well as its therapeutic potential in treating skin conditions such as psoriasis, eczema, and vitiligo. These effects are attributed to its ability to reduce oxidative stress, inhibit low-density lipoprotein (LDL) oxidation and platelet aggregation, and promote

vasodilation in blood vessels (Uikey et al., 2010; Anand David et al., 2016; Tripathi et al., 2023).

Our recent investigation further demonstrated its ability to facilitate pigment dispersion in reptilian melanophores via cholinergic receptor activation. These findings suggest that psoralen may serve as a potential therapeutic agent for managing hypopigmentary disorders such as vitiligo (Sultan and Ali., 2011). However, while previous studies have primarily focused on the biological and therapeutic significance of psoralen, there is limited literature on its qualitative and quantitative estimation, as well as its *in vivo* activity post-administration.

Based on these insights, the present study aims to utilize liquid chromatography-mass spectrometry (LC-MS) for the identification and characterization of psoralen in *P. corylifolia* seed extracts. Conventional methods such as column chromatography and high-performance liquid chromatography (HPLC) are often tedious, time-consuming, and require multiple chromatographic steps. Therefore, we also sought to develop and validate a rapid, sensitive, high-performance liquid chromatography with diode-array detection (HPLC-DAD) and time-of-flight mass spectrometry (HPLC-TOFMS) method for the efficient determination and quantification of psoralen in *P. corylifolia* seeds.

MATERIAL AND METHODS

Procurement of Reagent: Analytical grade methanol and ethanol were obtained from Qualigens Fine Chemicals, Mumbai, India. Acetonitrile (HPLC grade) and Water (HPLC grade) was procured from Sigma Aldrich (USA). Deionized water was purified with Milli-Q water system (Millipore, Milford, MA, USA). Standard psoralen was given as a gift sample from Dr. Syed Khalid Yousuf, Scientist, Medicinal Chemistry Division, Council of Scientific & Industrial Research-Indian Institute of Integrative Medicine (CSIR-IIIM), Srinagar. Fresh Seeds of Psoralea corylifolia were purchased and collected from Vindhya Herbals Sanjeevani Ayurveda, Bhopal, a Government of Madhya Pradesh enterprise. At the time of collection, seeds were chosen from healthy plants. The phenotypically superior samples of healthy and uninfected plants were chosen. Collected samples were stored in zip locked polyethylene bags until used.

The plant sample was identified and authenticated by Botanical Survey of India, Kolkata. The voucher specimen number - CNH/Tech.II/2022/52 was deposited at the herbarium of the Botanical Survey of India, Kolkata. Preservation of plants material was done according to the standard protocol following the literature, technical reports, and manuals.

Extraction of crude extracts of *Psoralea corylifolia* seeds: Extraction of crude extract from the seeds of *Psoralea corylifolia* has been done as per the method of Husain et al. (2016) with minor modifications. Soxhlet extraction method was performed for the preparation of crude extracts from the seeds of *P. corylifolia*. For the preparation of methanolic extract of *P. corylifolia*, fresh seeds of *P. corylifolia* were dried at room temperature in shade and then crushed into coarse powder. The whole or coarsely powdered plant material was extracted by 200 ml of methanol solvent for 10-12 hrs at 60°C in a Soxhlet apparatus (Khera Instruments, Pvt Ltd Delhi, India). The crude extract was filtered and evaporated to dryness on rotary evaporator (Khera Instruments, Pvt Ltd Delhi, India) and accurately weighed for further analysis.

Preparation of standard and sample solutions

Standard Solution: A standard stock solution of psoralen was prepared by dissolving 10mg of psoralen in acetonitrile and made up the volume to 10 ml in a standard volumetric flask. The solution was sonicated for 25 min. The standard solution of psoralen was further diluted as per the requirement by diluting the stock solution to obtain a concentration of 100 µg/mL.

Preparation of Sample Solution: The sample solution was prepared by weighing dried extracts (10mg), in a 10 ml volumetric flask containing acetonitrile. The solution was sonicated for 25 min, and the final volume was made with acetonitrile. Each sample solution was filtered through a 0.45 µm membrane filter into HPLC sample vial before HPLC injection. From the test sample, 1 ml was pipetted out and diluted up to 10 ml with acetonitrile for HPLC analysis. The stock solution was further diluted sufficiently to get a sample solution with a drug concentration of 100 µg/mL. 20µl of standard and sample were injected to HPLC system and then the chromatogram was recorded and the retention time of psoralen was determined in comparison with standard.

Instrumentation and conditions

HPLC Chromatographic conditions: The High-Performance Liquid Chromatography analysis was done on a chromatographic system (Waters, Massachusetts) as per the method of Shailajan et al., (2012) with slight modifications. The separation used was a thermo C 18 (250 × 4.6 mm, 5 µm), and the column temperature was maintained at 30°C. The diode array detector recorded UV spectra in the range of 200–400 nm, and the HPLC chromatogram was monitored at 247 nm. The mobile phase consisted of acetonitrile (A): water (40:60 v/v) (B). A gradient program was used as following: 0–20 min, 15–55% A; 20–30 min, 55–70% A; 30–35 min, 70–85% A, with a hold time of 10 min. The flow rate was 1 ml/min and the injection volume was 20 µL.

HPLC-TOFMS analysis: The Liquid Chromatography Mass Spectrometry (LC-MS) analysis was carried out as per the method of Tan et al., (2015) with slight modifications. The above HPLC conditions were used for HPLC-TOFMS analysis. The HPLC system was directly connected to the TOF mass spectrometer via an electrospray ionization (ESI) interface with the stream splitting ratio at 2:1.

The TOFMS analysis was performed using a full scan mode and the mass range was set at m/z 10 to 3000 in a

positive ion mode. The components in *P. corylifolia* were identified rapidly by HPLC-TOFMS according to their accurate molecular mass and molecular formulae matching against the formula database of *P. corylifolia*. The mass spectrometric parameters were optimized as follows: in the positive ion mode (ESI+), capillary voltage, 4500 V; nebulizer,40 psig; drying gas, 7.0 L min−1; gas temperature, 200°C; fragmentor voltage 160 V; skimmer voltage 60 V; collision cell RF 130 V. TOFMS was used for the detection and determination of accurate molecular masses of different compounds.

RESULTS AND DISCUSSION

Under the optimized chromatographic conditions, psoralen standard and crude extract of the seed of *Psoralea corylifolia* were separated in the HPLC chromatogram. The characteristic retention time (Rt) chromatograms of the psoralen standard and crude extract of seed of Psoralea corylifolia are shown in Figures 1 and 2, respectively, in which the Rt of psoralen was obtained at 3.856 and Rt of crude extract of seed of *Psoralea corylifolia* was obtained at 3.688. Two unidentified peaks were also obtained in the chromatogram of the crude extract of *Psoralea corylifolia* seeds and the Rt of peaks are 8.193 and 11.653, respectively as shown in Figure 2.

Figure 1: Chromatogram of Psoralen standard

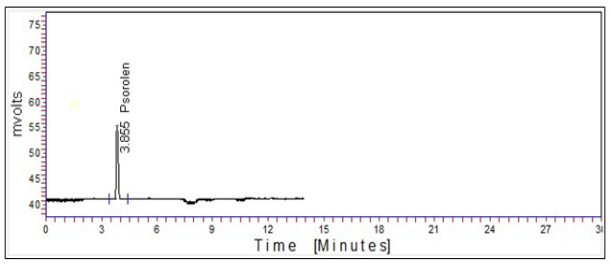
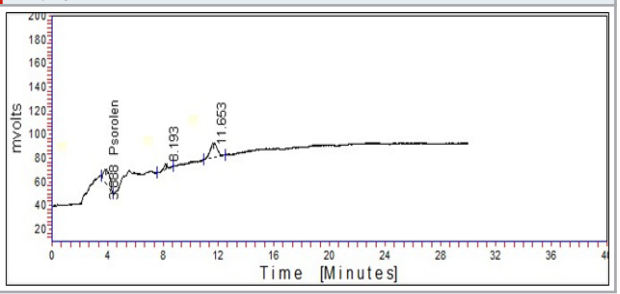


Figure 2: Chromatogram of crude extract of seed of *Psoralea corylifolia.*



Characterization of other compounds in *Psoralea corylifolia* by HPLC-TOFMS: To further confirms the unidentified peaks from the extract of *Psoralea corylifolia*, the crude extract was directly injected into the TOF mass spectrometer to optimize the spectrometric parameters, including capillary voltage, nebulizer gas pressure, drying gas flow rate, gas temperature and fragmentor voltage. Positive ion mode was set for the identification of compounds in Psoralea corylifolia. The total ion chromatogram (TIC) of *Psoralea corylifolia* extract was acquired by HPLC-ESI-TOFMS in the positive ion mode (Figure 3). Our interest is to confirm the presence of psoralen in standard and test sample, the retention time ratio of presence of psoralen in standard was obtained at 13.679:14.142 and the retention time ratio of presence of psoralen in tested extract of *P. corylifolia* was obtained at 13.337:14.365. The results of mass determination accuracy for all detected peaks are summarized in Table I. The result showed that the exact mass of psoralen content of *Psoralea corylifolia* (m/z 391.1) was identical to that of the standard psoralen ([M-H] + m/z 392.2). Five compounds in the TIC chromatogram from TOFMS were characterized by matching against literature searched as illustrated in table 1.

Differentiation of isomers in *Psoralea corylifolia* extract: Some compounds with identical molecular weights may not be distinguished only according to the accurate mass measurement capability of TOFMS, which included psoralenoside, isopsoralenoside and isopsoralen with approximate molecular weight of 324 at peaks near 5.177:5.554, 5.554:5.777 and 14.365:14.931 respectively. Since these molecular pairs cannot be distinguished by mass spectrometry without fragmentation, other analytical technique HPLC-DAD was further employed to analyze psoralen from same MWs of other isomers of *Psoralea corylifolia*. The HPLC-DAD data of the isomers in the positive ion mode are shown in figure 4 of crude extract of *Psoralea corylifolia*.

From the results of HPLC-TOFMS, it was confirmed that characteristic compound (psoralen) of standard and tested sample was identified at Rt of 13.679:14.142 and 13.337:14.365 respectively. HPLC DAD analysis employed further confirms the presence of psoralen in standard at Rt time 13.896 and the presence of psoralen in test compound at Rt 13.956 which is highly comparable which is shown figure 4 and illustrated in table 2. The quantity of psoralen in crude extract was found to be 75.27 % as calculated by dividing the peak area of psoralen by total peak area and multiplied with 99% purified standard.

Until now, no studies about the identification and quantification of psoralen in plant extracts using MS/MS detection have been described. Only Shailajan et al. (2012) and Tan et al. (2015) have reported on psoralen MS, but in the selected ion mode (SIM), which is less specific than multiple reaction mode (MRM) and high-resolution mass spectrometry identification. Our results matched the findings of Rakhmankulov and Korotkova, (2015). They pioneered the use of biologically active compounds. They reported that the seeds and roots were the richest sources of furanocoumarins (psoralen and angelicin).

Our findings are in line with the study of Shailajan et al. (2012), where they develop a reverse-phase high-performance liquid chromatography—photodiode array detector (RPHPLC—DAD) method for quantification of psoralen from *P. corylifolia* and its related formulations. Separation and detection of psoralen from various herbal formulations were achieved on a reversed-phase Cosmosil C18 column using acetonitrile: distilled water (40:60 v/v;

flow rate: 1.0 mL/minute) and the PDA detector (247 nm).

The present findings are correlated with the findings of Luan et al. (2018), who worked and standardised the high-performance liquid chromatography-diode array detector (HPLC-DAD) fingerprint method to present the comprehensive phytochemical profile of the *Psoraleae fructus*. Thirteen major compounds were separated and identified by HPLC coupled with time-of-flight mass spectrometry (HPLC/TOF-MS), namely psoralenoside, isopsoralenoside, psoralen, isopsoralen, neobavaisoflavone, bavachin, corylin, bavachromene, psoralidin, isobavachalcone, bavachinin, corylifol A, and bakuchiol.

Figure 3: The total ion chromatogram (TIC) of Psoralea corylifolia extract was acquired by HPLC-ESI-TOFMS in the positive ion mode. Psoralen was detected at retention time 13.966.

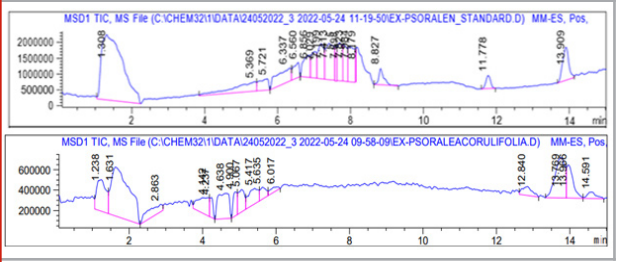


Figure4: HPLC-DAD graph of Psoralea corylifolia in the positive ion mode

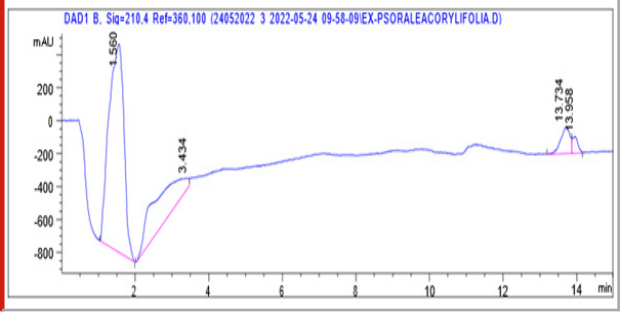


Table 1. HPLC-TOFMS Data of the Compounds in *Psoralea corylifolia*

No	Time of flight	identification	formula	m/z ratio (peak value)
1	13.679:14.142	Psoralen (from standard)	$C_{17}H_{18}O_9$	392.2
2	5.177:5.554	Psoralenoside (from test compound)	$C_{17}H_{18}O_9$	324.1
3	5.554:5.777	Isopsoralenoside (from test compound)	$C_{17}H_{18}O_9$	324.1
4	5.777:6.120	Daidzein (from test compound)	$C_{15}H_{10}O_4$	208.1
5	13.337:13.901	Psoralen (from test compound)	$C_{17}H_{18}O_9$	391.1
6	14.365:14.931	Isopsoralen (from test compound)	$C_{11}H_6O_3$	323.1

Table 2: HPLC DAD data of crude extract of *Psoralea corylifolia*. Retention time (Rt)

No.	Rt (min)	Identification	Area %
1	13.896	Psorolen from standard	4.225
2	13.958	Psorolen from test sample	2.078
3	13.734	Psorolen from test sample	5.389

Therefore, in this work, a reliable and powerful analytical method by using a combination of HPLC-DAD and HPLC-TOFMS for rapid identification of psoralen in *P. corylifolia* was established. Five peaks in the HPLC chromatogram were rapidly identified by comparing their related retention times and accurate mass measurements with the standards. Using HPLC-TOFMS and literature search, apart from psoralen, four other components were characterized tentatively. Since isomers that cannot be differentiated by HPLC-TOFMS, it is feasible to apply HPLC-DAD to differentiate them based on their characteristic fragment ions. By right of this combinative technique, psoralen and four other coumarins were identified rapidly, which provided essential data for further pharmacological and clinical studies of *P. corylifolia* and facilitated the rapid quality control of the crude drug. The fully validated HPLC-DAD and HPLC-TOFMS method can be successfully applied for the determination of psoralen in *Psoralea corylifolia*, which can be used as a potent therapeutic agent for the treatment of hypopigmentary disorders.

CONCLUSION

A simple, sensitive HPLC-DAD and HPLC-TOFMS

technique was demonstrated and justified for the characterization and quantitative estimation of the main coumarins psoralen in the seed extract of *Psoralea corylifolia*. The HPLC-TOFMS results confirmed the existence of psoralen in the crude extract of *Psoralea corylifolia* seeds. It is concluded that the higest amount of psoralen was found in the seed extract of of *Psoralea corylifolia*.

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Availability of data and material: All the data generated and analyzed during the study are included in the main manuscript.

Competing interests: The author declares no competing interests.

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Phytochemical Profiling of *Withania somnifera* for Qualitative and Quantitative Study of Bioactive Component withaferin a by High-Performance Liquid Chromatography

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ABSTRACT
Over the past few years, the attention of people has changed from man-made (synthetic) to natural medicines. *Withania somnifera's* phytochemical analysis has identified the presence of withanolides, which are pharmacologically active steroidal lactones. The two main withanolides that were isolated from *Withania somnifera* in India were withanolide D and withaferin A, both of which demonstrated cytotoxic and anticancer effects. The present investigation aimed to evolve a standard phytochemical profiling of *Withania somnifera* for the presence of withaferin A. The dried root sample was minced and ground with a mechanical grinder. The bioactive reference standard withaferin A was used. Methanol was used to extract the active fraction. By comparing the spectra of standard withaferin A with the root powder of Withania somnifera extracted using methanol, the peak purity of withaferin A was determined. The calibration curve was established for peak area vs concentration of Withaferin A. From the standard stock solution, six different concentrations (0.0 ppm, 1.25 ppm, 250 ppm, 5.0 ppm, 10.0 ppm, and 20.0 ppm) were used for preparing six six-point calibration curves. The comparative analysis of standard withaferin A and the sample has been determined. The sample (*Withania somnifera* root powder) and standard solutions were run in methanol mobile phase systems. It was found that after running the sample and standard withaferin A, it had a good resolution, a sharp and symmetrical peak at retention times 13.685 min. and 13.600 min. respectively. Estimation of Withaferin A can be used by the pharmaceutical industry as an appropriate bioactive marker compound for phytochemical profiling and quality control of *Withania somnifera*. Phytochemical profiling of *Withania somnifera* is essential to assess the quality, purity, efficacy, and safety of the bioactive component (withaferin A). The HPLC chromatogram of *Withania somnifera* root powder extract corresponding to standard Withaferin A was shown at a retention time of 13.685 min, at 227 nm wavelength. The quantitative evaluation of Withaferin A present in the sample was 0.10%.

KEY WORDS: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY, QUALITATIVE DETERMINATION, QUANTITATIVE DETERMINATION, WITHAFERIN A, *WITHANIA SOMNIFERA*.

INTRODUCTION

Over the past few years, the attention of people has changed from manmade (synthetic) to natural medicines. With increasing demand and acceptance of natural products, it is essential to maintain their quality for the benefit of human beings (Sharma et al., 2007). Many quality control tools are at hand, which are used to confirm the quality of herbal drugs before being marketed and consumed by consumers and patients. Both quantitative & qualitative data are essential for deciding the quality of them. The analytical technique HPLC (high-performance liquid chromatography) is commonly used for qualitative and quantitative determinations of natural bioactive fractions for quality control (Balekundri and Mannur, 2020.

Ashwagandha, or *Withania somnifera (L.)* Dunal, is a typical adaptogen that has long been utilized in Ayurvedic therapy in India. *Withania somnifera's* primary active ingredients are withanolides, and the root is frequently used as a medication with a variety of pharmacological actions that can be used to treat skin cancer, diabetes mellitus, insomnia, and neurasthenia. (Xiaoxing Liu et al , 2024)

Ashwagandha *(Withania somnifera)* is most precious herbaceous plant in the conventional systems of Indian medicine having many valuable effects (Gupta and Rana,

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2007). This plant is also called "Indian Winter cherry" or "Indian Ginseng" (Singh et al., 2011). It is one of the xeromorphic plants that grew up in desiccated and rainforest area (Meher et al., 2016)

The active chemical component of *Withania somnifera* is withaferin A (Saleem et al., 2020). It has life life-persisting, regenerating effect and is a beneficial therapy for cancer cells (Joshi, 2016).

Some marketed preparations of *Withania somnifera* are Ashwagandharista, Himalaya ashwagandha, Stresswin, Stresscom, MuscleBlaze Ashwagandha 1000mg Tablet, Inlife Ashwagandha Capsules, Himalaya massage oil, Ancient Apothecary, KSM 66 Ashwagandha, Vigomax, Baidyanath Ashwagandha Amrita 450 ml, Vital plus, Amrutha kasthuri and Brento etc. are available in market.

W. somnifera's phytochemical analysis identified withanolides, which are pharmacologically active steroidal lactones, (Lavie, et al 1972, Glotter, et al 1973).A class of alkaloids called withanine, which was separated from the plant's roots, makes up 38% of the alkaloids' total weight, (Atal et al 1975)

The two main withanolides that were isolated from *W. somnifera* in India were withanolide D and withaferin A, both of which demonstrated cytotoxic and anticancer effects. (Yoshida, et al 1979).The steroidal lactones known as withanolides were present in the methanolic extract. (Lavie et al 1964) (Lavie, 1966)(Lavie and Kirson, 1968). Depending on their geographic distribution, distinct chemotypes of *Withania somnifera* had varying amounts of substituted steroidal lactones. (Schmelzer et al 2008) (Lavie, et al 1972)

Lately, demand for Ayurvedic medicine has risen more in recent years in the world market because the bioactive fraction of *Withania somnifera* (Withaferin A) are nontoxic,safe and cost effective with fewer side effects (Arun et al 2013).

According to demand and popularity of Ayurvedic preparation, it is important to develop Phytochemical profiling of the bioactive fraction of *Withania somnifera* for safety, efficacy, and quality control of Ayurvedic preparation. Phytochemical profiling provides more safety and increase the faith of customers in ayurvedic preparations.

It is challenging to initiate the quality control parameters for phytochemical profiling due to the multifaceted nature and in-built variability of the bioactive fraction constituents of *Withania somnifera* (withaferin A).

The present investigation aimed to evolve a standard phytochemical profiling of *Withania somnifera* for the presence of withaferin A.

MATERIAL AND METHODS

Plant sample: Dried Ashwagandha *(Withania somnifera)* root samples were obtained from the Government Ayurvedic College and Hospital Herbarium, Patna, Bihar,. Dried root sample was minced and ground with a mechanical grinder (Hanil Co. Seoul, South Korea) into a mesh size 120 mm and stored at 4 °C, until further analysis.

Reagents and Standards: The bioactive reference standard withaferin A was purchased from ELECTROCRAFTS (FBD), Faridabad, Haryana, India. All chemicals and solvents used were of analytical grade or HPLC grade and obtained from E-Merck and other reputed companies.

HPLC Analysis Instrumentation HPLC instrumentation & chromatographic conditions: HPLC analysis was carried out on Shimadzu Model No. CBM-20A facilitated with Shimadzu's Lab Solutions software. Shimadzu High Performance Liquid Chromatographic system was equipped with a quaternary pump, Auto autosampler, an oven, and a detector. Separation was achieved on RP-C18, a model LC-20AD of length 150mm column. The mobile phase was methanol and used in an isocratic mode with a flow rate of 1.8ml/min, 10µl of the test sample (triplicate) was injected to HPLC system.Autosampler Model SIL-20AC, Sample Rack 1.5 mL 105 vials, Rinsing Volume 500 uL, Needle Stroke 52 mm, Control Vial Needle Stroke 52 mm, Rinsing Speed: 35 uL/sec, Sampling Speed: 15 uL/sec , Purge Time: 25.0 min, Rinse Dip Time : 3 sec and Cooler Temperature 150C Was used. Oven Model CTO-10ASvp, Oven Temperature 32 °C, Maximum Temperature 850C was used.

PDA Model SPD-M20A, Lamp D2, Start Wavelength 190 nm, End Wavelength 800 nm and Cell Temperature 400C was used.

Standard preparation: Stock solution of standard compound (Witheferin A) was prepared by dissolving 1.0 mg standard in 1.0 ml methanol. The stock solution as per requirement was diluted further.

Sample preparation: 19.7mg powdered Ashwagandha root was dissolved in 10.0 ml of methanol. From this solution 0.1ml was taken and dissolve in 0.9ml methanol.

Serial No.	Concentration(ppm)	Peak area
1	0	0
2	1.25	2431.12
3	2.5	5026.89
4	5	11385.1
5	10	23141.02
6	20	38015.15

The following optimal chromatographic settings were used to achieve good separations and an appropriate retention duration of Withaferin A in isocratic elution(Meena et al., 2021): –

Column: C18 ,150mm.
Detection: 227 nm wavelength.
Detector: Photodiode Array (PDA).

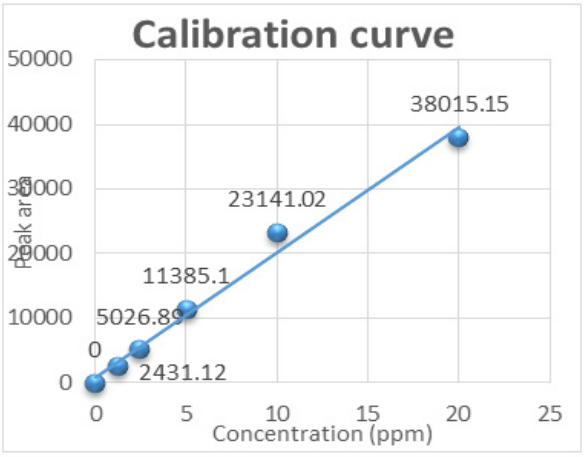
Mobile phase: Methanol
Flow rate: 1.8 ml/min.
Injection volume: 10 µl.
Mode of Operation: Isocratic elution.
Retention Time (Std.): 13.600
Retention Time (Test): 13.685
Run time: 30 min

RESULTS AND DISCUSSION

Calibration curve: From the standard stock solution, six different concentrations (0.0 ppm, 1.25 ppm, 250 ppm, 5.0 ppm, 10.0 ppm, and 20.0 ppm) were used for preparing six point calibration curve. Each of the standard solutions were run through the HPLC, and the respective peak areas were recorded. A calibration curve was created for the peak area versus Withaferin A concentration. Calibration peak summary is presented in Table 1, and the calibration curve is sketched in Figure 1.

Isocratic elution of the formulation extract produced a distinctive HPLC chromatogram with a smooth, clean baseline and good resolution where the marker peak was easily detected. In the chromatogram, the marker chemical Withaferin A is seen at retention times of 13.600 for the standard and 13.685 for the sample. The calibration curve, which was linear (r2 = 0.9853) in the concentration range of 0 ppm to 20 ppm, was created by measuring the peak area. Figure 2 displays the HPLC chromatogram of the sample that corresponds to standard Withaferin A at a wavelength of 227 nm and a retention period of 13.685 min.

Figure 1: HPLC Calibration curve of withaferin A standard



Quantitative estimation of withaferin A in sample by HPLC: By using methanol as mobile phase a sharp, reproducible and symmetric peak at retention time 13.685 mins. for standard and 13.600 mins. for sample (Fig.3) were obtained. Using the below mentioned formula, the quantitative assessment of Withaferin A in the sample was determined to be 0.10%.

$$\% \text{ of Withaferin A} = \frac{\text{Wt. of extract obtained} \times \text{Dilution of extract} \times \text{amount obtained by HPLC}}{\text{Wt. of sample} \times \text{Wt. of extract for HPLC analysis}}$$

Figure 2: HPLC chromatogram of sample

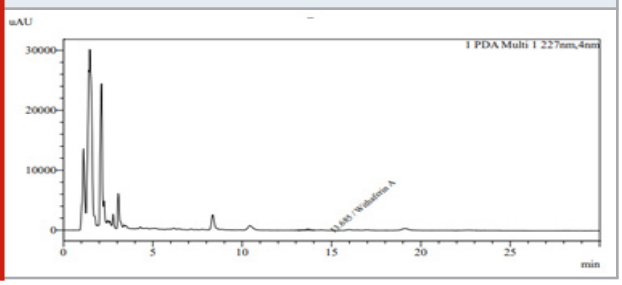


Figure 3: HPLC chromatogram of standard withaferin A

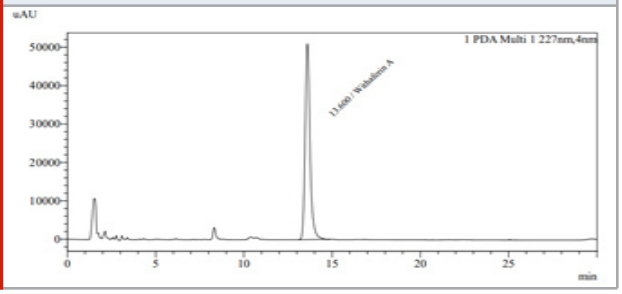


Figure4: Comparative HPLC chromatogram of sample (red) and standard withaferin A(black) at retaintion time 13.6

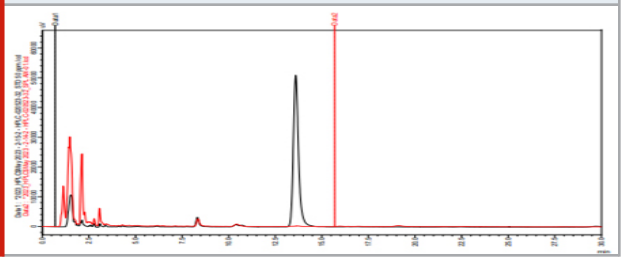
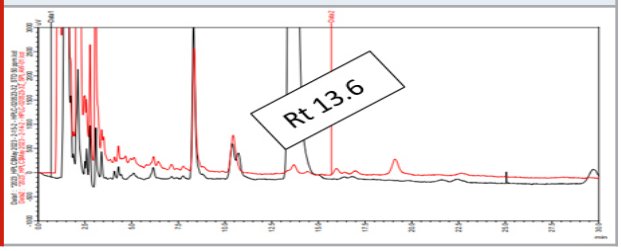


Figure 5: Zoomed view of comparative HPLC chromatogram of sample (red) and standard withaferin A (black) at retention time 13.6



Comparative analysis of standard withaferin A with the sample by HPLC: By comparing the peak obtained for standard withaferin A and that obtained for standard sample, it was found that the peak obtained for standard withaferin A and that for standard sample overlapped each other at retention time 13.6. This indicates that the sample contain withaferin A because at the retention time 13.6 standard sample showed a peak that is matched with the standard withaferin A peak. The comparative analysis of

standard withaferin A and standard sample is depicted in figure 4. Zoomed view of comparative analysis of standard withaferin A and standard sample was depicted in figure 5.

The sample and standard solutions were run in methanol mobile phase systems. It was found that after running the sample and standard Withaferin A, a good resolution, sharp and symmetrical peak at retention times 13.685 min. and 13.600 min. respectively was obtained.

CONCLUSION

Development of standard and reliable quality protocols for the Soxhlet extracted root powder of *Withania somnifera* by using modern techniques of analysis is extremely important. The generated HPLC result with bioactive marker compound will be used as a consistent analytical tool in the routine quality control of *Withania somnifera*. Estimation of Withaferin A can be used by pharmaceutical industry as an appropriate bioactive marker compound for phytochemical profiling and quality control of *Withania somnifera*.

Phytochemical profiling of *Withania somnifera* is essential in order to assess the quality, purity, efficacy, and safety of the bioactive component (withaferin A). The HPLC chromatogram of *Withania somnifera* root powder extract corresponding to standard Withaferin A was shown at a retention time of 13.685 min, at 227 nm wavelength. The quantitative evaluation of Withaferin A present in the sample was 0.10%.

Declarations

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Data availability statement: Data included in the article / referenced in the article.

Declaration of interest statement: The authors declare no conflict of interest.

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