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Molecular Characterization of *Fusarium oxysporum* f. sp. *fragariae* Isolated from Strawberry crops *Fragaria ananassa* Duch in Ecuador

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ABSTRACT:
Cultivation of strawberry has acquired great importance for consumption, promoting the increase of its production in Ecuador. However, the process of importing plant material from producer countries to improve domestic production has contributed to the dissemination of the fungus *Fusarium oxysporum* f. sp., fragariae. The objective of this study was to identify the presence of the pathogen by the application of molecular methods to Fusarium strains isolated in the province of Pichincha. Ninety-two diseased strawberry plants and 92 asymptomatic plants were analyzed. Of these samples, 13 fungal isolates with the characteristics of the genus Fusarium were identified. The isolates were analyzed at the molecular level by Polymerase Chain Reaction (PCR) through the amplification of the ITS regions of the rDNA sequences and the EF-1 is a gene, these were sequenced to elucidate the phylogenetic relationships between the isolates. Twelve strains were identified as F. oxysporum f. sp., fragariae. So, the present research contributes to the search for control alternatives to avoid the dissemination of the pathogen in strawberry plantations in Ecuador.

KEY WORDS: Molecular characterization, *Fusarium oxysporum*, strawberry

INTRODUCTION

The strawberry (*Fragaria ananassa*) has become a very important industrial crop worldwide, it can be said that this plant has the most varied and complex management possibilities, which has allowed for unusual development in the productive areas. The strawberry demand in the world is increasing, not only because of its flavor but also because of its health benefits. Markets like Europe and the United States register a high consumption, and most of the worldwide production of this fruit originates there [1].

In Ecuador, intensive strawberry cultivation began in 1983, mainly in the province of Pichincha [2], and in recent years the cultivated area has increased to 1,200 ha in the main strawberry varieties Albion, Monterrey and Diamante in greater proportion in the province of Pichincha with 400 ha, Tungurahua 240 ha and in the provinces of Chimborazo, Cotopaxi, Imbabura and Azuay with 40 ha, being thus the livelihood of the majority of fruit growers in these sectors, which are cultivated between 1300 and 3600 m above sea level with average temperatures of 15 °C. In the country, 12% of the strawberries harvested are exported while the rest of the production is used to meet domestic demand, which means the international market has a large field for exploitation [3].

The specific pathogenicity of *Fusarium oxysporum* f. sp., *Fragariae* causes a negative impact on agriculture worldwide as they are causative agents of vascular wilt and basal rot of a large variety of plants, in Ecuador this fungus affects the production of fruit crops such as babaco [4], strawberry, kidney tomato, among others, attributed to the difficult diagnosis of its worldwide dissemination [5].

Currently, the development and use of new molecular biology technologies based on Polymerase Chain Reaction (PCR) can be used for the application of molecular markers [6]. Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphism (AFLPs), among others, are used in the analysis of genetic DNA polymorphism, which in conjunction with the development of bio-informatic software designed for the analysis of sequences, allows to obtain efficient and timely information applicable to a large number of samples, being more specific, objective, sensitive and faster techniques than traditional methods [7]. The main objective of this research is to identify strains of *Fusarium oxysporum* f. at the molecular level using the ITS region and the ET-1 gene.

MATERIALS AND METHODS

Sampling was carried out in strawberry producing areas located in the province of Pichincha, from which 92 symptomatic and 92 asymptomatic plants were obtained; these plants were in phases of vegetative development and fruiting. The analyses were conducted in Agrocalidad laboratories according to the following procedure:

Seeding of plant material and substrate: Longitudinal cuts of the stem were made and 1-2 cm segments containing areas with and without vascular wilt lesions were removed. After this process, three sets of four segments were seeded in PDA medium, then incubated at 24 °C for 7 days. Isolates which present asexual structures of Fusarium sp., were selected [8].

Preservation of the pathogen: In the Petri dishes of monosporic cultures, 10 mL of sucrose (10%) and 10 mL of peptone (20%) were added with a handle (Digralsky), the mycelium and the poured liquid were homogenized. Paper squares (0.5 cm2) were placed in the solution, letting it rest 8 minutes; then enough paper segments embedded in the dilution were introduced into eppendorf tubes and cooled to 6 °C. After massification, enough biomass was obtained for the extraction of DNA [9, 10].

Molecular analysis (phylogenetic test): For this analysis, DNA was extracted from the mycelium using liquid nitrogen by macerating it in the mortar. The maceration was deposited in eppendorf tubes by adding 1 mL of extraction buffer and 0.5 mL of chloroform, iso-amino alcohol in a 24: 1 ratio. The tubes were introduced into the bain-marie, at 55 °C for 30 minutes while inverting them every 10 minutes. They were let to rest for 5 minutes, then centrifuged at 14000 rpm for 10 minutes.

The supernatant was transferred to a new sterile tube, adding 1 mL of isopropanol. It was allowed to freeze at −20 °C for 20 minutes, then centrifuged for 3 minutes at 10000 rpm. When the pellet was formed, rinsings were performed with 70% ethanol once or twice, then the pellet was allowed to dry for 25 minutes and inoculated into TE buffer and RNAse, then left in the environment for 30 minutes. DNA purity was assessed using a specific miniaturized spectrophotometer (nanodrop, Roche LC1536). Amplification analysis of the gene of interest by PCR. The primers EF1, EF2, ITS1, ITS4 were used in this study (table 1). For each PCR reaction, a volume of 25 µL was used; with concentrations of 1.5 mM MgCl2, 0.2 mM of each dNTP; 0.5 µM of each primer 1.25 U / 25 µL Taq polymerase (FLEXI), PCR buffer (Green FLEX 1X). The amplification was carried out in a thermocycler according to the following conditions: initial denaturation at 94 °C for 5 minutes, 35 reaction cycles at 94 °C for 30 seconds, annealing at 53 °C for 1minute, initial extension at 72 °C for 1 minute and a final extent at 72 °C for 10 minutes. The PCR product was analyzed by 0.5% agarose gel electrophoresis previously prepared in buffer TBE 1X (Trisborate, EDTA), a molecular weight marker of 100 bp was used and the gel was run for 30 minutes at 100 volts.

Table 1. Nucleotide sequences used for the amplification of ITS (s) and TEF-1α regions

Region	Initiator	Sequence	Author
Internal Transcribed Spacer (ITS)	ITS-1 ITS-4	5'-TCCGTAGGTGAACCTGCGG-3' 5'-TCCTCCGCTTATTGATATGC-3'	Martin and Rygiewicz (11)
Translation Elongation Factor (TEF-1α)	EF-1 EF-2	3'-ATGGGTAAGGARGACAAGAC-5' 3'-GGARGTACCAGTSATCATGTT-5'	O'Donnell et al. (12)

The gel was then visualized in a UV light transilluminator. PCR products with nonspecific bands were purified by means of the Band-stab PCR process until clear bands were obtained. All amplified products were sent to sequence MACROGEN (Seoul, Korea); the results were compared with the GenBank database [13]. Sequences were compared using a BLAST (Basic Local Alignment Search Tool) alignment that allowed gender and species identification (www.ncbi.nlm.nih.gov/BLAST).

RESULTS AND DISCUSSION

After isolation in PDA medium confirmed by microscopic morphology, only 52 samples showed

contamination by the fungi: Pestalotia sp., Fusarium sp., Rhizoctonia sp., and Mycosphaerella sp. The presence of spores belonging to the fungus Pestalotia sp., was evidenced in 46 isolates (88.52%); the presence of asexual structures of Fusarium sp., was determined in 13 isolates, representing 25% of the total samples; a minimum percentage (1.52%) with the fungus Rhizoctonia sp., and after analysis of the leaf area of the straw four isolates of Mycosphaerella sp., were identified (7.69%). It should also be noted that there were samples from which more than one isolate was obtained.

Phylogenetic analysis based on ITS fragments. It was carried out by a PCR with primers ITS1 - ITS4, DNA of the 13 treatments and two possible pathogens was characterized by optical microscopy as Pestalotia sp., and Rhizoctonia sp. A band was evidenced in the gel at a height of 570 base pairs for the 13 treatments, including Pestalotia sp. In the case of Rizhoctonia sp. the band has a slightly higher molecular weight equivalent to 650 bp, Treatment AB8 and negative control (C-) showed no visible band (Figure 1).

Figure 1: Amplified fragment of ITS regions from 13 treatments. Lane M: molecular weight marker (100-bp); lanes 1: AB12, 2: AB13, 3: AB1, 4: AB2, 5: AB3, 6: AB4, 7: AB5, 8: AB6, 9: AB7, 10: AB8, 11: AB9, 12: AB10, 13 : AB11; lane 14: Pestalotia sp; Lane 15: Rhizoctonia sp. Lane 16: negative control.

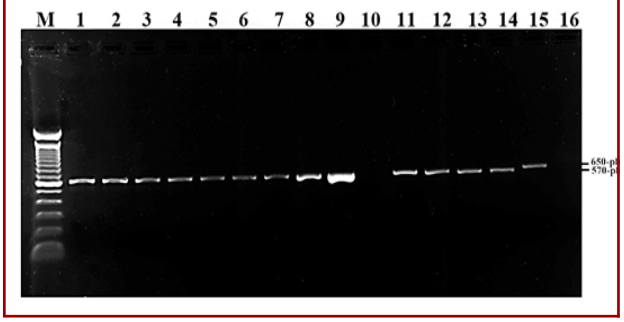
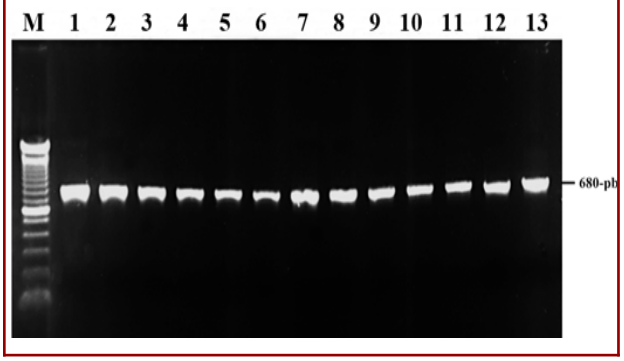


Figure 2: Amplified fragment of EF-1α region of the 13 isolates. Lane M: molecular weight marker (100-bp); lanes 1: AB12, 2: AB13, 3: AB1, 4: AB2, 5: AB3, 6: AB4, 7: AB5, 8: AB6, 9: AB7, 10: AB8, 11: AB9, 12: AB10, 13: AB11.



Phylogenetic analysis based on the EF-1α region. The amplification and sequencing of the EF-1α region was performed with the EF1-EF2 primers plus the DNA of the 13 treatments. After PCR amplification, bands of 570 bp were visualized for the 13 treatments and the presence of non-specific bands in each of the isolates was observed, so that the Band-stab PCR technique was performed in all isolates. Amplification of the products extracted with the Band-stab PCR technique enabled the visualization of 680 bp bands in the 13 Treatments (AB1- AB13), with the following DNA concentrations: 80 ng / µl (AB2, AB3, AB4, AB5, and AB8), 100 ng / µL (AB1, AB6, AB7, AB9, AB10, AB11, AB12 and AB13) (Figure 2).

Other authors have previously studied the ITS and EF-1α regions, demonstrating their effectiveness in solving some generated ambiguities and locating taxonomically new species of the genus Fusarium [14]. Although the use of universal primers allows us to study inter-specific variability and to establish identifications when comparing the sequences obtained with others previously deposited in the GenBank database, it should be emphasized that the best tool to quickly and unequivocally identify at a lower cost the different species of fungi associated with vascular wilt is the use of specific primers (15), but everything will depend on the purity of the isolates and the PCR products, hence several procedures should be performed the best result is reached [16].

Evaluation of the special form of *Fusarium oxysporum* with specific primers. Specific oligonucleotides designed for *Fusarium oxysporum* f sp. *Fragariae* Fofra-1 and Fofra-2 [17], used in the PCR reaction, did not show amplification in the 13 DNA samples. Thus, we must consider that the isolates used in this research did not amplify because there are differences in evolution between species from one locality to another [18, 19].

On the other hand, the ANOVA analysis revealed no significant statistical differences between the treatments in terms of distribution, indicating no variability among the variables. The coefficient of variation was 19.46%, which is considered tolerable and acceptable for field evaluation.

In conclusion, the molecular evaluation identified *Fusarium oxysporum* f. sp. *fragariae* as the pathogen responsible for vascular wilt in strawberry plants. During the assessment of pathogenesis, *Fusarium oxysporum* f. sp. *fragariae* was detected in all 13 treatments exhibiting vascular wilt symptoms, confirming its presence in Ecuador. This study marks a significant step in recognizing the microbiota associated with strawberry cultivation in Ecuador. It underscores the importance of using molecular techniques for pathogen detection. These tests revealed a high level of diversity among the identified fungal genera and species, providing valuable information for diagnosis and management strategies in strawberry farming.

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Conflict of Interest Statement: The authors declare that they have no competing interests.

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Exploring Impact of Parent Child Relationships on Holistic Development and Well- being: An Overview

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ABSTRACT:
Parent-child relationships play a vital role in shaping a child's development and well-being. Parent-child relationships are fundamental to the emotional, cognitive, and social development of children. This review paper explores the multifaceted effects of parenting quality, attachment, communication styles as well as parental involvement and discuss the consequences of positive and negative parents-child relationship on child outcomes. It also provides an overview of existing literature from development psychology and family studies. Besides, this paper highlights the crucial role of secure and supportive parent-child interactions in promoting optimal well-being and development in children.

KEY WORDS: Parent-child relationships, psychological well-being, and holistic development.

INTRODUCTION

The parent-child relationship is a central, aspect of a child's life, influencing their development, well-being, and future outcomes. The quality of this relationship can also influence children's emotional regulation, academic success, social competence and regulation of mental health. A positive, nurturing environment lays the foundation for healthy development, while dysfunctional relationships can predispose children to various psychological and behavioural challenges. So strengthening parent-child relationships is a mutual gain that significantly impacts the wall-being of both parents and children. This bond creates a ripple effect within the family, influencing dynamics and shaping the overall health and happiness of all its members. Several research studies that suggests, that 75 per cent of the time spent with our kids in our life time will be by age 12, emphasizing the crucial nature of these early shaping. Strengthening parent-child relationships is crucial for building resilient families and serving as catalyst for positive rearing outcomes in children. Thus creating a supportive environment through deepened bonds and enhancing children's well-being and development.

Theoretical Frameworks: Attachment theory suggests that the quality of the parent child relationship influences child development. Accordingly, John Bowlby's attachment theory suggests that early interactions with caregivers shape internal working models that influence future relationships [4]. Secure attachment has been linked with better emotional regulation and resilience in children [1].

Bronfenbrenner's Ecological System Theory: Urne Bronfenbrenner's ecological systems theory emphasizes the interaction of different environmental systems, with the family unit, playing a central role [5]. The microsystem, which includes the immediate family, school, and peers, plays a crucial role in shaping a child's experiences. John Bowlby's attachment theory points that early interactions with caregivers shape internal working models that influences future relationships [4]. Secure attachment has been linked with better emotional regulation and resilience in children [1].

Dimensions of Parent-child Relationships

Emotional Warmth and Responsiveness: Parental warmth and responsiveness foster trust and emotional security, were

as studies showed that irresponsive parenting is associated with lower levels of anxiety and depression in children [8].

Discipline and Control: Authoritative parenting-marked by high warmth and firm control has consistently been linked to the best developmental outcomes [2]. In contrast, authoritarian or permissive styles may hinder autonomy or lead to poor self-regulation.

Communication Patterns: Open and empathetic communication enhances problem-solving and reduces conflict [11]. It also helps children develop language skills and emotional intelligence.

Impact on Developmental Domains

Cognitive Development: Parental involvement in educational activities improves academic performance and cognitive skills [10]. Language-rich environments created through parent child interactions contribute to literacy and problem-solving behaviour in children.

Social Development: Secure parent-child relationships model on social behaviours like empathy and cooperation foster social development in children. Similarly children from supportive homes often display better peer relationships [12].

Emotional and Mental Health: Parental warmth and availability serve as buffers against stress and mental health issues. Conversely, neglectful or abusive relationships increase the risk of anxiety, depression, and behavioural disorders [6].

Cultural and Socioeconomic Considerations: Culture is defined as " the forms of traditional behaviour which are characteristic of a given society or of a group of societies, or of a certain race, or of certain area, or of a certain period of time [7], while socio-economic considerations are factors related to the social and economic standard of individuals or groups, including their education, income, occupation, and living conditions. These factors significantly impact various aspects of life and parenting practices one of them. Cultural norms influences parenting goals, while economic stressors can strain parent-child interactions [3]. Support system and parenting interventions can mitigate negative outcomes in disadvantaged families.

Interventions and Support Programs: Evidence based programs such as the Positive Parenting Program (Triple P) and Parent-child Interaction Therapy. (PCIT) have shown effectiveness in enhancing parenting skills and improving child outcomes [13, 9].

Consequences of Positive Parent – Child Relationships: Positive parent-child relationships are associated with numerous benefits, these are given below:

1. **Better emotional regulation:** Children with positive parent-child relationships tend to have better emotional regulation skills;
2. **Improved social skills:** Positive parent-child relationships foster social skills, such as cooperation and empathy.
3. **Enhanced cognitive development:** Supportive parent-child relationships are linked to better cognitive development and academic performance.
4. **Increased resilience:** Children with positive parent-child relationship tend to be more resilient in the face of adversity

Consequences of Negative Parent-child Relationships: Negative parent child relationships can have long-term consequences, these are mentioned below:

1. **Increased risk of mental health problems:** Children with strained or neglectful parent-child relationships are at higher risk of developing mental health problems.
2. **Poor social skills:** Negative parent-child relationships can lead to difficulties with poor social skills, such as aggression or withdrawal etc.
3. **Decreased academic performance:** Children with negative parent-child relationships may experience decreased academic performance and motivation.
4. **Increased risk of behavioural problems:** Negative parents child relationships can contribute to behavioural problems such as substances abuse or delinquency.

CONCLUSION

9 Parent-child relationships play a critical role in shaping child development and well-being, as it provides foundational influence on the holistic development of the children. Understanding the factors that influence parent-child relationships, such as parenting styles, attachment, and parental involvement, can inform the strategies to promote positive relationships and mitigate the consequences of negative relationships. By fostering positive parent-child relationships, parents, caregivers, and policymakers can promote healthy child development and well-being for healthy outcomes among children.

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Ameliorative Effect of Emilia Sonchifolia Plant Extract on Cisplatin Induced Renal Injury in Albino Wistar Rats".

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ABSTRACT:

The present study was aimed to explore the ameliorative effect of Emilia sonchifolia plant extract (ESPE) on cisplatin (CIS) induced nephrotoxicity in experimental rats. The animals were divided into 4 groups of 6 animals each. Normal (saline 10 ml/kg p.o) for 9 days, cisplatin (8mg/kg i.p on the 5th day of treatment), test group of low dose (ESPE 250 mg/kg p.o), test group of high dose (ESPE 500 mg/kg p.o) for 9 days in combination with cisplatin (on 5th day of treatment). After completion of 9 days dosing with test drug, on 10th day (after 6 days from CIS administration), the experimental rats were anesthetized by ether inhalation blood samples were collected from each rat by intra cardiac puncture and used for the estimation of biomarkers (creatine, urea and uric acid). Kidney of each animal was isolated, one part used for evaluation of antioxidants (GSH, SOD and catalase) and the other for histopathology. ESPE explored a significant ($P<0.01$) protection against CIS-induced nephrotoxicity by suppressing serum creatinine, urea and uric acid and by elevating the antioxidant parameters GSH, SOD and catalase at different doses compared to CIS treated group. Thus, investigational finding suggests that ESPE possess potential benefits against nephrotoxicity induced by anti-cancer drug CIS.

KEY WORDS: Cisplatin, Emilia Sonchifolia, Renal protective, Cytotoxic drug, Oxidative stress, Creatinine

INTRODUCTION

Nephrotoxicity is an intrinsic adverse effect of certain anticancer drugs. Anticancer drugs have a narrow therapeutic index and therapeutic dose of such drugs usually produces significant nephrotoxicity. Cisplatin (CIS), cis-diamine dichloro platinum (II), is one of the most powerful antineoplastic agents used to treat a wide range of human malignancies such as testicular, ovarian, bladder, head and neck, esophageal, small cell lung cancer, cervical, bladder and other genito-urinary tumours. The toxic effects of cisplatin in kidney affects approximately 25–35% of patients treated with a single dose, thereby limiting its use in higher doses and compromising its chemotherapeutic efficacy. CIS treatment is hampered by significant side effects such as neurotoxicity, nephrotoxicity, myelosuppression, ototoxicity, nausea and vomiting. The most serious and usually dose limiting toxicity of cisplatin is renal and it is associated with its metabolism [1-3].

CIS -induced nephrotoxicity is also closely associated with an increase in lipid peroxidation in the kidney tissues. This antitumoral drug also causes generation of reactive oxygen species (ROS), such as superoxide anion and hydroxyl radical that deplete the GSH (reduced glutathione) levels and inhibit the activity of antioxidant enzymes in renal tissue. This ROS may produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage [4]. In allopathy till date there is no effective drug reported to treat the nephrotoxicity of cisplatin. Several studies have focused currently on traditional herbal medicines to evaluate novel therapeutic drugs for

acute kidney injury (AKI) therapy. Various herbal medicines, including pomegranate, Prosthechea michoacana, Zingiber officinale, red ginseng etc. have protective effects against cisplatin-induced acute kidney injury with vivo experiment. Emilia sonchifolia is a soft annual herb which grows up to 40 cm in height, leaves are simple, lyrate-pinnate with large terminal lobe, flowers purplish with corymbose heads. Fruits are oblong containing many seeds.

The plant occurs in open field and waste lands. The plant is effective in treatment of fever, tonsillitis. The juice is used for eye infections. The herb is useful in conditions like cough, bronchial disorder, piles, worm infections, diarrhea, swelling and diabetics. The other reported activities of the plant include anti-anxiety, hepatoprotective, anti-cataract and anti-convulsant activities. Tribal uses the juice of crushed whole plant for wound healing [5,6].

MATERIAL AND METHODS

Animals: Albino Wistar rats of either sex weighing 180-250g were procured from animal house of Shree Devi College of pharmacy, Mangalore and housed at 25° ± 5°C, relative humidity 50 ± 5% in a well-ventilated animal house under 12:12 h light dark cycle. All the rats were provided with commercially available standard pellet diet, water and libitum. All the studies conducted were approved by Institutional Animal Ethical Committee (IAEC), Shree Devi College of Pharmacy, Mangalore (SDCP/IAEC/15/2020) according to prescribed guidelines of committee for the purpose of Control and Supervision of Experiments in Animals (CPCSEA), Government of India.

Chemicals and reagents: Cisplatin was purchased from Sigma Aldrich, Bangalore and biochemical kits from Robonik India Pvt Ltd in Mumbai. Other chemicals and reagents used were analytical grade and were procured from standard companies.

Preparation of Emilia Sonchifolia Plant Extract (ESPE): The plant, E. Sonchifolia was collected from Kerala, India. E. sonchifolia whole plants were washed with distilled water immediately after collection. The plants were chopped into small pieces, air dried at room temperature (26 ± 1) °C for about 10 days and ground into powder (grinder purchased locally) to store in an airtight container. Plant powder was soaked in n- hexane solvent and kept in the shaker for 48 hours at room temperature. The extract was collected and concentrated at 40°C under reduced pressure using a rotary evaporator. The dried extract was stored at 4°C until further use. The remaining residue was extracted again with the fresh solvent to ensure complete extraction and performed phytochemical analysis.7-8

Experimental Design: The present study was designed for 10 days. After acclimatization for one week, the male rats were randomly categorized into four equal groups in separate **polypropylene cages with six rats each. Group I:** Normal (saline 10 ml/kg p.o) for 9 days Group II: CIS (8mg/kg i.p) on the 5th day of treatment. Group III: Low dose (250 mg/kg, p.o) ESPE + CIS on the 5th day of treatment. Group IV: High dose (500 mg/kg, p.o) ESPE + CIS on the 5th day of treatment. After completion of 9 days dosing with test drug, on 10th day, the experimental rats were anesthetized by ether inhalation and blood was collected from each rat by intracardiac puncture for the estimation of biomarkers (creatine, urea and uric acid). Then animals were sacrificed and kidneys were isolated, one part used for evaluation of antioxidants (GSH, SOD and catalase) and the other for histopathology [9-10].

Table 1. Effect of ESPE on serum biomarkers in CIS-induced nephrotoxicity

TREATMENT	CREATININE (mg/dl)	UREA (mg/ml)	URIC ACID (mg/ml)
NORMAL	0.958±0.009	37.858±0.032	1.587±0.021
CIS	4.457±0.019###	70.808±0.048###	6.312±0.029###
ESPE 250+CIS	3.362±0.176**###	59.785±2.643**###	4.936±0.416*###
ESPE 500+CIS	2.087±0.373***##	55.761±3.063***###	4.229±0.548**###

n=6, values are expressed in Mean ±SEM, one way ANOVA followed by Tukey Kramer multiple comparison test. *p<0.05, **P<0.01, ***P<0.001 when compared to cisplatin ##P<0.01, ###P<0.001 when compared to normal.

Assessment of renal protective activity of ESPE - Evaluation of serum markers and endogenous antioxidant parameters: The concentration of blood markers such urea, uric acid, and creatinine in Heparinized serum samples were measured with semi-auto analyser (Robonik) and commercial kits. Each rat's kidney half was homogenized in a solution of saline (0.05M, pH 7.4) and ice-cold 10% trichloroacetic acid phosphate buffer. For fifteen minutes, the renal homogenates were centrifuged at 15,000 rpm. The generated supernatants were utilized in colorimetric measurements of GSH, SOD, and catalase. The GSH content of the kidney tissue was assessed using Ellmann's technique. Aebi approach was used to evaluate the activity of the catalase enzyme. Additionally, the Kakkar et al. approach was used to evaluate the SOD activity in renal tissues [11-13].

Histopathological study: Specimens of kidney tissues of each group were fixed in 10% of buffered formalin and processed with paraffin wax. For histopathological examination, 5 micrometer sections were stained with hematoxylin and eosin (H&E) for the examination using light microscope [14].

Figure 1: Effect of ESPE on serum creatinine and uric acid in CIS induced nephrotoxicity

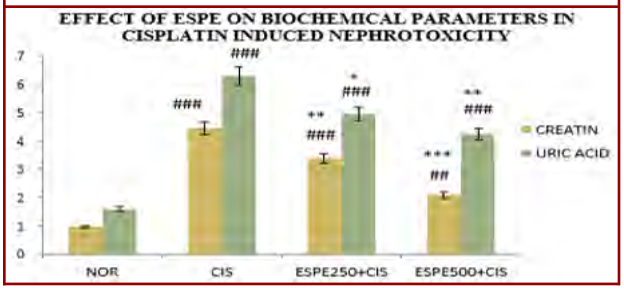
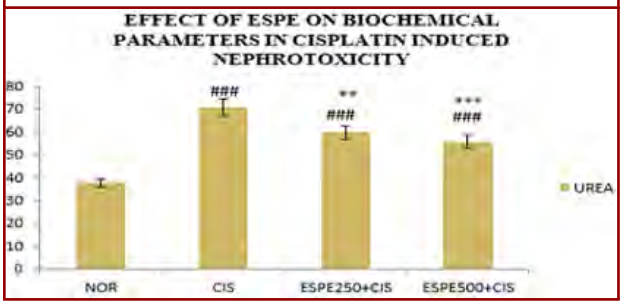


Figure 2: Effect of ESPE on serum urea in CIS induced nephrotoxicity n=6, values are expressed in Mean ±SEM, one way ANOVA followed by Tukey Kramer multiple comparison test. **P<0.01, ***P<0.001 when compared to cisplatin ###P<0.001 when compared to normal



Data and Statistical analysis: The data obtained by the various parameters was statistically evaluated by one-way analysis of variance (ANOVA) followed by Tukey-Karmer multiple comparison tests using Graph Pad Prism software. The definitive results as expressed in mean ± standard error of mean and $p< 0.05$ was considered to be statistically significant [15-16].

RESULTS

Effect of ESPE on renal function biomarkers: A significant (P<0.001) protection was explored on serum markers in ESPE pre-treated animals against CIS -induced disruption on renal system (Table 1 & Fig 1,2). A significant (P<0.001) rise in serum creatinine, urea and uric acid content was exhibited in CIS alone treated group compared to the normal group. The pre-treatment with ESPE at both doses (250 mg/kg and 500 mg/kg) groups showed a significant (P<0.01 & P<0.001) depletion in the level creatinine and urea compared to CIS alone treated groups. And The pre-treatment with ESPE (250 mg/kg and 500 mg/kg) groups explored a significant (P<0.05 & P<0.01) decline in the level of Uric acid compared to CIS alone treated groups.

ESPE ameliorates the CIS mediated decrease in antioxidant defense of renal system: The pre-treatment with ESPE revealed beneficial role on endogenous antioxidants against CIS -induced nephrotoxicity (Table 2 & Fig 3). A significant (P<0.001) decline in GSH was observed in CIS alone treated groups compared to normal groups. The pre-treated with ESPE (250 mg/kg and 500 mg/kg) animals exhibit a significant (P<0.05 & P<0.01) elevation in GSH compared to CIS alone treated groups. There was significant (P<0.001) fall in SOD and catalase was found in CIS alone treated animals compared to normal rats. The pre-treated with ESPE (250 mg/kg and 500 mg/kg) groups were explored significant (P<0.01 & P<0.001) rise in SOD and catalase compared to CIS alone treated animals.

Histological Analysis: The renal cortex of normal rats revealed a normal corpuscular and tubular histological structure. In CIS-treated rats, degenerative changes were noticeably observed in renal tissues. These changes were in the form of luminal dilatation with excessive cast accumulation; the renal corpuscles showed dilated capsular space with condensed and even degenerated glomerulus. Inflammatory cells infiltration and vascular congestion were noticed within the renal cortex and pyknotic nuclei were present in renal tubules. The kidneys of rats treated with low dose ESPE (250 mg/kg) + CIS and high dose ESPE (500mg/kg) + CIS revealed less histological damage in renal corpuscles and renal tubules. Mild tubular degeneration with luminal dilatation and inflammatory cell infiltration were seen within the renal cortex. (Fig 4).

DISCUSSION

The present study was aimed to evaluate the protective effect of ESPE on renal toxicity induced by CIS in wistar rat and the study revealed highly significant protective effects of ESPE against CIS.

CIS is widely used to treat a variety of tumors, such as the head and neck region, testis, liver, prostate, ovary, lung, and cervix. Renal damage, the most common deleterious effect limiting cisplatin medicines, affects around 30% of individuals. According to certain research, ROS reactions play a crucial role in mediating CIS-induced renal toxicity. Herbs and plants are naturally occurring resources with a long history of therapeutic use. The human search for a potent nephroprotective agent has led people to seek out appropriate sources, such as the traditional medical system that has less adverse benefits. The antioxidant-rich plants Rubia cordifolia, Hybanthus enneaspermus, Pongamia pinnata, Echina pallidum, Ginkgo biloba, and Nigella sativa shown a notable defense against nephrotoxicity caused by cisplatin. The current study also sought to reduce nephrotoxicity caused by cisplatin therapy by using ethanolic extracts of ESPE concurrently with treatment [17,1].

In CIS treated group induced renal toxicity, there is significant (P<0.001) elevation of urea, creatinine and uric acid when compared with normal rats. Serum creatinine and urea are termed as the major indicators or of nephrotoxicity and hepatorenal

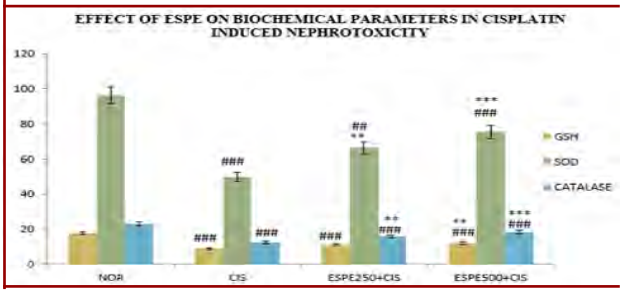
syndrome. Serum creatinine is the endogenous substance of muscle metabolism and eliminated unchanged by the renal system. The increase in creatinine level in the blood may be due to the abnormality of glomerular filtration process in the kidney. Urea is the waste substance generated during protein metabolism of by Urea cycle by the liver. The deficiency of GFR and a decrease in blood volume leads to increase in urea level in serum.

Table 2: Effect of ESPE on antioxidants in CIS induced nephrotoxicity

TREATMENT	GSH (ηM/g wet gland)	SOD (U/mg wet gland)	Catalase (U/mg wet gland)
NORMAL	17.509±0.058	96.262±0.007	22.75±0.012
CIS	8.737±0.006###	49.566±0.007###	12.08±0.017###
ESPE250+CIS	11.006±0.615*###	66.332±2.649**###	15.607±0.726**###
ESPE500+CIS	11.777±0.950**###	75.380±5.974***##	18.052±1.202***###

n=6, values are expressed in Mean ±SEM, one way ANOVA followed by Tukey Kramer multiple comparison test. *p<0.05, **P<0.01, ***P<0.001 when compared to cisplatin ##P<0.01, ###P<0.001 when compared to normal.

Figure 3. Effect of ESPE on antioxidants in CIS induced nephrotoxicity in rats.
n=6, values are expressed in Mean ±SEM, one way ANOVA followed by Tukey Kramer multiple comparison test. *p<0.05, **P<0.01, ***P<0.001 when compared to cisplatin ##P<0.01, ###P<0.001 when compared to normal

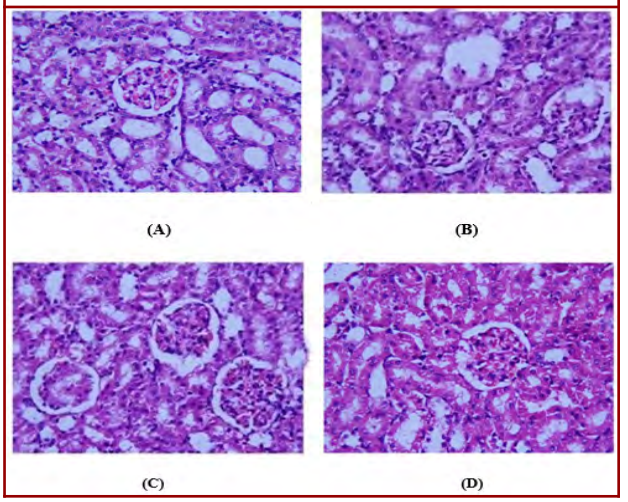


Uric acid is by-product produced during purine biotransformation by the enzyme urate oxidase (uridase). The increase in uric acid level might be due to kidney urate elimination and inefficiency of glomerular filtration in nephrons. Further, it may lead to hyperuricemia due to more tubular reabsorption, mediated by urate exchanger and voltage sensitive urate channel in proximal tubules [18-19]. The pre-treatment with ESPE successfully amend the increased levels of creatinine, urea and uric acid induced by CIS.

Oxidative stress damage and development of reactive oxygen species (ROS) were suggested in the pathogenesis of CIS and other cytotoxic drugs induced nephrotoxicity. Cellular injury causes due to disruption of dynamic equilibrium between pro-oxidant and free radicals scavenge by antioxidants. In present study, CIS administration leads significant decline in GSH level. The depletion in GSH content in renal tissues after CIS therapy is due to the toxic metabolite acrolein. Acrolein binds GSH in the plasma membrane and disrupts antioxidant defense system and increase the ROS and results in the necrosis of the tubular cells of kidney.

Figure 4: Histopathological slides of Rat kidney tissue in CIS induced nephrotoxicity

(A)Normal kidney section with normal renal corpuscle and renal tubules
(B)Rats treated with CIS with congested shrank and completely degenerated glomeruli, debris in the lumen of some renal tubules, and pyknotic nuclei in renal tubules.
(C)and (D) Treatment with ESPE 250 mg/kg and 500 mg/kg normal renal corpuscle and renal tubule more or less like normal structure



In the current work, a significant decline in SOD and catalase level was observed after CIS administration. SOD and catalase are the vital antioxidant enzymes which converts oxygen molecules into non-toxic products. Increase in ROS leads to decrease in SOD level. Elevation in the level H2O2 is due to the inhibition of catalase action. The depletion of catalase in turn also prevents the SOD action. The decline in these antioxidant enzymes is mainly due to more ROS and lipid peroxidation [20-22]. The pretreatment with ESPE lower and higher doses significantly

($P<0.01$) increases the level GSH, SOD and catalase after CIS treatment.

The findings of serum markers and antioxidant parameters were correlated with histopathological examination. The CIS therapy exhibit pathological abnormality such as decrease in the size of Bowman's capsule, congestion and dilation of blood vessels, inflammatory infiltration [23]. The pre-treatment with ESPE at both doses successfully augmented the toxic effects induced by CIS renal system by exploring beneficial effects such as, no change in the size of Bowman's capsule, slight inflammation and no congestion of blood capillaries.

CONCLUSION

The present investigation reveals the potent renal protective activity of ESPE (250 mg/kg and 500 mg/kg, p.o) against cisplatin induced renal injury in experimental rats. The prophylactic treatment and efficacy of herb could be attributed to its potential antioxidant property and free radical scavenging activity, modulation in serum markers and protection of histopathological features. Further research is required to establish the fact clinically.

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Conflicts of Interest: There is no Conflict of Interest.

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Investigation of Dose-Dependent Cholinesterase Inhibitory Activity of *Lycopodium clavatum* Spore Extracts: Implications for Neurodegenerative Disease Therapy

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ABSTRACT

Neurodegenerative disorders, particularly Alzheimer's disease (AD), present significant global health challenges, with cholinesterase enzymes, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), serving as critical therapeutic targets. The limitations of current synthetic pharmacological interventions, characterized by undesirable side effects and high costs, have intensified the scientific and clinical pursuit of novel enzyme inhibitors derived from natural products. This investigation aimed to systematically evaluate the inhibitory effects of extracts obtained from Lycopodium clavatum spores on both AChE and BChE enzymatic activities. The experimental approach involved preparing ethanol extracts from commercially acquired Lycopodium clavatum spores, followed by the assessment of enzyme activity using the standardized Ellman's colorimetric assay. 42. The findings revealed a clear dose-dependent inhibition of both AChE and BChE by the Lycopodium clavatum spore extracts. Specifically, at a concentration of 1 mg/ml, the extract demonstrated significant inhibition of AChE (49.85 ± 1.33%, $P<0.001$) and BChE (71.05 ± 0.25%, $P<0.01$). The calculated inhibitory concentration 50% (IC50) values further indicated potency, with 1.082 µM for AChE and a notably lower 0.019 µM for BChE.

The observation that Lycopodium clavatum spores are a common and inexpensive material that allows for economic scaling is particularly noteworthy, especially in the context of potential large-scale production for various applications, including responses to bioterrorism threats. This characteristic elevates the significance of this research beyond chronic disease management, suggesting a strategic value for rapid-response public health interventions due to its affordability and widespread availability. Furthermore, the deliberate focus on inhibiting both AChE and BChE is a critical aspect of this study. While AChE is a primary therapeutic target in early AD, BChE is increasingly recognized for its compensatory role, particularly in later stages of the disease or when AChE activity declines. An agent capable of inhibiting both enzymes could offer a more robust and comprehensive therapeutic strategy, potentially addressing a broader spectrum of cholinergic deficits and pathological progression in neurodegenerative conditions. The significantly lower IC50 for BChE (0.019 µM) compared to AChE (1.082 µM) further suggests a potent effect on BChE, which could be exploited for specific therapeutic advantages. In conclusion, Lycopodium clavatum spore extracts present as a promising natural candidate warranting further rigorous investigation for their potential therapeutic application in the development of novel treatments for neurodegenerative diseases, particularly AD.

KEY WORDS: Lycopodium Spores, Acetylcholinesterase, Butyrylcholinesterase, Neurodegenerative Diseases, Alzheimer's Disease.

INTRODUCTION

Lycopodium: Botanical Description and Ethnobotanical History: Lycopodium is a distinct genus of moss belonging to the family Lycopodiaceae, commonly referred to as club moss or ground pine (1). This ancient plant is characterized by its flowerless, vascular nature, existing as either terrestrial or epiphytic forms. Its morphology includes widely branched, erect stems that are densely covered with small, simple, needle-like or scale-like leaves. Each leaf possesses a single, unbranched vascular thread, a characteristic feature distinguishing it from more complex vascular plants (2). A key reproductive feature of Lycopodium is its production of a single type of spore, which is borne on the upper surface of specialized leaves known as sporophylls, arranged in a cone shape (3).

Historically, harvested Lycopodium spores, commonly known as lycopodium powder, have been utilized in various traditional practices (4). In traditional Austrian medicine, for instance, it found application as teas or compresses for a range of ailments, including disorders of the locomotor system, skin conditions, liver and bile issues, kidney and urinary tract problems, infections, rheumatism, and gout. However, it is important to note that claims regarding the efficacy of these traditional uses remain largely unproven by modern scientific standards.

Beyond medicinal applications, Lycopodium has also seen non-medicinal uses, such as its controversial employment in chemical warfare testing programs by some governments (5). Its highly flammable nature also led to its use by magicians for special effects and by pharmacists in earlier times for coating pills, while homeopaths used it for respiratory conditions (6).

The juxtaposition of Lycopodium's historical uses—some unproven, some even misused—with its modern scientific investigation underscores a critical aspect of natural product research. The explicit mention that traditional efficacy claims are "unproven" and its historical use in "chemical warfare testing programs" highlights a tension between anecdotal or historical applications and the imperative for rigorous, evidence-based scientific assessment. This historical context provides a strong justification for the current study's rigorous scientific approach (7).

It emphasizes the critical importance of subjecting natural products, irrespective of their traditional heritage, to modern pharmacological scrutiny to establish genuine efficacy, safety profiles, and precise mechanisms of action (8). This study, by employing controlled laboratory methods, directly addresses the gap between historical claims and validated scientific understanding, ensuring that potential therapeutic benefits are substantiated and potential toxicities, which are also noted in literature, are thoroughly understood and mitigated.

Cholinesterase Enzymes: Physiological Roles and Pathological Significance: Enzymes are fundamental biological catalysts, protein in structure, that accelerate myriad biochemical reactions essential for the metabolism of living organisms. Among these, cholinesterase enzymes play a pivotal role in neurotransmission and are of particular interest in neurodegenerative diseases (9).

Acetylcholinesterase (AChE; EC: 3.1.1.7) is a crucial member of the serine hydrolase family. Its primary physiological function is the rapid hydrolysis of the neurotransmitter acetylcholine (ACh) in the synaptic cleft, thereby terminating cholinergic signaling. Due to its central role in cognitive function, AChE has emerged as a significant therapeutic target for pharmacological interventions in neurodegenerative pathologies, most notably Alzheimer's disease (AD) (10,11).

Butyrylcholinesterase (BChE; EC 3.1.1.8) is a non-specific cholinesterase enzyme capable of hydrolyzing a diverse range of choline-based esters. In humans, BChE is predominantly produced in the liver and circulates in blood plasma, with its synthesis encoded by the BChE gene. It shares considerable structural and functional similarity with neuronal AChE, also known as erythrocyte cholinesterase. The analysis of BChE activity in plasma is clinically valuable as a liver function test, indicating pathological processes such as hypercholinesterasemia and hypocholinesterasemia. The enzyme has an approximate half-life of 10-14 days (12).

The central cholinergic pathway is critically involved in essential cognitive functions such, as learning and memory. Neurodegenerative disorders are often characterized by severe cholinergic deficiencies, which directly contribute to profound cognitive impairment. Consequently, the inhibition of brain cholinesterases has become a cornerstone therapeutic strategy for managing cognitive decline in various neurodegenerative conditions, including AD, senile dementia, ataxia, myasthenia gravis, glaucoma, and Parkinson's disease (13).

The description of BChE as a "nonspecific cholinesterase" that is nonetheless "very similar to neuronal acetylcholinesterase" highlights a nuanced understanding of its therapeutic significance. While AChE is the primary focus for early AD intervention, BChE activity has been observed to increase in later stages of AD, potentially compensating for declining AChE function or even contributing to pathology. Therefore, inhibiting BChE, despite its "nonspecific" label, could offer a crucial therapeutic advantage, especially in advanced disease states or when patients exhibit reduced responsiveness to selective AChE inhibitors. The inclusion of BChE as a target in this study implicitly acknowledges this evolving understanding of its role in neurodegeneration. Furthermore, its utility as a liver function test implies a systemic presence and function, necessitating careful consideration of

potential systemic effects when developing BChE-targeting therapeutics (14).

The Growing Imperative for Natural Product-Derived Therapeutics: The global scientific community is engaged in an intensive search for novel and more efficacious drug candidates to address unmet medical needs (15). In this endeavor, natural products have emerged as a significant source of interest for therapeutic agents (16). This burgeoning focus on natural compounds is largely driven by the inherent limitations of conventional modern pharmaceuticals. These limitations include the frequent occurrence of undesirable side effects, the potential for increased toxic effects on patients with long-term use, and the high economic cost associated with many synthetic drugs (17).

In contrast, plant-based treatment methods offer compelling advantages, notably their affordability and typically minimal to absent side effects. This favorable profile has led to a recent resurgence in the preference for naturally sourced medicines. Consequently, modern medical science has begun to accord greater importance to these traditional and plant-derived treatments, positioning plants as a significant subject of contemporary scientific research (18).

Study Rationale and Specific Objectives: Building upon the established roles of cholinesterases in neurodegeneration and the growing interest in natural product-derived therapeutics, the current study was specifically designed to investigate the inhibitory effect of plant-derived Lycopodium spores on both acetylcholinesterase and butyrylcholinesterase enzymes. The overarching objective is to discuss the potential applicability and usability of these extracts in the therapeutic management of neurodegenerative diseases (19).

MATERIAL AND METHODS

Plant Material Acquisition and Extract Preparation: Lycopodium clavatum spores used in this investigation were acquired from a reputable commercial supplier, Sigma-Aldrich. For the preparation of extracts, the spores were meticulously dissolved in 200 ml of 60% ethanol to facilitate the extraction of bioactive compounds. The resulting crude extracts then underwent a stringent purification process. This involved filtration through 0.22 µm sterile filters (Sartorius, Germany) to remove particulate matter and microorganisms, ensuring a cleaner extract.

Subsequently, the extracts were concentrated and dried using a lyophilizer, operating at a temperature of -49°C under a 3000 mT vacuum, which effectively removes solvent while preserving thermolabile compounds. Once dried, the extracts were stored at 4°C to preserve their integrity and stability until further use.

Comprehensive detailing of these experimental procedures, from extract preparation to assay conditions, is not merely a formality but a critical determinant of scientific validity. The meticulous reporting of methodology is paramount for ensuring the reproducibility of the study by independent researchers, a fundamental principle of the scientific method. Slight variations in solvent concentration (60% ethanol), filtration pore size (0.22 µm), lyophilization parameters (-49°C, 3000 mT vacuum), or storage conditions (4°C) can significantly alter the chemical profile and concentration of active compounds in natural extracts.

Similarly, precise assay conditions are critical for consistent enzyme kinetics and accurate inhibition measurements. This rigor not only validates the current findings but also enables direct comparability with future studies and facilitates the eventual translation of in vitro results to vivo models, laying a robust foundation for drug development. It implicitly addresses the inherent variability of natural products, a challenge that is further discussed later in this report.

Chemicals, Enzymes, and Reagents: Commercially sourced Acetylcholinesterase (EC 3.1.1.7, Type-VI-S, derived from electric eel) and Butyrylcholinesterase (EC 3.1.1.8, derived from equine plasma) were obtained from Sigma-Aldrich for the enzymatic assays. The specific substrates used were acetylthiocholine iodide for AChE and butyrylthiocholine chloride for BChE. 5,5'-dithio-bis-nitrobenzoic acid (DTNB), a chromogenic reagent essential for colorimetric detection, was also purchased from Sigma (St. Louis, MO). All buffers and other chemical reagents utilized throughout the study were of extra pure analytical grade, ensuring high purity and minimal interference in the experimental results. Galanthamine (Reminyl® from Johnson & Johnson), a clinically approved cholinesterase inhibitor, served as the positive control for comparative analysis, providing a benchmark for the observed inhibitory activity.

The deliberate choice of Galanthamine as a standard drug serves a crucial comparative purpose. Galanthamine is an established, FDA-approved cholinesterase inhibitor widely used in the clinical treatment of mild to moderate Alzheimer's disease. By comparing the inhibitory effects of Lycopodium extracts directly against Galanthamine, the study provides an immediate and clinically relevant benchmark for the extract's potential therapeutic efficacy. This comparative analysis allows researchers to gauge whether the natural extract's potency is comparable to, or significantly different from, a drug already proven effective in humans. This comparison is vital for prioritizing promising natural compounds and guiding subsequent drug development efforts, indicating whether the extract possesses sufficient in vitro activity to warrant further, more costly in vivo and clinical investigations.

Cholinesterase Inhibition Assay Protocol: The inhibitory activity of the Lycopodium extracts against cholinesterases was quantitatively determined using the well-established colorimetric method developed by Ellman et al. (1961)(20). For the acetylcholinesterase (AChE) inhibition assay, a precise

protocol was followed: 140 µl of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µl of enzyme preparation, and 20 µl of the test compound solution (dissolved in methanol) were combined in each reaction vessel. This mixture was incubated at a controlled temperature for 30 minutes to allow for potential enzyme-inhibitor interactions. Following this incubation period, 10 µl of DTNB was added, and the enzymatic reaction was initiated by the addition of 10 µl of acetylthiocholine, the specific substrate for AChE.

For the assessment of butyrylcholinesterase (BChE) activity, a modification was made to the substrate: 10 µl of butyrylthiocholine chloride was substituted as the substrate, while maintaining the other components and conditions of the assay.

The hydrolysis of both acetylthiocholine and butyrylthiocholine was monitored spectrophotometrically. The formation of the yellow 5-thio-2-nitrobenzoate anion, which results from the reaction between DTNB and the thiocholines catalyzed by the enzymes, was measured at a wavelength of 412 nm. Methanol was employed as a negative control in these experiments to account for any potential solvent effects on enzyme activity.

Data Analysis and Statistical Evaluation: The percentage of enzyme inhibition was calculated based on the Michaelis–Menten model, a standard kinetic model widely used for enzyme reactions. Galanthamine, serving as the standard drug, was tested at two concentrations: 10 µg/ml and 1 mg/ml, both dissolved in methanol, to provide a comparative reference for inhibitory potency. All experimental analyses were performed in triplicate to ensure reliability and reproducibility of the results. The data obtained expressed as means, were statistically compared using the Student T-Test. A P value of less than 0.05 ($P<0.05$) was predefined as the threshold for statistical significance, indicating a low probability that the observed differences occurred by chance. An enzyme inhibition graph, illustrating the percentage of activity, was generated to visualize the dose-response relationship across five different concentrations of the Lycopodium spore extract.

RESULTS

The investigation into the inhibitory effects of Lycopodium clavatum spore extracts on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes yielded clear dose-dependent results, summarized in the following tables and figure.

Table 1. Anticholinesterase activity of Lycopodium clavatum spore extracts against AChE and BChE.

		% inhibition		
	AChE (10 µg/ml)	**BChE (10 µg/ml)**	**AChE (1 mg/ml)**	**BChE (1 mg/ml)**
Lycopodium clavatum	1.56 ± 0.67	11.78 ± 0.31***	49.85 ± 1.33***	71.05 ± 0.25**

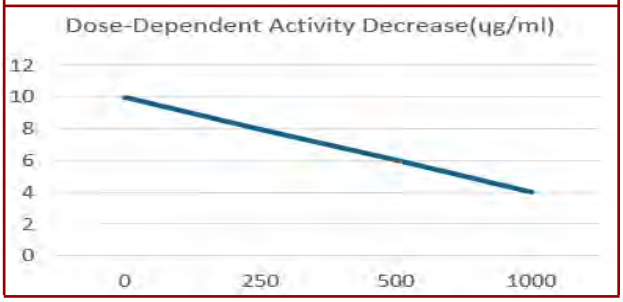
Values are expressed as mean (n=6). P>0.05; * P<0.05; ** p<0.01; *** P<0.001.

Dose-Dependent Anticholinesterase Activity of Lycopodium clavatum Extracts: Table 1 presents the anticholinesterase activity of Lycopodium clavatum spore extracts against both AChE and BChE at two distinct concentrations.

At the lower concentration of 10 µg/ml, the extract exhibited minimal inhibition of AChE (1.56 ± 0.67%). In contrast, BChE inhibition at this concentration was statistically significant, reaching 11.78 ± 0.31% ($P<0.001$). Upon increasing the concentration to 1 mg/ml, a substantial and statistically significant inhibitory effect was observed for both enzymes: AChE inhibition reached 49.85 ± 1.33% ($P<0.001$), and BChE inhibition was even more pronounced at 71.05 ± 0.25% ($P<0.01$).

The striking difference in IC50 values between AChE and BChE is a key finding that suggests more than just general inhibition. The IC50 for BChE (0.019 µM) is considerably lower than that for AChE (1.082 µM). This differential potency is a crucial aspect for future therapeutic development, indicating that the active compounds within the Lycopodium extract may possess a higher affinity or a more effective inhibitory mechanism specifically for BChE.

Figure 1: Enzyme inhibition percentage graph against ethanol extract concentrations of Lycopodium spores.



If BChE inhibition is a primary therapeutic target, for instance, in later stages of AD or specific BChE-related pathologies, Lycopodium could be an exceptionally potent

natural source. Conversely, if selective AChE inhibition is desired, further fractionation and isolation of specific compounds would be necessary to identify and potentially optimize the compounds responsible for AChE inhibition, or to enhance their selectivity. These finding guides future research towards either developing a potent BChE-centric therapy or pursuing selective targeting based on the desired clinical outcome.

Visual Representation of Enzyme Inhibition: Figure 1 visually illustrates the enzyme inhibition percentage as a function of increasing ethanol extract concentrations of Lycopodium spores. This graphical representation reinforces the clear dose-response relationship observed in the quantitative data, showing that higher concentrations of the extract lead to greater enzyme inhibition.

Table 2. Enzyme IC50 values for *Lycopodium clavatum* spores' ethanol extract.

	IC50 (µM)
AChE	1.082
BChE	0.019

The combined data from Table 1 and Figure 1 clearly illustrates that significant inhibitory effects are observed predominantly at higher concentrations of the extract. The observation that significant AChE inhibition only occurs at higher concentration (1 mg/ml) is highly relevant for future in vivo studies and potential clinical applications. It indicates that a therapeutically effective concentration of the active compounds must be achieved.

This finding directly informs dosage considerations for subsequent animal models and human trials, suggesting that simply administering small, arbitrary amounts of Lycopodium might not yield the desired pharmacological effect. It also prompts further inquiry into the bioavailability, pharmacokinetics, and effective concentration of these compounds in vivo.

Inhibitory Concentration 50% (IC50) Values: Table 2 provides the IC50 values, which represent the concentration of the extract required to inhibit 50% of the enzyme activity, for both cholinesterase enzymes.

The IC50 value for AChE was determined to be 1.082 µM, while the IC50 value for BChE was notably lower at 0.019 µM. These values quantitatively confirm the extract's potency and its differential inhibitory effect on the two enzymes.

DISCUSSION

Lycopodium Characteristics, Bioactive Constituents, and Safety Considerations: Lycopodium, commonly known as club moss, is a plant that grows along the ground and primarily reproduces by producing spores (21). Lycopodium powder is widely recognized for its use in educational demonstrations, particularly due to its highly flammable nature, which magicians historically exploited for dramatic effects (22). However, it is crucial to acknowledge the potential for moderate toxicity if the powder is inhaled or ingested. The plant is known to contain Huperzine A, a bioactive amine that is a recognized cholinesterase inhibitor (23).

Safety remains a significant concern, as studies have indicated the potential for lethality at high doses, with reported lethal doses exceeding 4 mg/kg in male rats (24). Despite the observed medical advantages of Lycopodiaceae, there is limited existing literature detailing adverse effects such as chemical pneumonia resulting from lycopodium aspiration. This gap in knowledge underscores that the comprehensive risks associated with Lycopodium use are not yet fully elucidated (25).

Contextualizing Cholinesterase Inhibition Findings in Neurodegenerative Disease: The current findings of cholinesterase inhibition by Lycopodium clavatum extracts align with previous research. Notably, alphaonocerin, another acetylcholinesterase inhibitor, was first reported to be isolated from Lycopodium clavatum by Orhan et al. (2003) (26). This reinforces the plant's established pharmacological relevance.

The central cholinergic pathway plays a pivotal role in cognitive functions, including learning and memory. In neurodegenerative disorders, severe cholinergic deficiencies contribute significantly to cognitive impairment, making cholinesterase inhibiting a cornerstone therapeutic strategy. This approach is applied in the management of various conditions such as Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis, glaucoma, and Parkinson's disease. While cholinesterase inhibitors are currently used for AD-type dementias, their utility is often constrained by associated side effects. This limitation has driven a broader discussion on the increasing appeal of natural plant products as viable therapeutic alternatives, largely due to their generally favorable side-effect profiles and greater accessibility. The potential role of various secondary metabolites, such as flavonoids, tannins, terpenoids, alkaloids, and phenols, found in natural sources in potentially mitigating disease progression is also gaining recognition (27,28).

Antioxidant Properties and Potential Synergistic Neuroprotection: Beyond cholinesterase inhibition, Lycopodium clavatum possesses inherent antioxidant properties. This is consistent with other known antioxidant-rich plants, such as Iceland moss (Cetraria islandica (L.) Ach.) and wild teasel (Dipsacus fullonum L.), whose antioxidant activities have been confirmed in various studies. Lycopodium contains key antioxidant compounds, including polyphenols, terpenoids, phenolic acids, flavonoids, alkaloids, and vitamins.

Specifically, apigenin, a flavonoid polyphenol with potent antioxidant activity, has been isolated from Lycopodium clavatum (29).

The presence of a wide array of bioactive compounds and the attribution of diverse pharmacological effects (cholinesterase inhibition, antioxidant, anticancer, anti-inflammatory, etc.) to Lycopodium extracts suggest that the observed cholinesterase inhibition is likely not due to a single isolated compound but rather a collective or synergistic effect of multiple compounds present in the crude extract. This underscores the inherent complexity and potential advantage of natural product pharmacology (30).

While the isolation and characterization of individual active compounds are crucial for understanding specific molecular mechanisms, the "whole extract" approach might offer synergistic benefits or a broader spectrum of action that a single isolated compound cannot replicate. For instance, simultaneous cholinesterase inhibition and antioxidant activity, both present in Lycopodium, could provide a more comprehensive therapeutic effect against multifactorial neurodegenerative diseases where oxidative stress is a significant pathophysiological contributor (31). This suggests that future research should not only aim to isolate the primary cholinesterase inhibitors but also investigate potential synergistic interactions among the various constituents and how their combined actions contribute to the overall therapeutic potential, moving towards a multi-target therapeutic strategy.

Broader Pharmacological Activities and Chemical Diversity of Lycopodium: Beyond their cholinesterase inhibitory activity, Lycopodium spores have been observed to possess a range of other biological effects, including anticancer, immune modulatory, anti-inflammatory, and hepatoprotective properties (32). They also influence various systems, such as the reproductive and central nervous systems (33). The alkaloids found in Lycopodium are significant due to their diverse biological activities and unique chemical structures, though many of these compounds remain underexplored (34). A comprehensive analysis has identified other compounds within the plant, including vanillic, coumaric, ferulic, and syringic acids, as well as huperzine A, lycopodine, lycoflexine, Alpha-onocerin, and sporopollenin.

A critical consideration in the study of natural products is the inherent variability in active compound levels (35). The time of collection, geographical location, specific extraction procedures, and storage conditions of the plant material can significantly affect both the quantitative and qualitative profiles of their active compounds (36). This inherent variability poses a significant challenge to achieving consistent pharmacological effects. For Lycopodium extracts to be considered as legitimate therapeutic agents, robust standardization protocols for sourcing, cultivation (if applicable), extraction, and quality control are essential.

Without such standardization, batch-to-batch inconsistencies in active compound profiles could lead to unpredictable potency, variable efficacy, and increased risks of adverse effects, making regulatory approval and widespread clinical adoption extremely difficult. This highlights that while the current study demonstrates promising in vitro activity, the path to a clinically applicable drug requires substantial effort in developing rigorous standardization methods to ensure consistent therapeutic outcomes and patient safety (37). Supporting evidence from other studies, such as the reported significant reduction in tumor incidence in carcinogenically poisoned mice treated with Lycopodium clavatum spore extract, showing decreased levels of biomarkers in both liver and spleen tissues, further underscores the plant's diverse pharmacological potential.

Comparative Analysis with Related Ethnopharmacological Studies: The findings of this study are consistent with and further supported by other ethnopharmacological investigations. Konrath et al. (2012) (38). explored the medicinal and therapeutic potentials of two Lycopodiaceae species, Lycopodium clavatum and Lycopodium thyoides, which have been traditionally used in South American folk medicine for central nervous system disorders. Their research, which evaluated alkaloid extracts for AChE and antioxidant activities, demonstrated dose- and time-dependent inhibition in various rat brain regions, including the cortex, striatum, and hippocampus, along with a reduction in lipid peroxidation. These comparative studies reinforce the notion that Lycopodium species possess multiple modes of action relevant to cognitive disorders, thereby lending scientific support to their traditional ethnobotanical uses.

The consistent referencing of Lycopodium's traditional uses across different cultures (Austrian, South American folk medicine) alongside its modern scientific investigation highlights a highly effective paradigm in modern drug discovery: ethnobotanical prospecting. Traditional knowledge, even if anecdotal or unproven, provides a targeted starting point for scientific inquiry, significantly narrowing the search space for novel bioactive compounds compared to random screening (39).

The current study, by demonstrating the biochemical basis (cholinesterase inhibition) for some of these traditional cognitive-enhancing claims, validates this approach (40). This not only accelerates the drug discovery process but also underscores the immense value of preserving indigenous and traditional knowledge systems as a vital resource for future pharmaceutical innovations, provided they are subjected to rigorous scientific validation for safety and efficacy.

Synthesis of Findings and Future Research Directions: The investigation unequivocally demonstrates that ethanol extracts of Lycopodium clavatum spores exhibit significant dose-dependent inhibitory activity against both acetylcholinesterase

and butyrylcholinesterase enzymes. This finding positions these natural extracts as promising candidates for continued research in the context of Alzheimer's disease treatment (41).

CONCLUSION

The present study provides compelling evidence that ethanol extracts derived from Lycopodium clavatum spores possess significant dose-dependent inhibitory activity against both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes. This finding underscores the compelling potential of these natural extracts as promising candidates for the development of novel therapeutic strategies aimed at combating neurodegenerative diseases, particularly Alzheimer's disease.

The explicit call for "further studies" in the conclusion, following promising vitro results, highlights a critical stage in drug development.

In vitro results, while foundational, do not directly translate to clinical efficacy or safety in living organisms. This underscores the complex and multi-stage nature of pharmaceutical development. The transition from in vitro enzymatic inhibition to a demonstrable in vivo therapeutic effect requires rigorous evaluation of numerous factors that cannot be assessed in a test tube. These include the bioavailability of the active compounds (how much reaches the target site), their metabolic fate (how they are processed by the body), their ability to cross biological barriers (such as the blood-brain barrier for central nervous system effects), and their potential systemic toxicities or side effects in a complex physiological environment.

The necessity for detailed mechanistic investigations further implies the need to identify the specific compounds responsible for the observed effects and to understand their precise molecular interactions within a living system. This highlights that the current findings are a crucial first step, but significant research investment is required to bridge the gap between laboratory observation and potential clinical application.

Therefore, the critical necessity for further comprehensive in vivo studies is paramount to validate efficacy and safety in biological systems. Concurrently, detailed mechanistic investigations are required to elucidate the precise molecular interactions underlying the observed inhibition. Furthermore, systematic active compound isolation and characterization are essential to fully unlock and optimize their therapeutic potential and establish a robust safety profile for future pharmacological development.

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Phytochemical and Therapeutic Evaluation of Psoralea corylifolia and *Withania somnifera:* A Comprehensive Review of Their Use in the Treatment of Hypopigmentary Disorders

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ABSTRACT

Hypopigmentary disorders, particularly vitiligo, represent complex dermatological conditions characterized by the loss of melanocytes or melanin. These conditions, though medically benign, often have significant psychosocial consequences and remain a therapeutic challenge due to complex etiologies involving autoimmune mechanisms, oxidative stress, and melanocyte dysfunction. Conventional treatment options, including corticosteroids, phototherapy, and immunosuppressants, frequently show limited efficacy and undesirable side effects, prompting increased interest in alternative, plant-based therapies. Traditional medicine systems such as Ayurveda and Unani have long utilized phytotherapeutic agents for their treatment. Among these, *Psoralea corylifolia* (Bakuchi) and *Withania somnifera* (Ashwagandha) have emerged as promising candidates due to their immunomodulatory, melanogenic, and adaptogenic properties. This review aims to compare the phytochemical profiles and therapeutic potential of these two herbs in the treatment of hypopigmentary conditions. A comprehensive literature search highlights key phytoconstituents such as psoralen in *P. corylifolia* and withanolides in *W. somnifera*, which exert melanocyte-stimulating and immune-regulating effects, respectively. The synergistic potential of these herbs, along with clinical and preclinical data, underscores their role in future integrative therapies for vitiligo and related disorders.

KEY WORDS:

INTRODUCTION

Hypopigmentary disorders, a category of dermatological conditions characterized by a reduction or complete loss of melanin pigment in the skin, significantly impact the quality of life of affected individuals. Among these disorders, vitiligo is the most prevalent, affecting approximately 0.5–2% of the global population worldwide [1,2]. It manifests as depigmented patches on the skin, primarily due to the progressive loss or dysfunction of melanocytes, the melanin-producing cells. While vitiligo itself is not physically harmful, its visible nature can lead to severe emotional distress, stigmatization, and reduced quality of life [3].

The etiology of vitiligo is multifactorial, involving complex interactions between genetic, immunological, oxidative, and neurogenic mechanisms [4]. The autoimmune hypothesis is strongly supported by clinical and histological findings, where melanocyte destruction is mediated by autoreactive cytotoxic T lymphocytes and inflammatory cytokines such as IFN-γ and TNF-α [5]. Concurrently, oxidative stress caused by elevated levels of reactive oxygen species (ROS) contributes to melanocyte damage and triggers immune responses [6]. These insights suggest that an effective therapeutic strategy must address not only melanocyte regeneration but also immune modulation and oxidative balance.

Currently, conventional therapies include topical corticosteroids, calcineurin inhibitors, phototherapy (PUVA, NB-UVB), and skin grafting techniques [7]. However, these approaches are often limited by relapse, variable efficacy, high costs, and adverse effects such as skin atrophy or carcinogenic risk with long-term UV exposure [8]. Consequently, attention has shifted toward herbal and phytotherapeutic interventions, especially from systems like Ayurveda and Traditional Chinese Medicine, which emphasize holistic treatment with fewer side effects.

In Ayurvedic dermatology, *Psoralea corylifolia* (Bakuchi) has long been utilized for treating leukoderma (Switra) due to its potent melanogenic properties. Its active constituents, notably psoralen and bakuchiol, have been shown to stimulate melanocyte activity when used in conjunction with sunlight or UVA radiation [9]. It also possesses antimicrobial and antioxidant properties, offering additional therapeutic benefits for skin health [10].

In contrast, *Withania somnifera* (Ashwagandha), although not primarily used for depigmentation in traditional texts, is valued for its adaptogenic, immunomodulatory, and antioxidant activities [11]. It contains bioactive withanolides and sitoindosides that regulate immune function and inhibit inflammatory cascades [12]. These properties make it a promising adjunct in managing autoimmune conditions, including vitiligo, by stabilizing immune responses and reducing oxidative stress [13].

Despite the therapeutic promise of both *Psoralea corylifolia* and *Withania somnifera*, a comprehensive, comparative review that highlights their individual and potential synergistic roles in the treatment of hypopigmentary disorders remains notably absent in current scientific literature. This review seeks to address that gap by critically examining the botanical characteristics and traditional medicinal applications of these plants, particularly in the context of skin pigmentation disorders such as vitiligo.

It further aims to analyze and compare their phytochemical compositions, identifying key bioactive constituents responsible for their therapeutic effects. Special emphasis is placed on exploring their roles in melanogenesis, immune regulation, and antioxidant defense, which are central to the pathophysiology of vitiligo and related conditions. In addition, this review synthesizes existing clinical and experimental evidence, evaluating the efficacy and limitations of each plant both as monotherapies and in potential combination regimens. Finally, it explores the safety profiles, toxicity considerations, and future therapeutic prospects of these botanicals within the expanding field of integrative dermatology. Through this comparative analysis, we aim to provide an evidence-based perspective on the viability of *P. corylifolia* and *W. somnifera* as complementary or alternative therapies in the management of hypopigmentary disorders.

Botanical Description And Ethnomedical Use

***Psoralea corylifolia* (Bakuchi):** *Psoralea corylifolia* Linn., commonly known as Bakuchi or Babchi, belongs to the family Fabaceae (Leguminosae). It is an annual or biennial herbaceous plant native to the Indian subcontinent and parts of China [14]. The plant reaches a height of approximately 60–120 cm and is characterized by:

- Leaves: Simple, alternate, broadly ovate with glandular dots.
- Flowers: Small, purple to bluish, papilionaceous flowers arranged in axillary racemes.
- Fruits: Small, flat, brownish pods containing a single seed.
- Seeds: Blackish-brown, kidney-shaped, aromatic, and oily; these are the primary medicinal part used [15].

It thrives in subtropical and tropical climates, often found growing wild in dry, sandy, or gravelly soil [16]. In Ayurveda, *P. corylifolia* is described under Kusthaghna (anti-leucoderma) and Kandughna (anti-itching) dravyas and is primarily used to treat vitiligo (Shwitra), leprosy, and other skin conditions. It is administered internally (powders, decoctions) and externally (oils, pastes) [17]. In Unani medicine, it is recognized for its skin-lightening and depigmenting action. In Traditional Chinese Medicine, known as Bu Gu Zhi, it is used to tonify kidney yang and enhance skin tone. Its photosensitizing compounds such as psoralen are used in conjunction with UV light in PUVA therapy [18].

***Withania somnifera* (Ashwagandha):** *Withania somnifera* (Linn.) Dunal, commonly referred to as Ashwagandha or Indian ginseng, belongs to the Solanaceae family. It is a perennial, erect shrub widely distributed across drier regions of India, the Middle East, and North Africa [19]. It typically grows to a height of 35–75 cm and displays:

- Leaves: Ovate, green, and covered with fine hairs.
- Flowers: Small, greenish-yellow, bell-shaped.
- Fruits: Tiny, orange-red berries enclosed in a papery calyx.
- Roots: Thick, fleshy, and aromatic — the most used part medicinally [20].

Ashwagandha is a Rasayana herb in Ayurveda, renowned for its rejuvenating, adaptogenic, and restorative properties [21]. It is used to balance Vata and Kapha, enhance vitality, reduce stress, and support immune function. Although not traditionally used for direct melanogenic effects, its immunomodulatory, antioxidant, and neuroprotective properties provide support for melanocyte function and survival [22].

Phytochemical Profile: Phytochemicals are bioactive compounds produced by plants that contribute to their medicinal properties. [29]. Both *Psoralea corylifolia* and *Withania somnifera* possess rich and diverse phytochemical

compositions that underpin their therapeutic effects, particularly in the management of skin disorders such as hypopigmentation. [30]. The seeds of *P. corylifolia* are pharmacologically potent and are known to contain a range of bioactive compounds, primarily furanocoumarins like psoralen and isopsoralen, which are well-documented for their melanogenic and photosensitizing properties.

Table 1: Traditional Uses of Psoralea corylifolia and *Withania somnifera* in Hypopigmentary Disorders

Plant Name	System of Medicine	Traditional Uses in Hypopigmentation	Key Formulations or Practices	References
Psoralea corylifolia	Ayurveda	Vitiligo (Switra), leukoderma, eczema	Bakuchi oil with sun exposure; mixed with cow's urine or turmeric	[23,24]
	Traditional Chinese Medicine (TCM)	Bu Gu Zhi for tonifying Yang, dermatological issues	Combined with warming herbs for skin diseases	[25]
	Unani	Skin discoloration, leprosy, infections	Decoctions, oils, and pastes	[26]
Withania somnifera	Ayurveda	Rasayana for immunity, stress relief; adjunct in vitiligo	Ashwagandha churna or extract; combined with Guggulu or Shatavari	[27]
	Siddha/Unani	Rejuvenator for systemic and neurological disorders	Part of compound herbal formulations	[28]

These compounds intercalate with DNA and, upon ultraviolet light activation, stimulate melanocyte proliferation and melanin production—an effect exploited in PUVA therapy. In addition to furanocoumarins, *P. corylifolia* also contains flavonoids such as bavachin, bakuchiol, and corylifolin, along with terpenoids, meroterpenes, and various essential oils, all of which contribute to its antioxidant, antimicrobial, and anti-inflammatory activities. [31].

Notably, bakuchiol—a meroterpene phenol structurally similar to resveratrol—exhibits retinoid-like activity and supports skin health by promoting collagen production and mitigating oxidative stress. [32].

Conversely, *Withania somnifera*, primarily valued for its roots, contains a unique class of steroidal lactones known as withanolides, including withaferin A, withanolide D, and withanone, which are responsible for its adaptogenic, immunomodulatory, and anti-inflammatory actions. [33]. These bioactive regulate multiple cellular pathways such as NF-κB and p38 MAPK, which are involved in inflammation and cellular immunity—critical processes implicated in the pathogenesis of autoimmune skin disorders like vitiligo.

Furthermore, *W. somnifera* is rich in alkaloids (e.g., somniferine, anaferine), sitoindosides, and other polyphenols, which provide neuroprotective and antioxidant effects, potentially shielding melanocytes from oxidative damage. Though not a direct melanogenic agent like *P. corylifolia,* the phytochemical profile of *W. somnifera* supports melanocyte survival, reduces autoimmune aggression, and helps restore pigmentation by improving the skin microenvironment. [34].

Together, the synergistic phytochemical landscapes of both plants—one promoting melanin production and the other preserving melanocyte integrity—offer a promising, multifaceted approach to managing hypopigmentary disorders.

Preclinical And Clinical Evidence: The pharmacological activities of *Psoralea corylifolia* and *Withania somnifera* in the treatment of hypopigmentary disorders have been substantiated by a growing body of preclinical and limited clinical evidence. [47]. In experimental studies, *P. corylifolia* has demonstrated significant melanogenic potential. In vitro assays on melanocyte cultures have shown that psoralen and isopsoralen stimulate tyrosinase activity, melanin synthesis, and increase the expression of MITF, confirming its direct role in melanogenesis.

Furthermore, studies on animal models, such as guinea pigs and mice, have revealed enhanced pigmentation following topical application or oral administration of Bakuchi extracts, particularly when combined with UVA exposure. These effects are attributed to the compound's ability to induce melanocyte proliferation and stimulate pigmentation pathways. The photosensitizing effect of psoralen, while therapeutically beneficial in controlled doses, necessitates careful administration to avoid adverse reactions such as phototoxicity or hyperpigmentation.

Table 2: Comparative Table of Major Phytochemical Classes in *Psoralea corylifolia* and *Withania somnifera*

Phytochemical Class	*Psoralea corylifolia*	*Withania somnifera*	Biological Significance	References
Furanocoumarins	Psoralen, Isopsoralen	–	Photosensitization, tyrosinase activation	[35,36]
Flavonoids	Bavachin, Isobavachalcone, Corylin	–	Antioxidant, melanogenesis stimulation	[37,38]
Terpenoids	Bakuchiol	Withanolides (Withaferin A, Withanolide D)	Antioxidant, anti-inflammatory, immune regulation	[39,40,41]
Alkaloids	–	Somniferine, Anaferine	Neuroprotection, stress modulation	[42]
Steroids & Sterols	–	Beta-sitosterol, Stigmasterol	Skin regeneration, inflammation control	[43]
Phenolic compounds	Psoralidin, Bakuchiol	Sitoindosides	Free radical scavenging, melanocyte protection	[39,44]
Essential oils & terpenes	Limonene, Caryophyllene	Minor constituents in leaves	Antimicrobial, penetration enhancement	[45,46]

Clinical evidence, though limited, has further supported its efficacy. Several open-label trials and case series conducted in Ayurvedic clinical settings have reported marked repigmentation in patients with vitiligo using topical formulations of Bakuchi oil or paste, often in combination with solar exposure or phototherapy. One study observed partial to complete repigmentation in over 60% of participants after 3–6 months of treatment, with relatively few side effects when monitored properly. [48].

In contrast, *Withania somnifera* has not been traditionally used for direct pigment stimulation but has demonstrated immunomodulatory and protective effects in preclinical models relevant to vitiligo. Animal studies have shown that withanolides can reduce oxidative stress-induced apoptosis in melanocytes, modulate cytokine release, and suppress the autoimmune responses typically implicated in melanocyte destruction. Additionally, Ashwagandha extract has been shown to improve skin health and resilience through antioxidant and anti-inflammatory mechanisms, which may indirectly benefit patients with hypopigmentary conditions by reducing the underlying pathological triggers. [49].

Although large-scale, placebo-controlled clinical trials are lacking, *W. somnifera* is commonly included in polyherbal Ayurvedic formulations for vitiligo and other autoimmune skin disorders. Some clinical observations suggest that oral supplementation of Ashwagandha improves stress-induced skin flare-ups and supports overall immune balance, which is particularly beneficial in vitiligo cases linked to psychological stress.

In summary, *Psoralea corylifolia* has demonstrated more direct and potent melanogenic effects, supported by both preclinical and clinical data, while *Withania somnifera* offers systemic support through its ability to modulate the immune system and protect melanocytes. The combination of these plants may thus offer a comprehensive and complementary approach to managing hypopigmentary disorders such as vitiligo. [50].

Safety Profile And Toxicology: While *Psoralea corylifolia* and *Withania somnifera* exhibit promising therapeutic potential in hypopigmentary disorders, their safety profiles require careful consideration, particularly when used in long-term or high-dose regimens. *Psoralea corylifolia*, despite its efficacy in promoting melanogenesis, is associated with a narrow therapeutic window. The primary concern lies in its photosensitizing effect, attributed mainly to psoralen and isopsoralen. Excessive or unmonitored exposure to ultraviolet (UV) radiation following psoralen application can lead to phototoxicity, resulting in blistering, hyperpigmentation, and in rare cases, skin carcinogenesis with chronic misuse.

Reports of contact dermatitis and allergic skin reactions have also been documented with topical use, especially when crude or non-standardized formulations are applied. Additionally, oral administration of high doses of Bakuchi extract has been linked to hepatotoxicity, as evidenced by elevated liver enzymes in animal studies and a few human case reports. These risks underscore the importance of using standardized extracts and conducting liver function monitoring during treatment. [51].

In contrast, *Withania somnifera* demonstrates a much safer toxicological profile, supported by extensive

use in traditional medicine and modern clinical trials. It is generally well tolerated in both short- and long-term use, with a low incidence of adverse effects. Mild gastrointestinal discomfort, drowsiness, or allergic reactions may occur in sensitive individuals. Acute and sub-chronic toxicity studies in animals have shown no significant organ toxicity even at high doses. Moreover, its adaptogenic and hepatoprotective actions may offer protective benefits when used in combination therapies. However, caution is advised in patients taking immunosuppressive drugs, sedatives, or thyroid medications, as Ashwagandha may potentiate or interfere with their effects. [52].

Overall, while *Withania somnifera* is considered a safe and supportive herb with minimal adverse effects, the use of *Psoralea corylifolia* requires a more cautious and regulated approach due to its potential for phototoxicity and hepatotoxicity. Appropriate dosing, patient education, and clinical supervision are essential to ensure the safe and effective use of these botanicals in treating hypopigmentary disorders.

Integrative And Therapeutic Potential: The therapeutic integration of *Psoralea corylifolia* and *Withania somnifera* offers a promising, multifaceted strategy for the management of hypopigmentary disorders, particularly vitiligo. Their complementary therapeutic properties form the basis for a synergistic approach that addresses both the underlying causes and the effects of melanocyte dysfunction. While *P. corylifolia* acts locally to induce pigmentation through activation of melanocyte proliferation and melanin biosynthesis, *W. somnifera* provides systemic support by suppressing autoimmune responses and protecting melanocytes from oxidative stress, which are primary pathological drivers in vitiligo. The use of these botanicals in combination—either in traditional formulations or as part of integrative therapeutic regimens—aligns with the principles of holistic medicine, targeting the disorder at multiple levels: cellular, immunological, and psychological. [53, 54].

From a formulation perspective, combining topical Bakuchi oil or psoralen-rich extracts with oral administration of Ashwagandha supplements could offer both localized and systemic benefits. This dual strategy may enhance treatment outcomes by promoting repigmentation while also stabilizing disease progression. Furthermore, the incorporation of these botanicals into polyherbal formulations or modern delivery systems—such as nanoemulsions, transdermal patches, and liposomal gels—may improve bioavailability, control dosing, and reduce adverse effects. [55].

Importantly, this integrative approach could also enhance patient adherence by minimizing side effects commonly associated with conventional therapies. In the broader context of dermatology, such plant-based integrative strategies may hold value not only for pigmentary disorders but also for other chronic inflammatory skin diseases. However, for this potential to be fully realized, further clinical validation, standardization of active constituents, and regulatory frameworks are essential. Ultimately, the integrative use of *P. corylifolia* and *W. somnifera* represents a rational, evidence-supported advancement in phytotherapeutic dermatology, blending traditional wisdom with modern biomedical insight. [56,57].

Future Perspectives: Despite the promising therapeutic potential of *Psoralea corylifolia* and *Withania somnifera* in the treatment of hypopigmentary disorders, several gaps and challenges limit their integration into mainstream dermatological practice. One of the foremost limitations is the lack of large-scale, randomized, controlled clinical trials to validate their efficacy and safety in diverse patient populations. Most available studies are preclinical, anecdotal, or based on traditional knowledge, which, while valuable, lack the rigorous methodological design required by modern evidence-based medicine. Additionally, the variability in phytochemical content due to differences in cultivation, harvesting, processing, and extraction methods poses a major challenge to standardization and dosage optimization.

The potential toxicity of *P. corylifolia*, especially in the context of phototoxic reactions and hepatotoxicity, further underscores the need for cautious formulation and clinical monitoring. In contrast, while *W. somnifera* exhibits a more favorable safety profile, its indirect role in melanogenesis means it is best employed as part of a broader therapeutic strategy rather than a standalone agent. Moreover, the mechanistic pathways of action, particularly synergistic or antagonistic interactions between phytochemicals in combination therapies, remain poorly understood and warrant deeper molecular investigation.

From a regulatory perspective, there is also a lack of clear guidelines for the formulation, labeling, and therapeutic claims of herbal products intended for dermatological use. This limits their global acceptance and commercialization. Nonetheless, the future remains promising. Advances in phytopharmaceutical technology, including nanocarriers, controlled-release systems, and biocompatible topical agents, are opening new avenues for the safe and effective delivery of plant-derived actives.

The development of standardized extracts with well-characterized phytoconstituents and the incorporation of biomarkers for therapeutic response can help bridge the gap between traditional knowledge and modern clinical applications. Interdisciplinary research that combines dermatology, phytochemistry, molecular biology, and pharmacognosy is essential to fully realize the therapeutic scope of *P. corylifolia* and *W. somnifera*. In the future, these botanicals may be integrated into personalized or adjunctive treatments for vitiligo and other pigmentary disorders, potentially transforming current therapeutic paradigms with a more holistic and patient-centered approach.

CONCLUSION

The management of hypopigmentary disorders, particularly vitiligo, remains a therapeutic challenge due to their multifactorial etiology, chronic course, and psychosocial burden. In this context, the use of traditional medicinal plants such as *Psoralea corylifolia* and *Withania somnifera* offers a compelling, holistic approach grounded in centuries of empirical use and increasingly supported by modern pharmacological evidence. *P. corylifolia* stands out for its direct melanogenic activity, primarily through its furanocoumarin content that stimulates melanocyte proliferation and melanin synthesis under controlled phototherapy. Meanwhile, *W. somnifera* contributes a complementary role by modulating the immune system, reducing oxidative stress, and promoting melanocyte survival—mechanisms that are crucial in autoimmune and stress-induced vitiligo.

Their phytochemical richness, encompassing compounds such as psoralen, bakuchiol, and withanolides, underpins their therapeutic efficacy and justifies their inclusion in integrative dermatological care. However, challenges such as standardization, toxicity management, and the absence of robust clinical data must be addressed through rigorous scientific inquiry and technological advancement.

The emergence of novel delivery systems and the application of systems biology approaches may further enhance their safety and efficacy, paving the way for their inclusion in evidence-based treatment regimens. In conclusion, the combined therapeutic potential of *Psoralea corylifolia* and *Withania somnifera* offers a promising natural alternative or adjunct to conventional therapies for hypopigmentary disorders. Continued interdisciplinary research and well-designed clinical studies are essential to validate their clinical applications and unlock their full potential in modern dermatological practice.

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A Comparative Review on Conventional and Nepheloturbidometric Methods for Estimation of Inorganic Contaminants in Bulk Drugs

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ABSTRACT:

Accurate estimation of inorganic contaminants present in bulk drugs is vitally important in order to guarantee quality, safety and regulatory compliance of pharmaceutical products. Traditional analytical techniques, including gravimetry, titrimetry and spectrophotometry have long been utilized for this purpose; however they often fall short in sensitivity, selectivity and time efficiency. Nepheloturbidometric methods, on the other hand, have increasingly come under scrutiny due to their greater precision, rapid analysis, and ability to detect trace levels of impurities via light scattering principles. This review compares conventional analytical methods with nepheloturbidometry as means for detecting common inorganic contaminants found in bulk drug substances, including sulfates, chlorides, phosphates and heavy metals. Key parameters, including detection limits, operational simplicity, sample preparation timeframes and requirements, reagent consumption estimates and instrumentation needs, are examined herein. Both approaches' advantages and limitations will also be reviewed within the context of pharmaceutical quality control standards as a guide for analysts when selecting their most effective method based on needs analysis needs, available resources and regulatory obligations.

KEY WORDS: Pharmaceutical Quality, Gravimetry, Titrimetry, Spectrophotometry, Nepheloturbidometric Methods, Conventional Analytical Methods

INTRODUCTION

Pharmaceutical quality control must address inorganic contaminants present in pharmaceutical substances, particularly bulk drugs. Impurities such as chlorides, sulphates and iron may originate in raw materials, reagents, solvents or manufacturing equipment and, if left unmanaged, can have adverse consequences for product safety, efficacy and stability [1]. Inorganic contaminants, although present only in trace amounts, can produce unwanted reactions, changes to drug activity or toxic side-effects that must be properly estimated when conducting pharmaceutical analysis [2]. Compliance with pharmaceutical standards such as those detailed by IP, USP or International Council for Harmonisation guidelines must also be ensured for industry.

These standards establish strict limits for inorganic impurities present in active pharmaceutical ingredients (APIs) and excipients [3,4]. Controlling impurities is integral for regulatory approval as well as protecting both public health

and product credibility. Traditional analytical techniques used for quantifying inorganic contaminants were gravity and volumetric analysis methods; although widely utilized, these can have limitations such as low sensitivity, operator dependency and time consumption [5] However, more recently nepheloturbidometry emerged as a rapid and reproducible solution to estimate trace contaminants when dealing with turbulent or colloidal systems [6].

Inorganic Contaminants in Bulk Drugs

Common Inorganic Impurities: Inorganic impurities, or non-carbon contaminants commonly encountered in pharmaceutical materials, may enter unknowingly during production, storage, and handling processes. Common examples are chlorides, sulfates, iron and heavy metals like palladium and platinum that remain after catalyst use [7]. While typically present only at trace amounts, inorganic impurities can significantly impair stability, efficacy and safety of drug products.

- Chlorides can enter drugs through hydrochloric acid or chloride-containing reagents. When present in excessive quantities, chlorides can corrode drug containers and interact negatively with drug molecules - this may compromise treatment processes as well as alter outcomes for users.
- Sulfates form when sulfuric acid or its salts are synthesized. They have the power to disrupt drug solubility and crystal growth [8].

Iron contamination of equipment or water sources often results in corrosion of sensitive drug molecules for degradation by oxidation reactions that lead to their breakdown and eventually their degradation [9, 10, 12].

Regulatory Standards: International regulatory bodies like the International Council for Harmonisation (ICH), World Health Organization (WHO), as well as national pharmacopoeias such as Indian, United States Pharmacopeia (USP), and British pharmacopoeias provide guidelines that specify allowable levels and limits of inorganic impurities found in medicines.

The ICH Q3D guideline offers an inclusive framework for evaluating elemental impurities, with particular attention given to heavy metals, residual catalysts and potentially toxic elements such as heavy metals. It categorizes 24 elements according to their toxicity/likelihood of occurring and then specifies permissible daily exposure limits via oral, parenteral or inhalation routes [10].

Indian Pharmacopoeia 2022 edition provides specific limits for chlorides, sulphates, iron and heavy metals present in bulk drugs and excipients. Chlorine levels should not exceed 0.5% while 0.1% maximum of sulphates depending on substance under test may be permitted [11].

WHO and other regulatory authorities endorse stringent impurity profiling requirements for active pharmaceutical ingredients (APIs) and excipients as part of Good Manufacturing Practices (GMP), to help ensure drug substances are suitable for long-term human use [12].

Sources of Contamination in Drug Synthesis and Storage: At various stages in drug manufacturing and postproduction handling, inorganic impurities may enter the process from sources. Examples may include:

Raw Materials and Reagents: Starting materials such as acids or bases may contain trace metal ions or inorganic salts that act as contaminants; impurities could also enter via solvents like water that have not been sufficiently purified [13].

Manufacturing Equipment: Stainless steel reactors, pipelines and mixing tanks may be used to leach iron, chromium and nickel into drug substances under acidic conditions with extended contact times [14].

Catalysts and Process Aids: Certain chemical synthesis processes utilise copper, palladium or platinum catalysts as formulation aids; should these remain present in their final product without proper removal [15], these could remain harmful components in it [16].

Storage and Packaging: Involvement with environmental elements like humidity, sunlight exposure or reactive containers may expose packaging materials to factors which cause leachable contaminants into them [16].

Conventional Analytical Methods: Estimating inorganic contaminants in pharmaceutical products has long relied upon traditional analytical techniques, including gravimetric, volumetric (titrimetric), colorimetric/spectrophotometric analyses. Although outdated in nature, such techniques remain essential tools in many quality control laboratories due to their cost efficiency, ease of implementation and historical reliability.

Gravimetric Analysis: Gravimetric analysis involves creating an insoluble precipitate of an analyte that is then filtered, dried and weighed to assess its concentration. For instance, chloride ions can form silver chloride precipitates while barium sulfate precipitations produces barium sulfate $BaSO_4$ with mass directly correlating with its analytic presence [17].

Gravimetric methods offer excellent accuracy and precision; however, to operate smoothly they require strict control of experimental conditions including pH, temperature and completeness of precipitation [18]. Interferring ions may decrease selectivity for this technique as well.

Volumetric (Titrimetric) Analysis: Volumetric or titrimetric analysis involves mixing an analyte with an appropriate standard titrant until an endpoint has been met, typically after some period of reacting time has elapsed. Examples may include:

- Mohr or Volhard's method for estimating chlorides by silver nitrate titration provides another reliable and accurate approach for measuring the levels of chlorides in solution. Complexometric titration with EDTA allows analysis of metal ions such as iron. Precipitation and redox titration techniques used in estimating sulphate and iron concentrations [5,11].
- Precipitation and redox titration techniques used in estimating sulphate and iron concentrations [5,11].

Volumetric analysis can be quick and relatively straightforward for routine laboratory applications; however, operator error may increase with color change endpoint detection or may lack the sensitivity necessary for precise trace-level quantification [19].

Colorimetric and Spectrophotometric Techniques: These techniques involve creating colored complexes between an analyte and specific reagents which can then be measured either visually (colorimetry) or with instruments (spectrophotometry). Examples:

- Iron can be estimated using o-phenanthroline or thiocyanate compounds that produce colored complexes measurable at specific wavelengths [6].
- Sulphates can be measured turbidimetrically through interaction with barium chloride to form barium sulfate suspensions that allow measurement.

Gravimetric and volumetric methods offer greater sensitivity and automation capabilities compared to alternative techniques, yet require high purity reagents with results vulnerable to light scattering, turbidity or matrix effects [20].

Strengths and Limitations of Conventional Methods: Overall, conventional analytical techniques offer cost-effective and suitable ways of conducting qualitative to semiquantitative analyses in settings where advanced instrumentation is unavailable; however, these conventional practices have increasingly been supplemented or replaced with instrumental and automated technologies which offer greater precision, reproducibility, sensitivity (especially at trace impurity levels), reproducibility etc [21,22].

Table 1. Outlines The Strengths And Limitations Associated With Various Conventional Analytical Methods For Inorganic Contamination Estimation.

Sl No	Method	Strengths	Limitations
1	Gravimetric	High accuracy, no need for calibration	Time-consuming, sensitive to interfering ions
2	Volumetric	Fast, simple, low-cost	Low sensitivity, subjective endpoints
3	Colorimetric	Moderate sensitivity, simple instrumentation	Affected by turbidity, interference from colors
3	Spectrophotometric	High sensitivity, quantitative	Requires calibration, affected by sample matrix

Conventional methods remain popular within academia, small industries and regulatory labs for both confirmatory testing as well as quality control purposes [23].

Nepheloturbidometric Method: Nepheloturbidometry is an instrumental analytical method widely utilized for quantitatively measuring suspended particles or precipitates present in solutions, providing a precise yet reproducible and cost-effective means of detecting inorganic contaminants such as chlorides, sulfates or iron compounds in pharmaceutical materials such as suspension suspension suspension suspension suspension or solutions.

Principle and Instrumentation: Nepheloturbidometry uses light scattered by suspended particles suspended in liquid to measure concentration levels of analytes such as BaSO4; when added as an analyte to samples containing these analytes using BaCl2, an insoluble precipitating agent such as BaCl2 precipitates fine particles which scatter light at specific angles - usually 90deg--resulting in proportionate light intensity related to concentration level [18, 19].

Nepheloturbidometry combines features from both types of measurements for improved detection across a wider concentration range [6].

Instrumentation typically consists of:

- A light source (usually a tungsten or LED lamp)
- A sample cell
- A photodetector placed at a right angle to the light source
- A readout system, often interfaced with software for calibration and quantification .

This method depends heavily on variables like particle size, light wavelength and sample clarity to achieve accurate analytes [5, 19].

Application in Detection of Inorganic Contaminants

- Nepheloturbidometry is used for quantifying various inorganic contaminants found in pharmaceutical products, such as:

- Chlorides may be measured by precipitating silver chloride with AgNO3 [11].
- Sulfates, identified through barium sulfate precipitation [24], may produce turbidity which reveals their identity [17].
- Finally, iron can often be identified indirectly via complex formation and precipitation reactions that involve ferric or ferrous ions [17].

This method of bulk drug analysis is especially suited for clarity-controlled solutions and particle behavior monitoring, making this an efficient approach to quality assurance of raw materials and finished products [20,22]. As it has become widely recommended in various pharmacopeial monographs for raw material testing as well as finished product quality checks [20,22], its value stands the test of time.

Advantages over Conventional Methods:
Nepheloturbidometric techniques offer multiple advantages when compared to conventional analytical methods:

Nepheloturbidometry's features make it an invaluable asset in environments involving large volumes or semiautomated workflow, including pharmaceutical industries and research labs [23].

Table 2. Outlines The Advantages Of Employing Nepheloturbidometric Method For Inorganic Contaminant Analysis.

Sl No	Advantage	Explanation
1	Higher Sensitivity	Can detect low levels of analytes (ppm or ppb range)
2	Faster Analysis	Shorter reaction and measurement time than gravimetric methods
3	Less Sample Preparation	Minimal heating or complex chemical reactions required
4	Automation-Friendly	Compatible with digital readouts, autosamplers, and software integration
5	Low Reagent Consumption	Requires small volumes, making it more economical

Limitations and Challenges

Notwithstanding its advantages, nepheloturbidometry presents several limitations:

- Interference from Colored or Opaque Samples: Solutions with strong hues may absorb light, interfering with measurements [25].
- Sensitivity to Particle Size and Aggregation: Variations in precipitate morphology can alter scattering behavior, producing nonlinear calibration curves.
- Requirement for Calibration and Standardization: This technique calls for precise preparation of standards; results may depend upon factors like light source, wavelength and cuvette cleanliness.
- Limited Use with Clear or Soluble Ions: Fully-dissolved species that do not form precipitates cannot be measured directly [26].

Temperature, pH and reagent purity all can have an effect on particle formation and light scattering; hence it is imperative that careful consideration be given when setting experimental conditions.

Comparative Evaluation of Methods: Assessing analytical techniques for quantifying inorganic contaminants requires taking a close look at various performance parameters. In this section we compare conventional (gravimetric, volumetric, spectrophotometric) to newer nepheloturbidometric methods in terms of sensitivities, precisions, costs, times-efficiencys and sample suitabilitys.

Sensitivity, Accuracy, and Precision: Sensitivity refers to a method's ability to detect low concentrations of an analyte. Nepheloturbidometry typically offers greater sensitivity, with its detection capabilities reaching down into ppm or sub-ppm range - exceeding even gravimetric or basic titrimetric methods' reach [5,18].

Gravimetric methods tend to be highly accurate due to mass measurement for analysis; volumetric methods may introduce subjectivity when endpoint detection takes place and thus reduce accuracy [7].

Precision in terms of results can generally be improved using instrument-based approaches like nepheloturbidometry and spectrophotometry, especially when combined with automation [19].

Time, Cost, and Ease of Operation: Analysis using gravity requires a significant amount of labor and is time-consuming and requires the filtration, drying and weighting that can require hours to complete [6]. Volumetric techniques can be a lot faster but are often constrained due to the manual process of titration and the possibility of errors by the operator [20].

Nepheloturbidometry provides rapid analysis (typically under 15 minutes per sample) and is therefore particularly suited to batch processing or automation, making it cost-effective even

in large scale operations . Furthermore, less reagent is used and no drying or weighing steps are necessary - further cutting costs in larger operations [11].

Nepheloturbidometry may require greater initial equipment costs compared to more traditional glassware-based techniques; however, its long-term operational effectiveness often outweighs this [23].

Table 3. Comparison of Analytical Techniques Based on Sensitivity, Accuracy, and Precision

Sl.No	Parameter	Gravimetric	Volumetric	Spectrophotometric	Nepheloturbidometric
1	Sensitivity	Low Moderate	Moderate	High	Very High
2	Accuracy	Very High	Moderate	High	High
3	Precision	High	Moderate	High	High

Table 4. Comparison of Time, Cost, and Ease of Operation

Sl No	Parameter	Gravimetric	Volumetric	Nepheloturbidometric
1	Time requirement	High	Moderate	Low
2	Reagent cost	Moderate	Low	Low
3	Equipment cost	Low	Low	Moderate
4	Ease of operation	Low	Moderate	Low

Table 5. Evaluation of Analytical Techniques Based on Sample Characteristics

Sl. No	Sample Type	Gravimetric	Volumetric	Nepheloturbidometric
1	Turbid / Colloidal solutions	Moderate	Poor	Excellent
2	Colored solutions	Poor	Poor	Moderate (if corrected)
3	Clear aqueous solutions	Good	Good	Moderate
4	Low-concentration analytes	Poor	Moderate	Excellent

Suitability for Different Sample Types: Conventional methods require clear, uncolored solutions with low viscousity for optimal performance. Volumetric analysis could fail with opaque or highly colored samples due to endpoint detection difficulties [25].

Gravimetric methods may not be ideal for low concentration analytes due to large sample sizes needed; on the contrary, nepheloturbidometry excels in analyzing dilute, turbid or colloidal solutions such as sulphate or chloride suspensions [24].

Nepheloturbidometry can also be advantageous when applied to samples where precipitate formation is key to analyte detection and visual changes such as cloudiness or turbidity are key analytical indicators [15].

However, very clear solutions or those without stable precipitates may not be suitable for direct nepheloturbidometric analysis [22].

Application in Academic and Industrial Settings: Analytical methods used to detect inorganic contaminants like chlorides, sulphates and iron play an integral part in both academic research and industrial quality control (QC). Method selection depends upon regulatory compliance requirements, available resources and sample nature - this section discusses both conventional and nepheloturbidometric applications for both environments.

Analysis of Real Samples (Store and Industry Sources): Real-time analysis of samples collected from college drug stores and industrial partners at academic institutions like MGCOP College provides students and researchers with hands-on training in drug quality assessment. Such samples often consist of active pharmaceutical ingredients (APIs) and bulk drugs that could contain trace inorganic impurities due to synthetic procedures, solvents or storage conditions [27].

Example: Sulfate and chloride impurities found in industrial API samples are often evaluated using gravimetric or

nephelometric techniques according to pharmaceutical guidance [11].

Iron contamination found in storage containers or processing equipment can often be detected using colorimetric or spectrophotometric techniques [5].

These analyses serve to confirm real world sample compliance with Indian Pharmacopoeia (IP) and International Council for Harmonisation (ICH Q3A/B) guidelines on elemental impurities [28].

Relevance of Each Method in Quality Control Labs: Industrial Quality Control Labs choose methods based on factors including speed, reproducibility, and compliance. Nepheloturbidometric analysis has become more and more preferred as an everyday method due to:

- Shorter analysis time
- Low reagent consumption and
- Compatibility with batch processing [18].

Conventional methods continue to be utilized widely when: mes Instrumentation is unavailable, Validation methods rely upon legacy methodologies or Highly accurate weight-based data is needed (for instance in gravimetric sulphate analysis) [6].

Quality Control labs often maintain method validation documentation that details linearity, precision and robustness when adopting novel instrument techniques such as nepheloturbidometry [23].

Integration into Standard Operating Procedures (SOPs): For any method to be successfully adopted consistently, it must be written into Standard Operating Procedures (SOPs). This involves:

- Calibration of instruments such as nephelometers) Preparation of standard solutions, and
- Defined steps for addition, incubation and measurement [20].

Academic labs utilize Standard Operating Procedures (SOPs) as part of training students and producing repeatable results, while in industries they are enforced under Good Manufacturing Practices (GMP), guaranteeing consistency across multiple production batches [29].

Nepheloturbidometric methods have become an integral component of medical monographs, encouraging their implementation into regulatory compliant standard operating procedures (SOPs) across academic and industrial settings.

Future Prospects and Recommendations: Pharmaceutical analysis has evolved in response to increasing demands for greater sensitivity, precision, regulatory compliance and cost-effectiveness. While traditional and nepheloturbidometric methods have long been relied upon for bulk drug contamination detection purposes, modern developments favor automation hybrid instrumentation standardization techniques as future directions of pharmaceutical quality control techniques. This section features such methodologies.

Emerging Techniques (e.g., Hybrid Methods, Automation): Integrating hybrid methods, which combine traditional and contemporary instrumental techniques to their maximum advantage, has become an emerging trend. One such example is coupling turbidimetric detection with spectrophotometric data analysis for increased both sensitivity and selectivity [30], while chemometric-assisted nephelometry has proven helpful for multicomponent analysis in complex matrices [31].

Automation is also becoming an essential aspect of analysis. Automated nephelometers with software-based calibration, data logging, and quality control checks have now made their debut in both academic and industrial lab environments [32], providing high throughput analysis without human error compromising reproducibility or increasing throughput rates .

Microfluidic platforms and lab-on-a-chip devices are being investigated as tools for on-site turbidimetric analysis in remote or field settings [33].

Need for Method Validation and Standardization: Method validation remains vital to ensure accuracy, precision, linearity and robustness in new techniques; validation should comply with ICH Q2(R1) guidelines which define parameters such as limit of detection (LOD), limit of quantitation (LOQ), specificity and system suitability [34].

Many laboratories, particularly academic institutions, still lack standardization of nepheloturbidometric protocols. Differences in instrument sensitivity, reagent quality and operator handling may result in inconsistent results; hence there is a growing demand to standardize such processes: Develop uniform Standard Operating Procedures, conduct inter-laboratory validations, and publish peer-reviewed performance comparisons between classical and modern methodologies [11, 20].

Potential Improvements in Current Practices:

1. Instrument Calibration: For consistent analysis, regular instrument calibration with pharmaceutical reference standards such as chloride, sulfate and iron is crucial [35].
2. Training and Capacity Building: Both academic and industrial labs should invest in technical training programs dedicated to modern analytical instrumentation as well as regulatory updates.
3. Eco-Friendly Practices: Subtlety reduction through eco-friendly analytical chemistry techniques such as miniaturized reagent systems and water-based reactions can increase sustainability during analysis [36].
4. Data Integration and Digitalization: Integrating instruments with Laboratory Information Management Systems (LIMS) will streamline processes and allow faster decision-making

within Quality Control environments.

These steps will not only advance current practices but will also promote global harmonization of analytical methods used for bulk drug contaminant analyses.

CONCLUSION

The accurate estimation of inorganic contaminants like chlorides, sulfates and iron in bulk drugs is of vital importance in assuring their safety, efficacy and regulatory compliance. This review compares traditional analytical techniques such as gravimetric, volumetric, and spectrophotometric approaches with the more popularly adopted nepheloturbidometric technique. Standard techniques offer ease of use and historical reliability; however, their results tend to take more time for analysis, require operator intervention for implementation, and offer less sensitivity at trace levels than their alternatives. Nepheloturbidometry provides rapid, sensitive and reproducible results making it ideal for quality control in academic and industrial laboratories alike.

Implementation requires instrumentation as well as standard protocols and validation - especially in resource constrained situations. Nepheloturbidometry should become even more integrated into standard quality control workflows as new advances emerge in automation, hybrid techniques and green analytical practices. As with any analytical method selection decision, selecting one ideally should depend on several considerations such as sample type, regulatory requirements, available infrastructure and required sensitivity. A balanced and validated approach incorporating both traditional and instrumental approaches may ensure high standards for safety and quality in drugs.

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Comparative Study of Selected Soil Samples From Industrial Areas of Kogi State

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ABSTRACT

The work reports the concentration of heavy metals and physiochemical parameters in some selected soil samples from industrial areas in Kogi State, Nigeria. The concentration of heavy metals that was worked upon on each of the samples were gotten using Atomic Absorption Spectrophotometer (AAS). The final concentration of Zinc (Zn) in Ajaokuta (Ajoa) sample was 53.44 mg/kg while that of Obajana (Obj) was 166.68 mg/kg. The concentration of Chromium (Cr) in Ajaokuta (Ajao) sample was 113.92 mg/kg while that of Obajana (Obj) was 31.04 mg/kg. The concentration of Nickel (Ni) in Ajaokuta (Ajao) sample was 5.42 mg/kg while that of Obajana (Obj) was 69.50 mg/kg. The concentration of Cadmium (Cd) in Ajaokuta (Ajao) sample was -3.14 mg/kg while that of Obajana (Obj) was -0.62 mg/kg. The concentration of Copper (Cu) in Ajaokuta (Ajao) sample was 59.88 mg/kg while that of Obajana (Obj) was 104.98 mg/kg. The concentration of lead (Pb) in Ajaokuta (Ajao) sample was 197.28 mg/kg while that of Obajana (Obj) was 98.64 mg/kg. The concentration of mercury (Hg) in Ajaokuta (Ajao) sample was -5.60 mg/kg while that of Obajana (Obj) was -5.40 mg/kg. The physicochemical results include; soil organic matter (%) for Ajaokuta (Ajao) which was 1.85 while for Obajana (Obj) was 1.47; pH for Ajaokuta (Ajao) was 2.670 while for Obajana (Obj) was 6.096; and Conductivity (µS/cm) for Ajaokuta (Ajao) was 184.5 while for Obajana (Obj) was 320.4. Comparing the concentration of each of the heavy metals with WHO (World Health Organization) and USEPA (United State Environmental Protection Agency), Zinc, Chromium, Copper and Lead were found to be above the permissible limit in sample Ajaokuta (Ajao) while Nickel, Cadmium and Mercury were below the permissible limit. In sample Obajana (Obj); Zinc, Nickel, Copper and Lead were found to be above the permissible limit, while Chromium, Cadmium, and Mercury were below the permissible limit set by WHO (World Health Organization) and USEPA (United State Environmental Protection Agency). The ranking of the occurrence of the metals from the least to greatest in sample Ajaokuta (Ajao) are Hg>Cd>Ni>Zn>Cu>Cr>Pb indicating that the concentration of lead in the location was high. The ranking of the occurrence of the metals from the least to the greatest in sample Obajana (Obj) is Hg>Cd>Cr>Ni>Pb>Cu>Zn indicating that the concentration of Zinc was high in the sample location. The two soil samples have Organic Matter value to be within the range of a typical agricultural soil, it is then good for agricultural activities, the pH indicates that the soil samples from the location was acidic and the electrical conductivity shows the amount of salts in the soil.

KEY WORDS: Soil, Heavy Metals, Ph, Organic Matter, Electrical Conductivity.

INTRODUCTION

Heavy metals are naturally present in the soil, geologic and anthropogenic activities have increased the concentration of these elements to the amounts that are harmful to both plants and animals. Some of these activities includes burning of fossil fuels, use of pesticides and fertilizers in agriculture, mining and smelting of metals, production of batteries and other products in industries, sewage sludge, and municipal waste disposal, therefore, beside the natural activities, almost all human activities also have potential contribution to produce heavy metals as side effects (20). Heavy metals have been defined by several researchers. The term heavy metal is a collective term that applies to group of metals and metalloids, with atomicity of density greater than 4g/cm3 or 5 times or more great than water (19).

The aim of this work is to evaluate the high level of heavy metals in the industrial areas of Kogi State as to ensure that the safety of the land is minimized of potential hazards. While the objectives are; to determine heavy metals contamination on soil samples in industrial areas. Determine the physicochemical analysis on the soil samples. Also compare the level of heavy metals on two different soil samples with the permissible limit by World Health Organization (WHO), United State Environmental Protection Agency (USEPA).

The Chemistry involves in heavy metal pollution of water, soil and air includes; mining activities and other geochemical processes often result in the generation of Acid Mine Drainage (AMD), a phenomenon that is commonly associated with mining activities. It is generated when pyrite (FeS2) and other sulphide minerals in the aquifer and present and former mining sites are exposed to air and water in the presence of oxidizing bacteria, such as Thiobacillus ferrooxidans, and oxidized to produce metal ions, sulphate and acidity(18).

2FeS2 + 7O2 + 2H2O 2FeSO4 + 2H2SO4
2FeSO4 + 2H2SO4 Fe2(SO4)3 + SO2 + 2H2O
Fe2(SO4)3 + 2FeAsS + 9/2O2 + 3H2O 2H3AsO4 + 4FeSO4 + S

METHODOLOGY

Sample Collection: A selected soil samples were collected from two different industrial areas within Kogi State taking note of the distance not far from the industries and the temperatures were taken at sample collection site. Each of the samples was properly labeled and transported in polythene bags to the laboratory where the soil samples were prepared for analyses.

List of Equipment Used: pH-meter, Conical flasks, 100ml Volumetric flasks, Beakers, measuring cylinder, Wash bottles, Hot plate, Spatula, glass stirring rod, Pipettes, Kjeldahl Digestion tube, Weighing-balance, Sieving pan, Test tubes, Oven, Water bath, Filter papers, digestion tubes, digestion tube rack, syringe, Google glass, Plastic funnels, Fume cupboard, Flame Atomic Absorption Spectrophotometer, manufactured by Thermo-Scientific Spectrometer model ICE-3000 AA02134104 v1.30.

Reagents: 0.01M CaCl2, pH 4.00 and pH 7.00 buffer solution, deionized water, concentrated hydrochloric acid, Nitric acid and the soil samples.

Procedure:
Determination of Soil pH

1. 10g of soil samples was put into each 50ml beakers. It was weighed to the nearest 1g only.
2. To the soil in one beaker was added 20ml of distilled water. This gives a soil: solution ratio of 1:2.
3. To the soil in second beaker was added 20ml of CaCl2. The use of this salt solution will demonstrate the effect of a neutral salt on the pH of the soil solution.

It was allowed to stand for 30mins, stirring occasionally with a glass stirring rod. This allowed time for aggregates to break down so that the solution equilibrates with all of the soil and for the sample to equilibrate with atmosphere CO2. The pH of the soil in each beaker was determined with the pH meter.

Digestion of the Soil Samples

1. The digestion tubes were sterilized by washing with soap rinsed with tap water, then distilled water, soaked in acid rinse and finally rinsed in distilled water
2. Put in an oven to dry at 60oc for some minutes.
3. A 5g of each sample was weighed using weighing balance and poured into a conical flask.
4. A 25ml of Aqua regia (a mixture of Hydrochloric acid (HCl) and Nitric acid (HNO3) in the ratio 3:1) was measured and poured into the flask containing the sample and was placed on a digester.
5. The digester was turned on at a temperature starting from 60oc to 180oc for one and a half hours.
6. Constant checking at 10 minutes interval in order to prevent it from drying until a clear solution was observed.
7. Then the mixture was cooled.
8. It was then filtered into a 100ml volumetric flask using filter paper and made up to mark with distilled water.
9. Elemental analysis was carried out using the Flame Atomic Absorption Spectrometer (F-AAS) manufactured by Thermo-Scientific Spectrometer model ICE-3000 AA02134104 v1.30, situated in Sheda Science and Technology Complex, Abuja and also in Albert Einstein Bioscience Centre National Cereals Research institute Badeggi, Niger State to determine heavy metals present with the aid of the standards.

Final concentrations of the metals in the soil samples were calculated using the following formula (Wodaje Addis and Alemayehu Abebaw, 2017):

$$\text{Concentration}\left(\frac{mg}{kg}\right) = \frac{\text{Conc. of sample} - \text{conc. of black x volume}}{\textit{Weight of sample}}$$

where V = Final volume (100 mL) of solution, and M = Initial weight (5 g) of sample measured.

Determination of Organic Matter: To determine soil organic matter (SOM), Loss on Ignition (LOI) method was used by the loss of weight of the soil sample heated at a high temperature. Temperature of Loss on Ignition (LOI) method is enough to burn organic matter of soil but should not decompose carbonates present in the soil (11). First soil samples were placed in oven at 105oC for 24hr, and then the samples were placed in muffle furnace (of Ncycraft Company) for 2hr at 360oC. Percentage Organic Matter (%OM) was calculated by comparing the weight of a sample before and after the soil has been ignited (17):

%OM = weight at 105oC (g) – weight at 360oC (g) / weight at 360oC (g) × 100

RESULTS AND DISCUSSION

Results of the final concentration of Zinc (Zn) in Ajaokuta (Ajoa) sample were 53.44 mg/kg while that of Obajana (Obj) was 166.68 mg/kg. The concentration of Chromium (Cr) in Ajaokuta (Ajao) sample was 113.92 mg/kg while that of Obajana (Obj) was 31.04 mg/kg. The concentration of Nickel (Ni) in Ajaokuta (Ajao) sample was 5.42 mg/kg while that of Obajana (Obj) was 69.50 mg/kg. The concentration of Cadmium (Cd) in Ajaokuta (Ajao) sample was -3.14 mg/kg while that of Obajana (Obj) was -0.62 mg/kg. The concentration of Copper (Cu) in Ajaokuta (Ajao) sample was 59.88 mg/kg while that of Obajana (Obj) was 104.98 mg/kg. The concentration of lead (Pb) in Ajaokuta (Ajao) sample was 197.28 mg/kg while that of Obajana (Obj) was 98.64 mg/kg. The concentration of mercury (Hg) in Ajaokuta (Ajao) sample was -5.60 mg/kg while that of Obajana (Obj) was -5.40 mg/kg. The physicochemical results include; soil organic matter (%) for Ajaokuta (Ajao) which was 1.85 while for Obajana (Obj) was 1.47; pH for Ajaokuta (Ajao) was 2.670 while for Obajana (Obj) was 6.096; and Conductivity (µS/cm) for Ajaokuta (Ajao) was 184.5 while for Obajana (Obj) was 320.4.

Heavy Metal Analyses: Comparing the concentration of each of the heavy metals with WHO (World Health Organization) and USEPA (United State Environmental Protection Agency), Zinc, Chromium, Copper and Lead were found to be above the permissible limit in sample Ajaokuta (Ajao) while Nickel, Cadmium and Mercury were below the permissible limit. In sample Obajana (Obj); Zinc, Nickel, Copper and Lead were found to be above the permissible limit, while Chromium, Cadmium, and Mercury were below the permissible limit set by WHO (World Health Organization) and USEPA (United State Environmental Protection Agency). That is, there is enrichment of the metals that were above the standards in the soil locations more than the others. The ranking of the occurrence of the metals from the least to greatest in sample Ajaokuta (Ajao) are Hg>Cd>Ni>Zn>Cu>Cr>Pb indicating that the concentration of lead in the location was high. The ranking of the occurrence of the metals from the least to the greatest in sample Obajana (Obj) is Hg>Cd>Cr>Ni>Pb>Cu>Zn indicating that the concentration of Zinc was high in the sample location.

Table 1. Results of Heavy Metal Concentration in Ajaokuta (Ajao) and Obajana (Obj)

Heavy Metals	Ajaokuta (Ajao) (mg/kg)	Obajana (Obj) (mg/kg)
Zinc	53.44	166.68
Chromium	113.92	31.04
Nickel	5.42	69.50
Cadmium	-3.14	-0.62
Copper	59.88	104.98
Lead	197.28	98.64
Mercury	-5.60	-5.40

Figure 1: Concentration of heavy metals in Ajaokuta (Ajao).

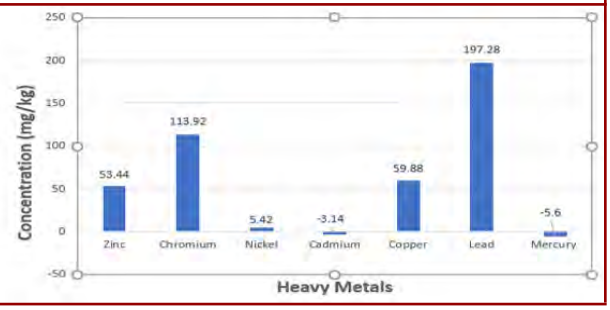
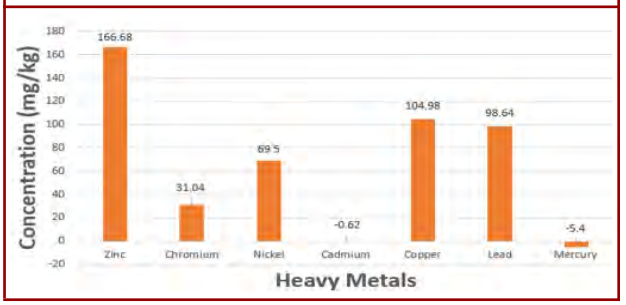


Figure 2: Concentration of heavy metals in Obajana (Obj)



Soil electrical conductivity is the amount of salts in soil (salinity of soil). It is an important indicator of soil health. The electrical

conductivity of the two samples, Ajaokuta (Ajao) and Obajana (Obj) are given in the table below;

Table 2. Results of Soil Organic Matter percentage of Samples Ajaokuta (Ajao) and Obajana (Obj)

Parameter	AJAO (%)	OBJ (%)
Organic Matter	1.85	1.47

Table 3. Results of Soil pH in Ajaokuta (Ajao) and Obajana (Obj) Samples

Parameter	Ajaokuta (Ajao)	Obajana (Obj)
pH	2.670	6.096

Figure 3: Soil pH of Ajaokuta (Ajao) and Obajana (Obj) respectively Soil Electrical Conductivity (µS/cm)

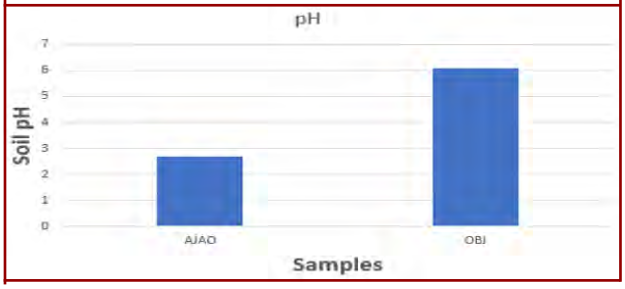


Table 4. Results of Soil Conductivity (µS/cm) for Ajaokuta (Ajao) and Obajana (Obj) Samples

Parameter	Ajaokuta (Ajao)	Obajana (Obj)
Conductivity (µS/cm)	184.5	320.4

Figure 4: Electrical conductivity in Ajaokuta (Ajao) and Obajana (Obj) respectively.

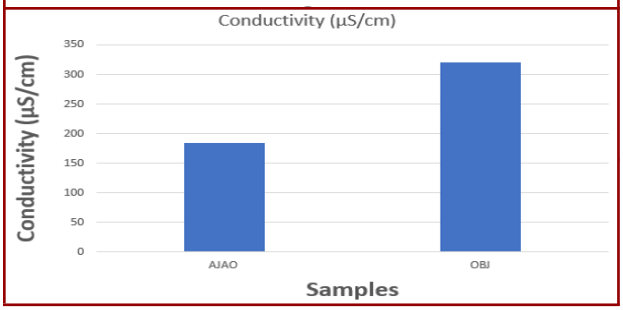
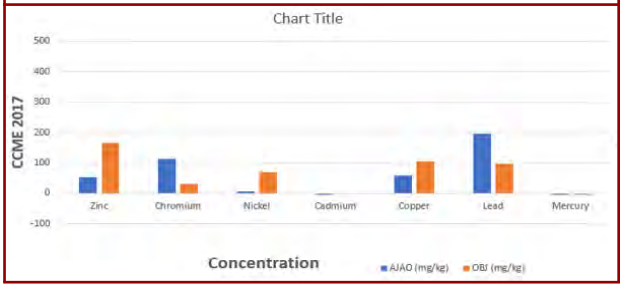


Table 5. Permissible Limits for Heavy Metals in Soil

Elements	WHO (1996) (mg/kg)	CCME (2007) (mg/kg)	USEPA (2002) (mg/kg)
Zn	50.00	500.00	1100.00
Cr	100.00	250.00	11.00
Ni	35.00	100.00	72.00
Cd	0.80	3.00	0.48
Cu	36.00	150.00	270.00
Pb	85.00	200.00	200.00
Hg	-	0.80	1.00

Figure 5: Permissible limits for the various heavy metals and their concentration in the soil.



CONCLUSION

Heavy metal contamination in soil is very dangerous to human health because of agricultural activities that will be carried out on the soil. The results above shows that there were presence of heavy metals in the soil locations and they were found in high concentration (such as, Zinc-53.44mg/kg, Chromium-113.92mg/kg, Copper-59.88mg/kg, Lead-197.28mg/kg in sample Ajaokuta (Ajao) and Zinc-166.68mg/kg, Nickel-69.5mg/kg, Copper-104.98mg/kg, Lead-98.64mg/kg in sample Obajana (Obj) except for Mercury and Cadmium therefore posing potential risk for inhabitants.

While the findings were geared towards providing baseline data on the current pollution states of the industrial areas, constant monitoring of the levels of contamination is deemed necessary, the industries in the areas should be cautioned and guarded against anthropogenic activities caused by industrial effluents, atmospheric deposition, erosion of geological matrix, domestic savage and mining wastes etc. would be suggested against soil contamination (18)

Recommendation

1. Considering the conclusion of this assessment, there is need for constant monitoring of heavy metal concentrations in the industrial areas of Ajaokuta (Ajao) and Obajana (Obj).
2. Concentrations of other heavy metals like Arsenic, Manganese, Aluminum, Cobalt, Tin and others should

be assayed.

3. The process of remediation could be carried out to get a healthy soil formation for every human activity.

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