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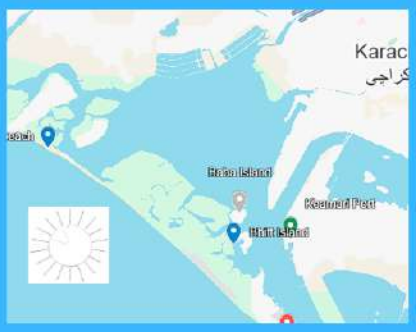

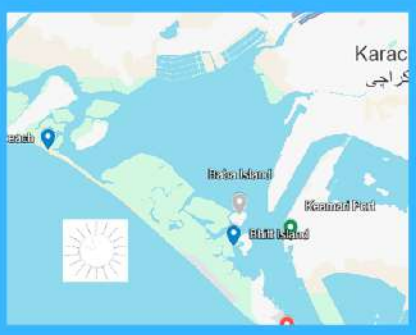

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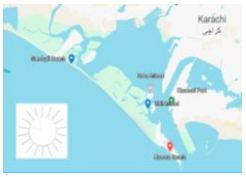

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Culturable Diversity of Halophilic Bacteria from Soil and Saline Water Samples of Arabian Sea, Karachi Port, Pakistan

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ABSTRACT: *Microorganisms capable of survival in extremely high salt concentration are termed as halophiles and a significant source of various enzymes such as hydrolases. Many hydrolases e.g., proteases, amylases and lipases are widely employed in industrial applications. In the current study, we isolated, screened and characterized halophilic bacteria from the shoreline of Arabian Sea, Karachi Port, Pakistan. The water and soil samples collected from Karachi Port; Pakistan were tested for the presence of halophilic bacteria using increasing salt concentrations. Isolated strains were described using various biochemical assays i.e., KOH production, ammonia production, catalase activity, and oxidase activity. For amylase and protease activity, formation of clearance zones – around bacterial growth – was observed, on starch and gelatin agar plates, respectively. Two bacterial strains, N20 (PQ460959) and M3 (PQ460960), with notable amylase and protease activities, were identified via 16S rRNA sequencing as Halobacillus salinus and Halobacillus fulvus, respectively. Further exploration of saline environments is crucial for discovering microbiota with potential applications in biotechnology and environmental remediation*

Keywords: Halophile, Saline water, Protease, Amylase, Hydrolase

INTRODUCTION

The Arabian Sea, with its unique saline properties and proximity to Karachi Port, offers a distinct habitat that may harbour diverse halophilic bacteria with unique adaptations. The bacterial species in such habitats that thrive in the presence of salt are referred to as halophiles (Kushner, 2020). Halophilic bacteria grow optimally in high salt concentrations and are catalytically efficient in surviving other extreme conditions i.e. high temperature, pH, etc. (Ahmed et al., 2020). According to the growth promotion showed by these strains on salt consumption, they are classified as moderate and extreme halophiles (Oren, 2024). Archaea capable of growth in up to 30% w/v salt concentration are termed as extreme halophiles (Kasirajan et al., 2021). While most bacterial domain members capable of growth in up to 15% w/v salts are termed as moderate halophiles.

For survival in such extreme habitats, halophiles produce various unique enzymes that are necessary for survival in such extremes (Wang et al., 2023). Halophiles are significant sources of amylase, protease and are being utilized for various biotechnological applications (Ahmed et al., 2020). Since, the production of hydrolases is an adaptation mechanism, these enzymes help in breakdown of complex macromolecules. Under extreme osmotic pressure, hydrolases also aid in nutrient acquisition (Cui et al., 2017). Various hydrolases have been found to confer cellular stability and flexibility, allowing halophiles to function in their salt-rich niches (Kushner, 2020). Exploration of other saline settings may lead to identification of novel halophiles with promising biotechnological applications (Oren, 2020).

This study was designed to isolate indigenous microbiota from the water samples of Arabian Sea and coastal soils of Karachi Port. Isolated bacterial strains were screened for presence of halophiles via KOH hydrolysis, ammonia production and amylolytic, proteolytic activity. Promising strains were identified via 16S rRNA sequencing.

MATERIALS AND METHODS

Sample Collection and Characterization

Halophilic water and sand samples were collected from Arabian Sea, Port of Karachi, Sindh, Pakistan. Samples were collected from eleven different sites at the Port of Karachi, i.e., Sandspit Beach, Manora Beach, Keamari Port, Baba Bit Island and labelled from 1-11 (Fig. 1).

Fig. 1. A map showing the location of the sampling sites used in the diversification study. Location tags mark the sites; Sandspit Beach, Baba Island, Bhitt Island, Keamari Port, and Manora Beach

Samples were collected aseptically, labelled and stored at 4^{o}C. Sample pH, temperature, colour, texture, odour, turbidity, appearance, etc. were observed and tabulated.

Isolation of halophilic bacteria

Dilutions in series, up to 10^{-5} were used to prepare samples. Pure water and 100 µl portions from also applied to nutritional agar (NA) plates in dilution. Then incubated for 72 hours at 30 °C. In terms of morphology, different bacterial colonies were chosen for additional research. Specific bacterial isolates were stored at -20 °C in a 20% glycerol solution.

Screening for Halophilic Behavior

Halophilic media was prepared using salt concentration of 1M NaCl and 2M NaCl. Isolated strains were streaked on plates and incubated at 37 ℃. After 24 h incubation, plates were checked for absence and presence of culture growth.

KOH hydrolysis test

Two drops of a 3% KOH solution were dispensed on a glass slide. One loopful of bacterial culture was added to the KOH solution using a sterile stick. The suspension thickened in 10-20 s. Formation of thin slime thread on pulling the suspension with sterile

toothpick indicated a positive result (MMM Ahmed et al., 2020).

Ammonia production

Isolated bacterial strains were cultured in 5 mL peptone broth. Media was incubated at 30 °C for 72 h. After incubation, 0.5 mL Nessler's reagent was added to the media. The change in colour from brown to yellow signified the formation of ammonia (James et al., 2014).

Screening for hydrolase producing bacteria

For amylase production activity, bacterial strains were streaked on N. Agar plates containing 20 g/L starch. After incubation, plates were flooded for 10-15 min with iodine solution. The zone of clearance was observed. Screening for protease production activity was observed by streaking bacterial strains on N. Agar plates containing 10 g/L gelatin. After incubation, plates were treated with mercuric chloride solution and zone of clearance was observed (Ahmed et al., 2020).

Characterization of bacterial isolates

Bacterial isolates were characterized via gram staining, catalase and oxidase activity (James et al., 2014).

1.1.1 16S rRNA Sequencing of Promising Strains

Strains were grown on Nutrient Agar medium. The 16S rRNA sequencing of samples was done commercially at Macrogen Inc., Seoul, Korea (https://dna.macrogen.com/eng/support/ces/guide/universal_primer.jsp). Sequences were attained from chromatograms using Chromas 2.6.6. software. Maximum homology was inspected against GenBank using BlastN. Sequences were submitted to NCBI GenBank. Evolutionary relationship of selected strains with related taxa were mapped out using MEGA 10.1.7 for dendrogram construction (umar et al., 2018). Neighbour-joining method was used for computing evolutionary distances, based on the Maximum Likelihood Composite (Saitou et al., 1987).

RESULTS

1.1.2 Isolation and Screening of Halophiles

Samples were characterized based on pH, temperature, colour, odour, texture. Microbial flora of samples collected from Arabian Sea; Karachi Port was enumerated by spread plate method. Calculations of log CFU/mL showed that highest diversity was obtained in samples 8 and 10 taken from Baba-Bhitt Island. The comparative enumeration on media N Agar and Halophilic media showed that almost all isolates were halophiles except for samples from Keamari Port 6, and 7 that showed no halophilic growth. While one sample from Manora Beach (5), showed no growth on either media (Fig. 2). A total of 107 strains were isolated

form all samples and named as N1-40, M1-M67.

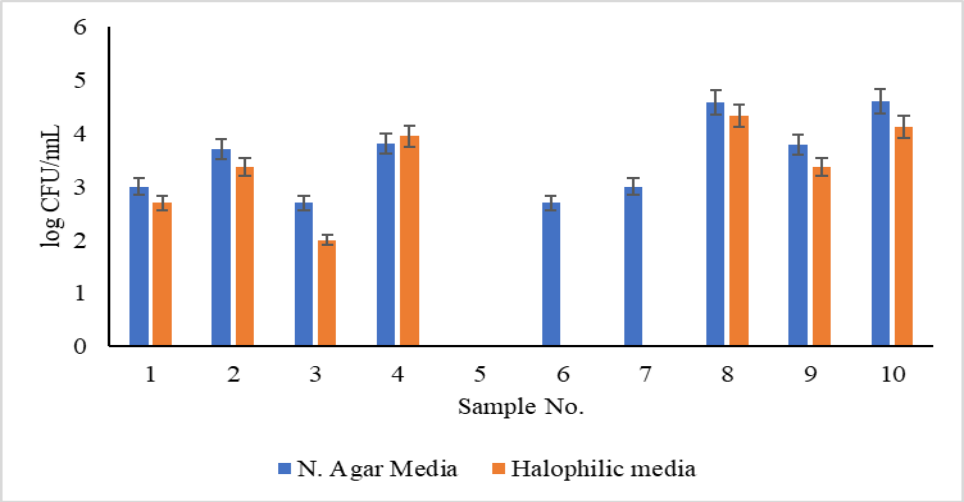

Fig. 2. Comparative Sample Enumeration Karachi Port samples on N. Agar and Halophilic Media

1.1.3 Screening of Halophiles

Screening for halophilic behaviour was done by observation of various parameters i.e., growth on 1M and 2M NaCl to screen moderate and extreme halophiles showed that out of isolates only 14 were moderately halophilic. Further screening tests showed only six among these strains, were capable of gelatin and starch hydrolysis (Fig. 3).

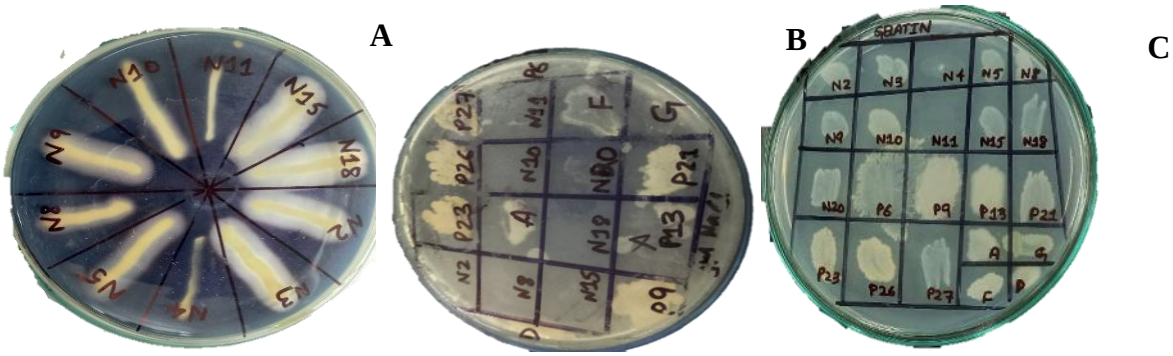

Fig. 3. Screening for Halophiles; A) Starch Hydrolysis, B) Growth on 1M and 2M NaCl, C) Growth on Gelatin

While only strains N20 and M3 were capable of KOH hydrolysis

and ammonia production (Table 1).

Table 1: Screening of Pure Cultures

Strain name	Growth on 1M NaCl	Growth on 2M NaCl	Gelatin Hydrolysis	Starch Hydrolysis	KOH Hydrolysis	Ammonia Production
N2	+	+	+	-	+	-
N3	-	-	+	+	+	-
N4	-	-	-	-	-	-
N5	-	-	+	-	+	-
N8	+	+	+	+	+	+
N9	-	-	-	-	+	+
N10	-	-	-	-	+	-
N11	+	+	-	-	-	-
N15	+	+	+	-	+	+
N20	+	+	+	+	+	+
M2	+	+	+	-	-	-
M3	+	+	+	+	+	+
M4	+	+	+	-	-	-
M5	+	+	-	-	-	-
M10	+	+	-	-	-	-
M11	+	-	-	-	-	-
M12	+	+	+	+	-	-
M16	+	+		-	-	-
M18	+	+	+	+	-	-
M21	+	+	+	-	-	-

1.1.4 16S rRNA Sequencing of Promising Strains

Strains N20 and M3 were identified via 16S rRNA sequencing as *Halobacillus salinus* (PQ460959) and *Halobacillus fulvus* (PQ460960), respectively. Neighbor-Joining method was used for dendrogram mapping. The optimum tree is shown to scale, and Maximum Composite Likelihood was used to calculate the branch lengths corresponding to evolutionary distances.

The evolutionary distance for computation of phylogenetic tree is expressed in terms of the base substitutions per site. This test included 18 nucleotide sequences while there was total 1599 sites in the complete dataset. Ambiguous sequences were eliminated using pairwise deletion (Fig. 4).

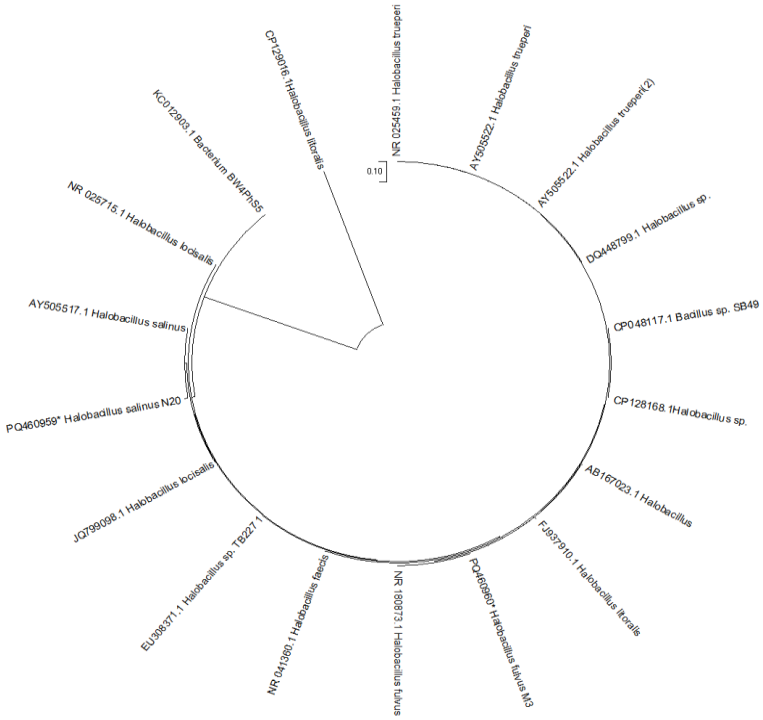

Fig. 4. Evolutionary relationship of closely related taxa with Strain N20 and M3. Neighbor-Joining approach was used to infer the evolutionary history in terms of the number of nucleotide substitutions per site. The optimal tree is presented to scale, and the branch lengths correspond to evolutionary distances determined with the Maximum Composite Likelihood approach. The analysis contained 18 nucleotide sequences while the codon sites given were first, second, third, and noncoding. All ambiguous sites in each sequence pair were removed (using the pairwise deletion option). The entire dataset had a total of 1599 sites.

DISCUSSION

The surface of Earth is covered with 70% water, most of which is comprised of extremely saline environments. Microbiota of various kinds thrive in such extreme environments of temperature, pH, and salinity, by adapting different survival techniques (Dutta et al., 2022). Halophiles thriving in high salt concentrations store potassium ions in their cytoplasm and counteract the osmotic pressure of their environment (Qiu et al., 2021). These halophile have also developed many osmo-protectants for maintenance of osmotic pressure (Oren, 2024).

Among the notable saline waterbodies on Earth surface, Arabian Sea has been the focus of many explorative studies for discovery of novel microbiota (Khan et al., 2021). Like many other saline waterbodies i.e., the Dead Sea, saltern crystallized ponds and hypersaline sun lakes, the Arabian Sea has been crucial in isolation of many industrially important halophiles (Didari et al., 2020; Dutta et al., 2022). In this study, the saline water samples collected from Arabian Sea and coastal soil samples from Karachi Port, were found to be an excellent source of halophiles. Approximately 107 different bacterial strains were isolated form these samples, ranging from moderate to extreme halophiles. These strains were culturally characterized and a variety of gram-positive, gram-negative, spore-formers etc. were observed. Similar results were observed in explorative studies conducted on Oman Sea (Hashemzahi et al., 2020), South China Sea (H Zhang et al., 2022), Southwest Indian Sea (Qiu et al., 2021) and many others.

Studies have also shown that halophile employ different adaptation mechanisms to overcome the harsh growth conditions in their extreme habitats (Deosthali et al., 2022). These include production of enzymes capable of bioremediation in oil contaminated waters (Ullah et al., 2021), plant growth promotion (F Orhan et al., 2020), potential in bio saline agriculture (Ghafar et al., 2022), etc. Strains isolated from various location of Karachi Port underwent further screening for production of hydrolases. Among 107 isolates, many strains showed strong amylolytic, and proteolytic activities. Studies have shown that hydrolases exhibit significant catalytic activity (Drissi Kaitouni et al., 2020). Recent studies have shown the impact of such hydrolases on the biogeochemical cycle via aid in nitrogen recovery

(Zhang et al., 2021), removal of nitrogen from saline waste water via various nitrification processes (Cui et al., 2021), and potential in bio-cleaning (Ruginescu et al., 2022).

Two strains N20 and M3 with promising results were sequenced via 16S rRNA sequencing. These strains belonging to *Halobacillus* genus have been known to survive in highly saline environments (Kong et al., 2024; Wang et al., 2019). During this study, strains M3 and N20 were grown on high concentration of NaCl up to 2M. Similar studies have shown that *Halobacillus* is capable of growth on 5 to 10% NaCl (Kim et al., 2023). Isolation of *Halobacillus* from saline samples of Arabian Sea water and coastal soils, is corroborated by similar studies conducted in such environments. *Halobacillus* species have been found in saline and hyper saline lakes (Didari et al., 2020; Ruginescu et al., 2020), solar salterns (Ali et al., 2024; Ding et al., 2020), salt lakes, etc. In their niche ecosystems, these *Halobacillus* species have been reported to contribute in organic matter decomposition (Ibrahim et al., 2020), nitrogen cycling (Yaradoddi Jayachandra et al., 2020), etc. Studies have also shown the importance of Halobacillus in various biotechnological industries i.e., detergent and food processing, (Xu et al., 2023) and bioremediation (Huang et al., 2006), and therapeutics (Sadaf Wajahat, 2024). The strains N20 and M3, therefore, have high potential for utilization in various biotechnological processes. This study broadens our understanding of microbial diversity in saline environments and highlights potential applications in industry, particularly for enzymes requiring high salt tolerance.

CONCLUSION

In this study, the Arabian Sea – an extremely hyper-saline environment – was explored for isolation of halophilic bacteria. Isolation of two halophilic strains N20 (*Halobacillus salinus*) and M3 (*Halobacillus fulvus*) with hydrolase activity, showed the importance of screening such extreme environments. Future studies should focus on optimizing enzyme production and exploring additional biotechnological applications of these strains, such as in saline wastewater treatment.

ACKNOWLEDGMENT

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ETHICAL STATEMENT

There are no ethical issues. No human or animal experiments were performed in this study.

CONFLICT OF INTEREST

Authors declare no conflict of interests.

DATA AVAILABILITY

The data that supports the findings of this study are available on request by the corresponding author.

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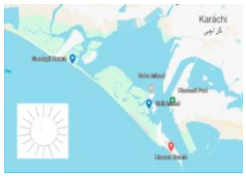

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Research Article
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Comparative Study on the Oothecae of Three Species of Mantodea (Dictyoptera: Insecta)

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ABSTRACT: *During present study, oothecae of three species, namely Hierodula transcaucasica (Brunner von Wattenwyl, 1878), Sphodromantis viridis (Forskal, 1775) and Mantis religiosa (Linnaeus, 1758), were collected and described based on their morphometric characteristics and illustrated features. Significant differences were observed in the dimensions of the oothecae, with S.viridis exhibiting the largest dimensions, measuring 24 ± 0.94 mm in length and 21.6 ± 0.51 mm in width. M. religiosa followed with dimensions of 21.2 ± 0.78 mm in length and 15.3 ± 0.67 mm in width. H. transcaucasica had the smallest length at 17.33 ± 1.23 mm. Additionally, substantial variations in shape and size were noted among the oothecae of the studied specimens. H. transcaucasica oothecae were typically globular in shape, predominantly dark brown with some appearing coffee brown. S. viridis oothecae had a prominently rounded proximal part and a slightly narrower distal end. On the other hand, oothecae of M. religiosa were elongated, typically pale or straw brown, with a thin residual layer. The study recommends the collection and artificial rearing of oothecae as a beneficial approach to promoting mantid culture for potential use as bio-agents in biological control programs.*

Keywords: Carnivores, Pest, Population, Ootheca, Morphometric, Pesticides

INTRODUCTION

Praying mantids, belonging to the order Mantodea, are a diverse group of approximately 2500 carnivorous predatory insects found mainly in warm, humid tropical and subtropical regions worldwide, with rare occurrences in colder areas (Greyvenstein et al., 2020; Otte et al., 2020; Fatimah et al., 2021). They also dwell in the forests, home gardens, pond bionetwork, grassland ecosystem, paddy ecosystem, mango and banana ecosystem (Fatimah et al., 2024). The family Mantidae is particularly rich in mantid species encompassing over 21 families within the order (McMonigle, 2013; Wieland, 2013; Patel and

Singh, 2016; Schwarz and Roy, 2019).They are sternly carnivores predators so they consume lot of agricultural pest insects such as wasps, grasshoppers, aphids, crickets, moths, spiders, beetles, jassids, mealy bugs, termites, white flies and many other small injurious vertebrates viz tiny birds, snakes, lizards etc (Prete et al., 2002; Symondson et al., 2002; Sathe and Patil, 2014; Fatimah et al., 2022). They are furious predators with vital 3-D vision for long distances and because of that they remained very valuable to the agriculturalists and horticulturists in the agronomic sector. In regions of lower Sindh, where pesticides are extensively used year-round, there are significant concerns about their adverse effects on human health, domestic animals and the environment. Pesticides can cause a range of pesticide-related diseases through direct and indirect exposure, including skin blisters, vomiting, cancer, abdominal pain, nausea, eye problems, severe cough, dizziness, and headaches. To mitigate these issues, in the agricultural fields to control pest populations it has been suggested that due to the predatory nature praying mantids proving to be effective bio-control agents against number of pest insects.

The Order Mantodea includes primarily carnivorous insects with diverse hunting capabilities, exemplified by species such as *Humbertiella indica* (Saussure, 1869), *Tenodera attenuate* (Stoll, l877), and *M. religiosa* (Linnaeus, 1758), (Khokhar and Soomro, 2009). Mantids possess unique morphological traits compared to other insects (Sultana et al., 2016), equipped with specialized adaptations for capturing prey, including a triangular head with large compound eyes, an ultrasonic ear, spiky fore raptorial legs, and powerful chewing mouthparts. They are characterized by their distinctive posture of holding their forelegs in a "praying" stance, hence their common name. Praying mantids vary in size from moderate to large, typically ranging from 50-65 mm in length, though some species can reach lengths of 85-90 mm (Harris and Moran, 2000). Their well-developed antennae and robust forelegs with spikes assist in capturing prey (Teyssier, 1997). Their large compound eyes enable them to detect both motionless prey and potential predators (Kral, 2012). While significant research has been conducted on the mantid fauna of Sindh, Pakistan, focusing on their taxonomy and behavior (Fatimah et al., 2016-2020; Fatimah et al., 2022), there remains a gap in understanding the variation and significance of oothecae among different mantid species. This study aims to explore and document these variations, which could contribute to further utilizing praying mantids as effective bio-control agents in agricultural and ecological contexts.

MATERIALS AND METHODS

Exploration of current research was conducted in the Entomological Bio-Control Research Laboratory, Department of Zoology, University of Sindh, Jamshoro during the years 2021-2023 to discover the comparison between the oothecae of three species *H. transcaucasica* (Brunner von Wattenwyl, 1878), *S. viridis* (Forskal, 1775) and *M. religiosa* (Linnaeus, 1758).

RESEARCH STUDY SITES AND SAMPLING

Oothecae were collected from the zones of lower Sindh, Pakistan, with surveys conducted across four different localities: Badin (24.6459^{o} N, 68.8467^{o} E), Tando Muhammad Khan (25.1256^{o} N, 68.5426^{o} E), Thatta (24.7475^{o} N, 67.9106^{o} E), and Karachi (24.8607^{o} N, 67.0011^{o} E), spanning the years 2021 to 2023 (Fig. 1).

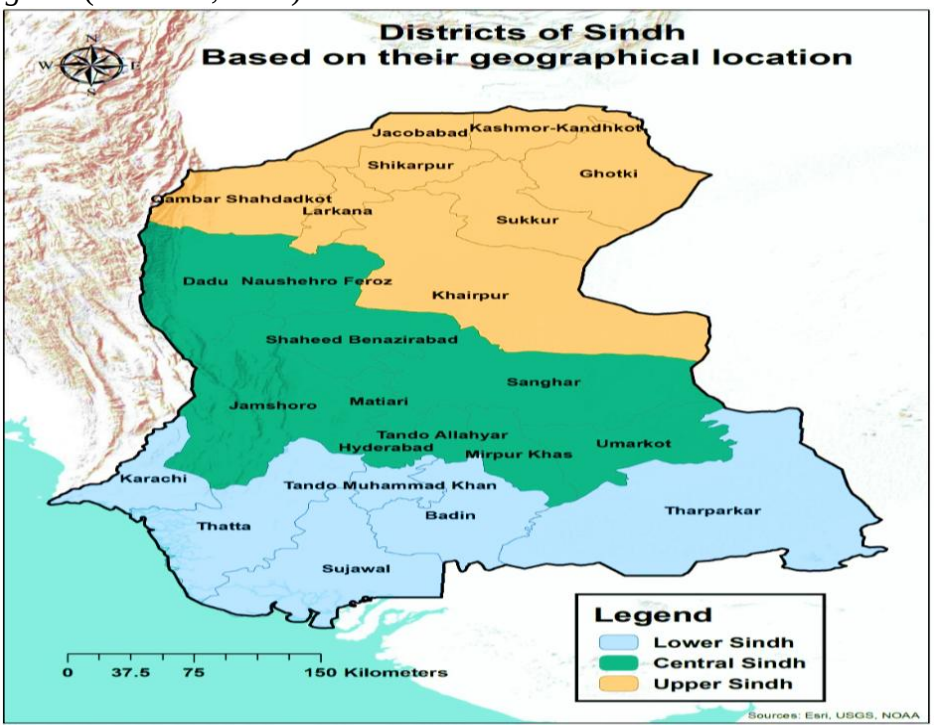

Fig. 1. Representing the Map of Study Areas (Badin, Tando Muhammad Khan, Thatta and Karachi) of Lower Sindh, Pakistan

A total of 276 egg cases (oothecae) were gathered during various months of these years. Locating oothecae proved challenging due to the female mantids' behavior of depositing their ootheca on secure substrates, making them somewhat difficult to spot immediately on plants or trees. The oothecae were carefully collected by hand-picking

or by cutting branches with a sharp knife several inches away from the egg cases to avoid damage. Those attached to walls, boards, lamp posts, or other flat surfaces were removed without harming the egg capsules (Table 1, 2, and 3).

Table 1. Total Number of Collected Oothecae from Fields and Rearing/ Culture

S.NO	*H. transcaucasica*	*S.viridis*	*M.religiosa*
1	4	11	3
2	4	9	2
3	6	9	2
4	8	9	4
5	5	10	8
6	4	8	7
7	3	8	5
8	4	6	5
9	7	6	5
10	5	4	9
11	5	4	6
12	5	4	6
13	9	7	10
14	9	5	5
15	3	11	7
	81	**111**	**84**

Table 2. Total Number of Collected Oothecae

S. No	Species	Mean ± SD (n=15)	Total Number of collected Oothecae
1	*Hierodula transcaucasica*	5.4±1.99c	81
2	*Sphodromantis viridis*	7.4±2.47[a]	111
3	*Mantis religiosa*	5.6±2.35[b]	84
	F. (0.05)	(6.1) 11.34*	276

Mean in the same column followed by the same letters are not significantly different from one another at 5% level of probability.

Table 3. Morphometric Observation on the Oothecae of Various Species of Mantodea

Species	Parameters (Mean ± SD)		
	Length (mm)	**Width (mm)**	**Weight (mm)**
Hierodula transcaucasica	17.33 ± 1.23	15.06 ± 0.70	0.27 ± 0.01
Sphodromantis viridis	24 ± 0.94	21.6 ± 0.51	0.38 ± 0.008
Mantis religiosa	21.2 ± 0.78	15.3 ± 0.67	0.42 ± 0.01

It has been seen from Table 3. The length and width of *Sphodromantis viridis* (Forskal, 1775) was significantly highest i-e 24 ± 0.94mm and 21.6 ± o.51mm respectively followed by *Mantis religiosa* (Linnaeus, 1758) i-e 21.2 ± 0.78 and 15.3 ± 0.67mm. While in the case of *Hierodula transcaucasica* (Brunner von Wattenwyl, 1878) its length was calculated minimum i-e 17.33 ± 1.23mm.

After collection, the oothecae were preserved in jars and warehoused in the laboratory for observation. This meticulous collection method ensured that the oothecae remained intact and suitable for detailed examination of their morphological characteristics and other relevant observations.

PRESERVATION

All plastic jars containing collected oothecae were maintained under controlled laboratory conditions. The temperature was consistently maintained between 39°C to 40°C, while humidity levels ranged from 85% to 92%. To ensure suitable conditions for the oothecae, they were misted with water daily. Upon arrival at the Entomological Bio-Control Research Laboratory, the oothecae were sorted and organized for further study. Observations commenced immediately and continued through the hatching of nymphs, allowing us to document and study the developmental stages and behaviors of the mantid offspring. This controlled environment provided optimal conditions for studying the lifecycle and behavior of the mantids, ensuring that the research could accurately capture and analyze key aspects of their development and ecological interactions.

DESCRIPTIVE STATISTCAL EXPLORATION

The ootheca is an egg case or foamy spongy bag in which the eggs are settle down into the rows. Primarily, at the time of construction it remained foamy in texture and white in color but as soon as contact with air it became hardened and turned into brown or golden brown. So, as it was completely dried the two statistical parameters like length and width

were calculated for analysis. The measurements were taken of field or wild collected as well as oothecae laid by reared species. Different parameters of morphological characteristics such as length (vertically) and width (horizontally) were measured by the help of ruler with measurement scale and geometrical dividers. After taking all the measurements, complete data was calculated by using excel sheet.

RESULTS

The present study focuses on the morphometric characteristics of oothecae from *H. transcaucasica* (Brunner von Wattenwyl, 1878), *S. viridis* (Forskal, 1775), and *M. religiosa* (Linnaeus, 1758), all belonging to the order Mantodea within the family Mantidae. Ootheca easily can be identified by these diagnostic features i-e; Shape, Size, External wall, Texture and color, Point of attachment, Egg chamber, Emergence area (Fig. 2).

MORPHOLOGICAL DESCRIPTION OF OOTHECAE

(A) *Hierodula transcaucasica* (Brunner von Wattenwyl, 1878)

During rearing phase mated female laid 7-9 oothecae (for 09-11 months). Oothecae of *Hierodula* (Deposited as well as wild collected) were ovoid in shape, russet brown in color, soft and spongy in texture with pointed residual process. During fabricating ootheca, it was semi-solid and foamy white in color while after hardening it transformed into brown. The proximal end was wide, oval and the distal end (residual process) was somewhat thin with pointy end. Ootheca grabs 14 transverse and 20-28 longitudinal rows. In the compartments of ootheca, all eggs were horizontally settled into the alignment. Compartments enclosed several eggs in their egg cells (05-400) but number of eggs into the egg case depends on the size of ootheca. Analytically, it was observed that well-nourished female laid massive sized ootheca as compared to low nurtured. Field collected oothecae of this species were also changed in size.

(B) *Sphodromantis viridis* (Forskal, 1775)

Oothecae were puffy, whitish brown at the time of construction. Within time it became hard and gradually changed into dark shimmering or tan, brown, bright prominent rows of compartments about 16-18 ventral and 32-36 dorsal on both sides, rounded or oval frothy along with top of spiky tip. After a few days it became gloomy brown, with brightly pale froth layer; sides were crenelated and emergence area small and concealed majorly in center. The proximal area was much rounded swollen, and the distal area was slightly narrow.

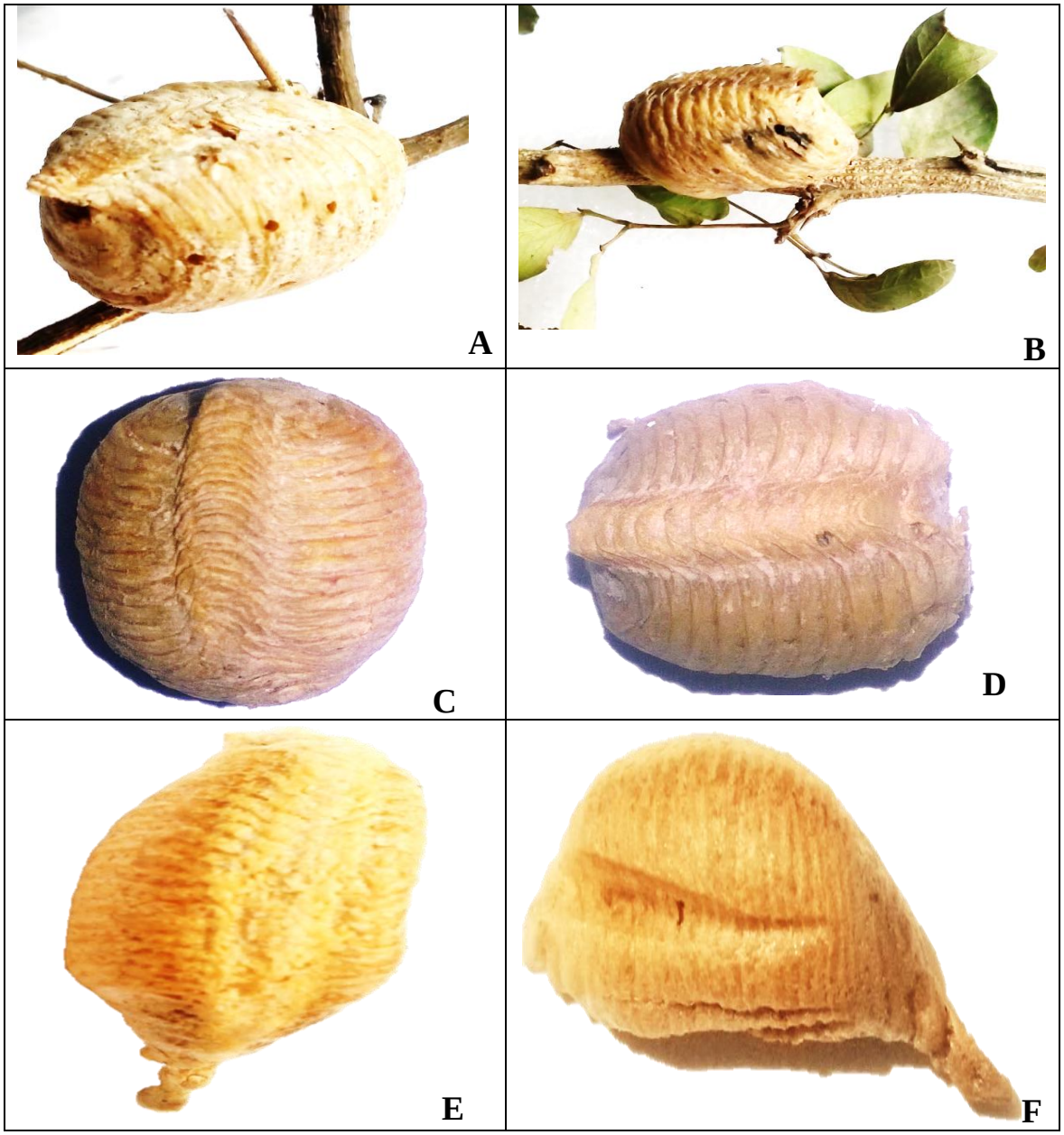

Fig. 2. *Hierodula transcaucasica* Ootheca. A-B, (lateral view); C-D, *Sphodromantis viridis* Ootheca (C, dorsal; D, lateral view); E-F, *Mantis religiosa* Ootheca (E, dorsal; F, lateral view

(C) *Mantis religiosa* (Linnaeus, 1758)

Oothecae were rich, elongated, straw brown with much larger residual layer. About 20-24 slightly proportioned outlines, showed glistening at glance. Two deep depressions or grooves from lateral sides were present. Ootheca was white colored at the time of building within time it became dried out and hard-bitten, initially turned straw yellow and later color transformed into bright whitish

brown. Oothecae were rich, elongated; pale brown with a thin much large residual layer which was somehow lengthy.

DISCUSSION

Morphologic or morphometric refers to the taxonomic quantitative examination of structure that consists of size and shape or it is the technique or process of measuring the external shape and measurement of an organism. Mantids are incredible insects in the insect world which keep distinctive place by showing brilliant habitual behavior of camouflage, head swivel of 180^{o}, ensnaring prey, sexual cannibalism and formation of distinct foamy or spongy type of egg case called "Ootheca". After mating female start producing egg cases in the season of autumn from the month of February to May continuously until she survives (Ramsay, 1990). The varieties of ootheca prominently differs from species to species that type of variation furthermore aids in the identification of species. In the summer season during mating, the male is eaten by female, while approach of female ferocious towards the male as his head come close to her raptorial forelegs shortly, she seizes the male and primarily eat her head. Though mating will continue without the head until her feast is finished. Copulation of mantids is completed in more than a few phases and after or before copulation the female devours the male. After a two-day mating phase, she begins producing an egg case and searching for protective substrates for depositing it, i.e., leaves, trunks, plant stems, shrubs, rocks or walls, or placed in soil or grass and sides of houses. The study displays that the direction of ootheca towards the North, probably to gain ultimate sunlight and heat to accelerating the eggs development (Bowie and Bowie, 2003). Then, well-nourished female with distended abdomen lay eggs in a frothy substance that solidifies into a protective case (Ootheca; plural Oothecae) where eggs remain throughout the winter. Females of some species guard their ootheca while others dump them in sand due to vulnerability of attack from numerous species of parasitic wasps and birds (Ene, 1964; Ramsay, 1990; Vyjayandi, 2007; Weiland, 2008). Adult female *Theopropus elegans* (Westwood, 1832) was observed as guard or protector of her newly laid eggs deposited within an elongated ootheca (Leong and Teo, 2008). An ootheca contain about 200-300 eggs in a bulky shield like case which protect the eggs from unpleasant climatic conditions and enemies. During rearing phase, it was observed that well-nourished females lay large ootheca while under-nourished females will lay small oothecae which contains a small number of eggs. Oothecae vary in size, shape and color (at the time white or creamy or green but after hardening turn into light or

dark brown). The female *H. patellifera* (Serville, 1839) was found depositing her eggs surrounded by a foamy, green ootheca. The ootheca turned hard and became brown within the day because of contact with air (Leong, 2009). Some species of mantids will construct large, round foamy ootheca; some will create short, thick ribbon-like ootheca. Also depending on the species of mantis, some fabricate only one or two ootheca, whereas others can secrete up to twenty during their lives. The entire outer arrangement has an uneven surface, a foam-covered oozing in contact with air rapidly became harden (Avigliano, 2009). Few species attached their oothecae to a flat surfaced area and some enfolded them around the parts of trees or even dumped them into the ground due to the attack of several species of bees or parasitic wasps. Also, it was recorded in a few species, the mother protects their egg cases by guarding them (Ene, 1964). The globular shape of ootheca with pointed end and size depends on the technique of depositing it by *Hierodula* species (Bischoff et al., 2001). The ootheca protects the eggs from microorganisms, parasitoids, predators, and adverse weather. The ootheca preserves a secured water balance through its porous surface and in dry weather protects the eggs against desiccation. In May and June, the eggs hatched steadily after two weeks. The juveniles or immature baby mantids hanged down within a single silk strand out of emerging area of an ootheca. Each ootheca has a compartment-like gap which enclosed tiny valve-like structures from where immatures came out. Both adult as well as nymph are predacious, they are prospective predators because of their weird habits selection for slaying their prey, camouflage for concealment against their own enemies, unique reproductive behavior of cannibalism and enclosing their eggs into the sac (Yadav, 2018).

CONCLUSION

It is concluded that the work on identified oothecae of praying Mantids belongs to the three genera *Hierodula* (Burmeister, 1838), *Sphodromantis* (Stal, 1871) and *Mantis* (Linne, 1758) was not described formerly from the zones of lower Sindh, Pakistan. Furthermore, morphological variance between oothecae were recorded for the first time with their pictures from four diverse areas: Badin, Tando Muhammad Khan, Thatta, and Karachi. This exploration of study provides cherished revelations on taxonomy, biology and diversity of Mantids in Pakistan.

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ETHICAL APPROVAL

No ethical approval is required

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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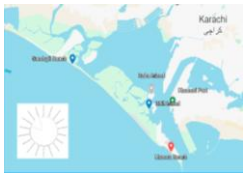

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An Augmented Review on Monkeypox (An Emerging Public Health Threat) in Pakistan

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ABSTRACT: *Monkeypox (MPX) is a zoonotic viral disease caused by the monkeypox virus, closely related to the variola virus (smallpox). First identified in monkeys in 1958 and humans in 1970 in the Democratic Republic of Congo, it is now endemic in parts of West and Central Africa, with rising cases in non-endemic regions. Symptoms resemble smallpox but are less severe. Transmission occurs through direct contact with infected animals, humans, respiratory droplets, bodily fluids, or lesion material. The 2022-2023 outbreak spread globally, with tens of thousands of cases linked to the West African variant reported in over 110 countries. Severe cases, mainly in immunocompromised individuals, emphasize the need for vaccination and antiviral therapies. In Pakistan, although no major outbreaks have occurred, suspected cases have prompted health authorities to enhance surveillance and diagnostic capacity. Public awareness, early detection, vaccination, and international collaboration remain critical to prevent outbreaks and control MPX both locally and globally.*

Keywords: Monkeypox, orthopoxvirus, Zoonotic concern, Animal reservoirs

INTRODUCTION

MPX has emerged as a growing public health concern in Pakistan, causing a great challenge to the healthcare system of the country. As a zoonotic disease the monkeypox has the potential to cause outbreaks with serious consequences for human health. Current global cases have reported its capability to spread in non-endemic regions, rising challenge about its potential effect in developing countries like Pakistan with limited resources for scrutiny, diagnosis, and containment. Addressing this danger needs serious measures, comprising increasing public awareness, supporting healthcare infrastructure, and implementing vigorous disease surveillance systems to avoid further spread. Reports state that the monkeypox virus (MPXV) is the cause of the zoonotic viral infection known as monkeypox (MPX), which manifests as a smallpox-like rash (Letafati and Sakhavarz, 2023; Koenig et al., 2022; Ejaz et al., 2024; Niu et al., 2023). However, the case fatality rate for MPX infection is much lower and varying between 1-10% depending upon the strain and region (Sklenovská and Van Ranst, 2018). Recent outbreaks of MPVX have commonly been identified amongst gay communities in prevalent areas, or bisexual and/or other men having sex with men who have had current sexual relation with a new partner or partner. Trans and gender-diverse communities were also affected, pregnant females were also found infected (WHO, 2023).

Monkeys shipped in 1958 from Singapore to Denmark for research purposes were the first to be shown to exhibit MPX (Hussain and Ghouse, 2022; Shaikh et al., 2023) marking the discovery of the MPXV during an outbreak in a laboratory setting. A second outbreak was then recorded in the United States, the Netherlands, France, Australia, the United Kingdom, and Europe (Meo and Klonoff, 2022). (Hussain and Ghouse, 2022). In 1970, a nine-month-old boy in the Congo became the first person to get MPX (Spirito et al., 2023; Mitjà et al., 2023). Following the initial human case, sporadic outbreaks were reported in some west and focal African countries, primarily affecting children in rural, rainforest areas. Clinical presentation and the results of genome sequencing were used to identify two clades of MPX virus isolates (Foster et al., 1972). From 1981 to 2017, the Democratic Republic of the Congo saw multiple outbreaks of the MPX virus clade 1, with high death rates (ranging from 1 to 12 percent) (Yinka-Ogunleye et al., 2019).

Most of these instances were not laboratory-confirmed because of the challenges posed by civil instability and the current health system. Furthermore, most patients lived in remote locations that were challenging to access. There were relatively few human MPX case reports in West Africa throughout this period, although a notable epidemic of 122 PCR-confirmed MPX virus clade 2 infections occurred in Nigeria in 2017 (Nguyen et al., 2021). Although not endemic to the region, the outbreak of MPX in Pakistan, has upraised important public health fears. The 1st confirmed cases of MPX were reported in 2023, possibly related to global traveling and cross-border human connections spreading through close contact with infected individuals, respiratory droplets, or contaminated materials (WHO, 2023; Fareed et al., 2024). Its emergence in Pakistan has highlighted gaps in surveillance/awareness systems like inadequate public awareness and limited diagnostic capabilities. Therefore, there is dire need to adopt effective management strategies (vaccination campaigns and public education etc) to curb the spread and mitigate the socio-economic impacts. Many factors, such as the expansion of urbanization with infringement into woodland and bog regions, the decline of resistance, the frequent hunting or butchering of bush meat for resources, and the end of smallpox vaccination in 1980, were blamed for the resurgence of MPX in Nigeria in 2017 and the steadily rising number of cases in the Popularity Republic of Congo. Despite this, little is understood about the worldwide neglect of African outbreaks (Chatterjee et al., 2022). In April 2023, the first confirmed MPXV case in Pakistan was reported, prompting prompt action. Due to inadequate healthcare arrangements, unfavorable economic conditions, and the current MPXV outbreak in 2024, Pakistan's healthcare systems are facing difficulties (Muneer et al., 2021). Therefore, to control the MPXV outbreak, a coordinated strategy involving the public, healthcare providers, and governmental and non-governmental groups is essential. Additionally, research is needed to identify the MPXV and create a thorough preventive procedure that works for pandemics in the future. Therefore, it is urgent to train healthcare professionals for early detection in the diagnosis and management of the MPXV to adopt prompt and stringent measures, mobilize resources, collaborate

with authorities, and educate those who are at risk (Table 1).

Table 1: Number of confirmed MPX cases by country

Year	Event	Description	Reference
2023-2024	Current Investigation	Efforts continue globally to monitor and control the spread of MPXV focusing on the development of vaccines.	WHO, 2023; Fareed et al. (2024).
2022-2024	Pakistan	The first confirmed MPX case in Pakistan was reported on April 19, 2023, by the NIH, Islamabad, involving a traveler from Saudi Arabia. In 2023, nine confirmed cases and one death were recorded. The first case of 2024 was reported in January, also linked to a traveler from Saudi Arabia. By August 27, 2024, two additional cases were identified, raising the total to 12 cases since April 2022.	WHO, 2023; WHO, 2024
2022	Global Public Health Emergency	Several cases of MPXV were reported across non-endemic countries and MPXV was announced a public health emergency.	WHO, (2022).
2017	Nigeria Outbreak	over 200 suspected cases of MPXV were reported in Nigeria with the largest since 1978.	Yinka-Ogunleye et al. (2018).
2003	American Outbreak	The 1st outbreak outside Africa reported in the USA, connected to imported *Cricetomys gambianus* (Gambian pouched rats) from Ghana.	Reynolds et al. (2006).
1980s-1990s	Sporadic Cases	Sporadic cases of humans were reported in the countryside, and rainforest regions of West and Central Africa.	Jezek et al. (1988).

1970	First Human Case	In the Democratic Republic of the Congo (Zaire), a 9-month-old boy was the first human to be diagnosed with MPXV.	(Ladnyj et al., 1972)
1958	Discovery	In Denmark, the MPXV virus was initially discovered in lab monkeys during two outbreaks.	(Breman and Henderson, 1977)

Etiology

Like the variola virus, which causes smallpox, the vaccinia virus, which causes smallpox vaccinations, and the cowpox virus, MPX is an Ortho poxvirus, which is a double-stranded DNA virus infection. Electron imaging of MPX-infected cells reveals a brick-shaped virion approximately 200 to 250 nm in size and is not distinguishable from variola or vaccinia virus virions (Kumar et al., 2023).

With over 200 kilobase-coordinates, the MPXV genome is enormous and encodes about 190 proteins that are used to build viral particles and control different host functions (Hassan et al., 2023). In different topographical regions of Africa, two specific clades of MPX have been widely identified (Okwor et al., 2023). Clades 1 and 2 are two different kinds of clades that have been found in West Africa (Karagoz et al., 2023).

Transmission

MPXV is usually transmitted to humans by infected animals (Girón-Guzmán et al., 2023) through bites and bodily fluids like urine, feces, saliva, or semen (Ali et al., 2023). Khattak et al. (2023) state that close, persistent skin-to-skin contact—particularly through contact with skin lesions such as blisters, pustules, rash, scabs, or wounds is the main way it can transmit from person to person. Particularly infectious are the blister fluid, the ulcers that emerge after the blisters rupture, and the scabs that cover them (Oiwoh et al., 2023). Viral transmission can happen by direct contact with infectious fluids, rash, scabs, and/or crusts from sores, saliva, or contaminated bodily fluids, including respiratory secretions, according to Ejaz et al. (2023) and Shaikh et al. (2023). The primary entry points for the virus include mucous membranes such as the anal and oral areas (Mukherjee et al., 2023). In the general population, MPX poses a reduced risk to public health. However, in Africa, where MPX has been documented historically and is still a common occurrence, the risk is moderate for the public, and it is moderate for males who have sex

with men and sex workers in all situations.

A total of 94,274 confirmed cases of MPXV, including 157 fatalities, were reported between January 1, 2022, and September 11, 2023. Of these, 2,432 took place in regions where monkeypox had previously been recorded, and 91,842 took place in regions where monkeypox had not. Seven historically reported MPX and 111 historically reported MPX were found in 118 countries and regions. Malaysia and the Lao People's Democratic Republic have reported their first MPX cases since the last situation report was published on August 14, 2023.

Pathobiology of MPXV

According to Karagoz et al. (2023), MPX enters the host through the nasal, oral, and cutaneous routes. Once the virus has reproduced in the host sites, it moves to lymph node locations (Safdar et al., 2023; Saadh et al., 2023). The morphological structures of known OPXVs and MPXVs are similar (Karagoz et al., 2023). The viruses are monoblock or oval, and their envelope is made up of a lipoprotein outer layer membrane (Karagoz et al., 2023). During the viral cycles, the host cytoplasm goes through several processes, including protein assembly, transcription, translation, viral DNA replication, and the discharge of mature virions. The poxvirus enters the host through micropinocytosis and fusion (Wei et al., 2023). The human MPX activity atomic system is depicted in this image. Brain discomfort, fever, chills, rashes, throat infections, and ulcers are some of the initial side effects that MPX patients experience (Sardana et al., 2023). Infectious germs frequently enter the body through macropinocytosis. Stages like DNA replication, recording, interpretation, and the appearance of fully developed virions come next. Once more, the mature virions may exhibit the same illness pattern. The fundamental pathophysiology, signs, and symptoms of MPX infection are depicted in this figure. MPX is a viral infection that can spread through the respiratory system through mucosal, pulmonary, or percutaneous sites, leading to primary viremia. Following the initial exposure, the virus enters the bloodstream, causes primary viremia, and then multiplies and spreads throughout the body. This phase, referred to as the prodrome period, is characterized by nebulous symptoms like fatigue, headache, fever, and muscle aches. The virus spreads to other organs and tissues during secondary viremia, which causes characteristic skin lesions to appear during the rash phase. These lesions progress from macules to papules, vesicles, pustules, and crusts, frequently accompanied by lymph node enlargement. The respiratory route of transmission highlights the importance of respiratory precautions in preventing the spread of MPX and

the importance of early identification and isolation of cases to mitigate its transmission, according to Sardana et al., 2023; Ahmed et al., 2023; 2023a; Wang and Lun, 2023).

The pathophysiology of MPX, a viral infection brought on by the MPX virus (MPXV), heavily relies on the JAK/STAT pathway. The JAK/STAT pathway, a signaling cascade involved in several physiological functions, including triggering immunological responses, is activated by the virus during infection. Activation of this pathway leads to the production of pro-inflammatory cytokines and chemokines, which support the inflammatory response and recruit immune cells to the infection site. Furthermore, the JAK/STAT system controls the expression of antiviral genes like interferons, which are important in preventing the transmission and replication of viruses. The precise mechanism by which MPXV modifies the JAK/STAT pathway to evade host immune responses and promote viral growth is still unknown, though. Understanding the relationship between MPXV and the JAK/STAT pathway may help develop novel therapeutic methods against MPX and associated viral infections (Mukherjee et al., 2023).

Effects MVPX on different organs

MPXV may affect the respiratory system (Beeson et al., 2023). Serious respiratory issues will result Severe respiratory complications may lead to asphyxia. MPXV can induce severe lung inflammation and/or pneumonia. Macaques produce more proteins in response to an immune response, such as C-reactive protein, vitamin D-binding protein, S100A8, S100A9, and fibrinogen, while producing fewer proteins involved in lung tissue metabolism and lubrication, such as ACTB, COL1A1, GSN, STMN1, TMSB4X, TUBB1, and VIM (Mukherjee et al., 2023).

In extreme situations, the virus may potentially affect other organs, including the heart, lungs, liver, spleen, and lymph nodes (see Fig. 3) (Falendysz et al., 2023). MPXV impacts the immune system, leading to immune dysfunction and inflammation (Saghazadeh and Rezaei, 2023). Common symptoms of MPX include tongue lesions, inflammation, and ulceration (Joseph and Anil, 2023), which frequently result in the formation of pus or edema (Mukherjee et al., 2023). Its migration to various body parts affects the lymphatic hub (Ahmed et al., 2023). Usually, it affects the facial and furthest locations rather than the storage container. The immunopathological activity of MPXV and its effects on different organs are depicted in this picture. (A) The cytoplasmic virus life pattern of MPXV. Enveloped virion (EV) enters the host following fusion with mature virion (MV). Waves of protein synthesis are produced by the beginning, middle, and late viral

mRNA during fully permissive viral reproduction. Infectious particle morphogenesis occurs after these waves. B) MPX has an impact on the mouth, skin, lymph nodes, liver, reproductive system, and respiratory system.

a-MPXV and Skin

The first symptom of MPX infection is skin flaring up within 13 days after the onset of fever (Cabanillas et al., 2023; Mukherjee et al., 2023). Rashes usually appear on the face and/or extremities more often than on the trunk. The WHO reports that 75% of cases affect the palms and soles of the hands and feet, whereas 95% of cases usually impact the face (Patel et al. 2023). The conjunctivae (20%), oral mucous membranes (70%), and genitalia (30%) are also impacted (Patel et al., 2023). After going through four stages (macular, papular, vesicular, and pustular) and crusting, the lesions, which are between 0.5 and 1 cm in size, finally dry up and fall (Cabanillas et al., 2023). When conditions worsen, lesions may grow together and result in extensive skin loss. Cellulitis often develops over the resultant skin lesions, which are susceptible to bacterial superinfection (Wang and Lun, 2023). According to a clinical report, 41% of lesions in MPX-infected individuals were mucosal, and 58% of lesions were vesiculopustular (Mukherjee et al., 2023).

b-MPXV and the lymphoid system

The World Health Organization states that one characteristic of MPX that differs from other disorders is lymphadenopathy. One clinical characteristic of MPX that can help distinguish it from smallpox or chickenpox is lymphadenopathy in the prodromal stage. Two stages of spreading are discernible. In primary viremia, the virus spreads to nearby lymph nodes; in secondary viremia, the virus spreads to distant organs and lymph nodes via circulation. The maxillary, cervical, and inguinal lymph nodes always enlarge when the immune system is first engaged, and this always occurs concurrently with the onset of fever. The contamination extends to the tonsil, mediastinum, and mandible lymph nodes during MPX illness. In his study of cynomolgus monkeys, he found that the most serious lesions were seen in the mandible and mediastinum; lymph nodes in the groin, armpits, and mesentery were also impacted. There are foamy macrophages in the enlarged medullary sinuses. He observed that most lymphoid hyperplasia was found in minor zones and periarteriolar lymphoid sheaths.

c-MPXV and liver

MPXV-positive people have viral antigens scattered throughout their livers, that even though MPXV-related liver lesions are not very common. When MPX-infected ground squirrels examined their livers, they found steatosis, centrilobular necrosis, and basophilic inclusion bodies in the hepatocytes. However, when two researchers examined MPXV-positive monkeys, they discovered that the monkeys exhibited a range of flat, gross liver lesions that ranged in diameter from 2 to 5 mm. Some of them died from hepatitis, and others developed ulcers. Regardless, histologic evidence linking MPXV to hepatitis was nonexistent. Occasionally, many degenerated Kupffer cells cause the sinusoids to become plugged with fibrin and cellular debris.

Clinical Manifestations

While MPXV usually results in a transient rash, its clinical manifestation is very similar to smallpox. The afflicted individual may be ill for two to four weeks during the three to seventeen-day incubation phase. In addition to a skin rash, Symptoms typically include fever, headache, decreased energy, sore throat, back discomfort, muscle aches and lymphadenopathy. Additional symptoms may include rectal pain, difficulty urinating, and severe swelling, which can persist until all wounds have healed and new skin has formed. Clinical instances are infectious and have the potential to spread the illness to other people. Pregnant women, children, and those with weakened immune systems are especially susceptible to mumps complications. The lesions are usually well-circumscribed, deep-seated, solid, or rubbery, and they often develop umbilication, which is a dot-like look on the top of the lesion. During the current worldwide epidemic" is vague and should be more specific (e.g., the ongoing 2022–2023 global outbreak). The rashes caused by MPXV may be limited to one or a few lesions and do not always extend to many different areas of the body. During the present pandemic, MPXV patients also experienced bleeding, rectal pain, or purulent or bloody feces. Lesions are commonly described as painful until they become itchy (crusts) during the healing process. Fever and other prodromal symptoms (such as chills, lymphadenopathy, malaise, myalgias, or headache) may appear before the rash.

Table 2: Different types of lesions formed due to MPXV, duration, and characteristics

Lesion	Duration	Characteristics
Enanthem		The initial indication of the rash is a red plaque that is 2–5 mm in diameter and has a flat base.
Macules	1−2 days	There are Flat, red lesions.
Papules	1−2 days	A hard lessonlesson that has a little bulging
Vesicles	1−2 days	Transparent fluid fills the lesions.
Pustules	5−7 days	pus-filled lesions that become yellow, monkeypox lesions, umbilication—a central depression that resembles an umbilical fossa—will be common. The patient will experience pain and itching during this time. The pustules last for five to seven days before the crust begins to form.
Scabs	7−14 days	By the end of the second week, pustules had turned into scabs. Scab shedding can begin as early as two to four weeks and last up to eight weeks.

Where scabs are being shed, they may leave behind erythema or pigmentation. Years may pass before the scar heals.

Complications

Additionally, respiratory complications and secondary infections (e.g., bacterial pneumonia) can arise from MPX. Pneumonia, some eye infections, and secondary skin or soft tissue infections are complications that can arise from an MPX infection. Hepatomegaly, myocarditis, scrotal edema, renal disruption, and encephalitis are examples of serious side effects.

Diagnosis

The following diagnostic methods are recommended:

- Clinical signs and symptoms
- Laboratory tests, such as blood tests and CBCs
- Histopathology of the skin
- Culturing cells
- The sample's immunohistochemistry
- Molecular identification methods include restriction-fragment-length polymorphism (RFLP), ELISA, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA).

Table 3: Diagnostic tests for Orthopoxvirus

Test	Characteristics
Viral cultures	Culturing the virus in cell cultures helps identify the species. A lesion sample is required, and careful handling is necessary due to the contagious nature of the virus
Immunohistochemistry	This makes diagnoses. Antigens specific to Orthopoxvirus Any biopsy sample can be utilized to make a diagnosis. For dealing, a technical crew is necessary.
Real-time polymerase chain reaction	This can identify the MPX virus and previous contact by looking for MPX-specific DNA markers. This calls for deft hands as well.
Anti- *Orthopoxvirus* immunoglobulins (IgM/IgG)	To investigate a recent or past exposure, this test looks for Orthopoxvirus antibodies.
	This test even can be used with smallpox vaccination.
	The essay is vague and requires a cold chain and blood collection. MPX
Immunohistochemistry	This tests for *Orthopoxvirus*-specific antigens
	A large laboratory setup and highly qualified technical personnel are required.

Treatment

MPXV has no known cure, although antivirals like Tecovirimat (ST-246), which were created to treat smallpox, be successful. to control symptoms and avoid complications, supportive care and therapy are essential.

Supportive treatment

Patients who experience pain, particularly from rashes or lesions on mucous membranes, need to receive supportive care. Supportive treatments may include NSAIDs for pain relief, saltwater gargles for throat lesions, stool softeners for rectal lesions, and local anesthetics for oral lesions.".

Specific treatment

In cases of secondary infections or severe disease that may be hemorrhagic, pharmacological treatment is advised. Patients with severe cases or secondary infections should be isolated and

hospitalized for proper care and monitoring. Table 4 provides a list of some suggested treatments.

Table 4: Recommended treatment of cases requires hospitalization and isolation

Drug	**Mode of action**	**Indication**	**Recommended dose**	**Adverse reaction**
Tecovirimat	Viral protein packaging disruption during the maturation stage	Severe cases of bleeding, Patients with weakened immune systems	25-40 kg @400mg BD 41-120 kg @600 mg TD	GIT disturbances including nausea vomiting etc.
Brincidofivir	Stopping the production of viral DNA	Serious situations, Patients who don't react to Tecovirimat	200 mg once a week for 48 kg and beyond 47–10 kg with an oral suspension of 200 mg Oral suspension of less than 10 kg BW at 6 mg/kg	The most frequent GIT disorders include nausea and vomiting.
Cidofovir	Stopping the replication of viral DNA	Serious situations, Individuals who are unable to take Tecovirimat	Adult patients weighing less than 10 kg BW should be premedicated with 2 grams of probenecid three hours before cidofovir, followed by 1 gram two and eight hours	Renal impairments

			following infusion.n @ 6 mg/kg	
Vaccinia immune Globulin	Not sure exactly	When no other form of treatment is working	As per CDC guidance	Other vaccine interactions

Preventive/precautionary measures

The affected person should stay in quarantine at home and refrain from needless socializing. To promote better preventing the spread of disease, it is essential to adopt good hygiene practices. Make it a habit to wash your hands regularly and confirm that open rashes are covered with a veil or bandage to lessen exposure. Keep your skin dry unless necessary to support the healing process and avoid touching the shared objects, disinfecting them regularly. For mouth sores, saline solutions (mouth washes) can provide help, whilst body bruises may benefit from scrubbing down or hot showers with Epsom salts or baking soda. Moreover, avoid from using over-the-counter medications i.e. paracetamol or ibuprofen without medical guidance, as these can inhibit the healing or mask symptoms.

To prevent the disease from spreading to others, individuals with MPXV should be screened and instructed to isolate themselves at home or, if necessary, in a hospital for the full duration of the infection period, which is from the beginning of clinical symptoms until the lesions heal and the scabs fall off (Koenig et al., 2022). Additionally, to stop the spread of disease in the presence of healthy people, lesions should be carefully covered and a medical mask worn. The bulk of MPXV cases in the current outbreak were recorded in men who engage in sexual activity with other men, which ought to be prohibited in these groups. To reduce the risk of contracting MPXV, an affected person should use condoms during intercourse and refrain from skin-to-skin or mouth-to-skin contact.

Physical therapists may need to diagnose monkeypox, and patients should be cautioned against self-diagnosing or self-isolating. Given the emergence of atypical MPXV appearances, such as restricted lesions and/or lack of a prodromal phase, physical therapists should treat any suspicious new rash as a probable MPXV case (CDC, 2022).

MPXV infection in Animals

Due to its zoonotic dissemination, MPXV is a viral illness of significant veterinary concern. Animals, especially rodents and non-human primates can contract monkeypox.

Animals may have respiratory difficulties, skin sores, and fever as symptoms. Direct contact with sick animals, their bodily fluids, or contaminated objects like bedding or cages can spread the infection.

The increasing global spread of epidemics, particularly recent outbreaks, has made this disease even more important. Because it is prevalent in some rodent populations, the disease is most frequently seen in Central and West African nations. Nonetheless, MPX infections have also been documented in other countries, such as the US, and are typically connected to imported exotic pets.

It is crucial to remember that animal-to-human transmission of the MPX virus is comparatively uncommon because animals can act as reservoirs for the virus. Infections in humans typically result from intimate contact with diseased individuals or direct interaction with contaminated animals. Transmission from person to person is also feasible, albeit usually restricted.

Monkeypox virus (MPXV) is not typically zoonotic for cattle and sheep. The primary reservoirs of MPXV are wild rodents and some primates, and zoonotic transmission primarily occurs between these animals and humans. There is no significant evidence that cattle or sheep are naturally susceptible to Monkeypox or play a role in its transmission.

Monkeypox primarily affects small mammals, such as rodents and squirrels, and non-human primates. These animals are more closely associated with the virus's zoonotic cycle. In contrast, cattle and sheep have not been observed as common hosts for MPXV.

It's important to note that different animals may exhibit different clinical presentations of MPX, with some species exhibiting more severe symptoms than others. Furthermore, different animal species may be susceptible to the virus, with non-human primates being the most vulnerable.

It's crucial to seek advice from a veterinarian or public health officials if you think an animal might have MPX. They can offer pertinent guidance on testing and handling practices to reduce the possibility of transmission to people and other animals.

MPXV Infection and the lymphatic system

The World Health Organization states that one characteristic of MPX that differs from other disorders is lymphadenopathy. One clinical characteristic of MPX that can help differentiate it from smallpox or chickenpox is

lymphadenopathy in the prodromal stage. Two stages of spreading are discernible. In primary viremia, the virus spreads to nearby lymph nodes; in secondary viremia, the viral load moves through the bloodstream to lymph nodes and other organs. The maxillary, cervical, and inguinal lymph nodes always enlarge when the immune system is first engaged, and this always occurs concurrently with the onset of fever. The contamination extends to the tonsils, mediastinum, and mandibular lymph nodes during MPX illness. In his studies of cynomolgus monkeys, he found that the most serious lesions were seen in the mandible and mediastinum; lymph nodes in the groin, mesentery, and armpits were also afflicted.

MPXV and liver

MPXV-positive people have viral antigens scattered throughout their livers, even though MPXV-related liver lesions are not very common. When MPX-infected ground squirrels examined their livers, they found steatosis, centrilobular necrosis, and basophilic inclusion bodies in the hepatocytes. However, when two researchers examined MPXV-positive monkeys, they discovered that the monkeys exhibited a range of flat, gross liver lesions that ranged in diameter from 2 to 5 mm. Some of them died from hepatitis, and others developed ulcers. Regardless, histologic evidence linking MPX to hepatitis was nonexistent. Occasionally, many degenerated Kupffer cells cause the sinusoids to become plugged with fibrin and cellular debris.

MPXV and gastrointestinal tract

Although MPXV-infected individuals often have modest gastrointestinal lesions, proximal fibroblasts and epithelial cells in the gastrointestinal system show varied growth. In the squamous region of the stomach, there were 24 mm foci of putrefaction, and the condition was white and somewhat elevated. The lesions were characterized by modest neutrophil and macrophage infiltration, fibroblast growth, and mucosal necrosis that developed into more severe lesions and lethal ulceration. Granulomatous-looking gross lesions were found in the gastrointestinal organs of both the ground squirrel and mouse MPXV models (Ramakrishnan et al., 2023).

Effect of high glucose level in T2D patients on severity of MPXV infection in Pakistan: Implication for public health

Globally, diabetes mellitus (DM) is a severe medical condition linked to both lifestyle choices and heredity (Sapra and Bhandari, 2022). Pakistan ranked amongst the top nations globally in terms of diabetes prevalence. According to the International Diabetes Federation Diabetes Atlas (2021), an estimated 33 million adults in

Pakistan were living with diabetes, accounting for 27.4% of the adult population. The disease is growing public health concern in the next few years because of rapid urbanization, unhealthy dietary habits, genetic predisposition, and sedentary lifestyles.The high prevalence is coupled with alarming rates of undiagnosed diabetes; approximately 25% of adults with diabetes in Pakistan remain unaware of their condition. MPX may lead to more severe symptoms and prolonged recovery in diabetic patients. Therefore, managing diabetes successfully and checking for infections is essential for reducing the risks of complications in individuals exposed to or infected with MPX.

The strategies and public health frameworks (including containment measures, early detection, and vaccination campaigns) applied during the COVID-19 pandemic in Pakistan can provide important insights into controlling monkeypox. For instance, the National Command and Operation Center which coordinated Pakistan's COVID-19 response, could similarly manage the response to monkeypox by ensuring effective communication, resource allocation, and swift decision-making.

The impact of MPXV on diabetes is not well recognized, that even though most viral infections, including the COVID-19 pandemic, are known to cause poor blood glucose control. According to reports, people with diabetes are more likely than healthy people to contract MPXV. Diabetic people are particularly vulnerable to the MPXV virus because of the effects of elevated glucose levels on DNA sensors (toll-like receptor 9), cyclic GMP-AMP synthase, melanoma 2 deficiency, and DNA-dependent protein kinase. According to several research, people with Type 2 diabetes are more likely to contract a virus (Abu-Ashour et al., 2018) due to weakened immune systems and potential complications from high blood sugar levels. Table 9 illustrates the impact of elevated blood glucose levels in individuals with type 2 diabetes on the intensity of their MPXV infection.

Table 5: Effects of high glucose levels in T2D patients on the severity of Monkeypox Virus (MPXV) infection

Parameter	**References**	**High Glucose Level in T2D Patients**	**Normal Glucose Level**	**Effect on MPXV Infection Severity**
Immune Response	Johnson et al., 2022	Impaired immune function	Normal immune function	Delayed or weakened immune response, leading to severe infection
Viral Replication	Gupta et al., 2021	Enhanced viral replication	Controlled replication	Increased viral load, worsening disease outcomes
Inflammatory Response	Zhang et al., 2022	Hyperinflammation (due to chronic condition)	Balanced inflammation	Heightened risk of complications like severe rash or organ damage
Wound Healing (Lesions)	Roberts and Wang, 2022	Delayed healing of the wounds	Normal wound healing	Prolonged presence of monkeypox lesions, increasing infection risk
Risk of Secondary Infections	Taylor et al., 2023	High	Low	Increased susceptibility to secondary bacterial or fungal infections
Hospitalization/ICU Admission	Brown et al., 2020	Higher risk	Lower risk	More likely to require hospitalization or intensive care
Duration of Infection	Ali et al., 2021	Prolonged duration of infection	Shorter recovery time	Extended recovery period with a higher risk of complications
Mortality Risk	Singh et al., 2023	Elevated mortality risk	Lower mortality	Increased likelihood of

			risk	fatal outcomes due to compromised health

T2D and Immune Dysfunction

Through inflammation and pattern recognition receptors, innate immunity defends the body against a range of diseases (Kawasaki et al., 2014). However, patients with uncontrolled blood sugar levels have significantly reduced immune component effectiveness, which results in delayed or impaired wound healing and increased susceptibility to infection. This immunological dysfunction results in altered production of TNF-alpha and IL-6 (Morohoshi et al., 1995) and insufficient control of infections (Chávez-Reyes et al., 2021). This could be a result of the binding of advanced glycation end products to proteins associated with IFN-gamma production (Imani et al., 1993). Diabetes macrophage patients exhibited decreased phagocytic activity.

T2D and antiviral mechanism

Uncontrolled blood sugar levels have been demonstrated to worsen viral infections (Marshall et al., 2020). such as CMV infection, influenza virus, human herpes virus 8, hepatitis B and hepatitis C viruses, SARS-CoV-2 infection, and herpes simplex virus (Lontchi-Yimagou et al., 2021). During the H1N1 infection, T cells lacking insulin receptors demonstrated reduced antigen-specific proliferation (Tsai et al., 2018).

In skeletal muscle, virus-induced interferon-gamma inhibits the insulin receptor (Sestan et al., 2018). According to Szturmowicz et al. (2021), COVID-19 in diabetic individuals exhibited a greater propensity to generate neutrophil extracellular traps, which could result in tissue injury. Peripheral blood mononuclear cells produced less interleukin-6, interleukin-10, and interleukin-2 and more transforming growth factor-β in response to elevated glucose concentrations (Reinhold et al., 1996). This implies that increased glucose levels can cause the synthesis of transforming growth factor-β, which can weaken immunity by interfering with the inflammatory response and ultimately resulting in infection. The relationship between T2D and antiviral systems is seen in Table 6.

Table 6: Relationship between T2D and antiviral mechanisms

Antiviral	References	T2D Impact	Normal	Effect on Viral

Mechanism			**Response**	**Infections**
Innate Immune Response	Watanabe et al., 2021	Delayed or suppressed IFN response	Rapid activation of interferons (IFNs) and immune cells	Increased susceptibility to viral infections due to weak immune defense
Cytokine Production	Lee et al., 2020	Dysregulated cytokine production (e.g., cytokine storm)	Balanced cytokine release	Exacerbation of viral infection severity due to hyperinflammation
Natural Killer (NK) Cell Function	Li et al., 2022	Impaired NK cell activity	Efficient virus-infected cell clearance	Reduced viral clearance, leading to prolonged infections
Adaptive Immune Response	Petrie et al., 2022	Impaired T-cell activation, lower antibody production	Effective T-cell and B-cell responses	Increased viral persistence and decreased ability to control infection
Glucose Uptake by Immune Cells	Chew et al., 2020	Hyperglycemia affects immune cell metabolism	Normal glucose uptake	Reduced immune cell function, compromising the antiviral defense
Viral Replication	Codo et al., 2020	Enhanced viral replication in high-glucose environments	Limited viral replication due to immune control	Higher viral load, prolonged infection duration, and greater severity
Antiviral Drug Efficacy	Kumar et al., 2021	Reduced effectiveness of certain antiviral drugs in T2D	Normal drug efficacy	Diminished response to antiviral treatment, requiring adjusted therapies

		patients		
Oxidative Stress	Menegazzo et al., 2018	Increased oxidative stress and cellular damage	Controlled oxidative stress response	Higher risk of viral-induced complications due to cellular damage

An altered toll-like receptor pathway may result from elevated glucose levels (Dasu et al., 2008). Diabetic mice's kidneys exhibit an up-regulation of the DNA sensor Toll-like receptor 9. and trigger the inflammasome that contains NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) (Shen et al., 2022). The development of IL-1b and IL-18 depends on the NLRP3 inflammasome. The immunological response is triggered when TLRs and NLRP3 identify viral components. In rats and humans with diabetes, NLRP3-dependent increases in IL-18 and IL-1β levels were observed (Wang et al., 2020). It has been demonstrated that elevated blood glucose levels suppress IRF-3 and MDA-5 expression (Kocic et al., 2010). Age and diabetes patients had greater levels of AIM2 expression, which was linked to an inflammatory response. However, it has been demonstrated that metformin reduces macrophage dysfunction in a way that is dependent on AIM2. Insulin resistance brought on by obesity and the chronic inflammatory response are linked to the cGAS-cGAMP-STING pathway (Bai et al., 2017).

CONCLUSION

It is essential to set up and implement a working agreement after the procedure, isolate individuals who have been exposed to or contaminated with MPXV and use postexposure vaccinations that may prevent the spread of the virus. Due of the COVID-19 epidemic, the world economy is currently facing challenges. The growth in MPX cases in the United States and around the world will instantly jeopardize anticipated financial progress. The financial problems will get more challenging if this MPX disease isn't stopped immediately.50 People have two distinct problems: social isolation and social shame. This situation has affected people for the last two years. Even the most affluent and educated members of the community still have a limited understanding of the disease's processes. People had disregarded quarantine orders and refused to be tested for the disease. In poorer countries, hand-washing habits are likewise dubious.

ETHICAL APPROVAL

No ethical approval is required

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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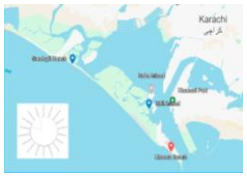

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Detection and Antimicrobial Resistance Profiling of Coliforms Isolated from Fresh Vegetables in Lahore, Pakistan

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ABSTRACT: *Vegetables are among the finest sources of high-quality nutrients including, carbohydrates, vitamins, fibers and minerals, having beneficial health effects. However consuming raw vegetables obtained from retailer shops renders them a reservoir of pathogenic bacteria due to unhygienic production, storage, and handling practices, and could be a major route of antibiotics resistance dissemination. The present work aimed at the evaluation of fresh vegetables for total microbial load and coliforms presence and determination antibiotic susceptibility of the isolated strains. The selective media were used for the targeted isolation of coliforms and antimicrobial resistance profiling of the isolates was performed using disc diffusion assay to determine the current resistance patterns of the bacterial isolates. Escherichia coli and Salmonella were the top isolated strains from the vegetable and salad samples. The antimicrobial resistance patterns showed an increased resistance to most of the tested antibiotics highlighting the critical issue of the dissemination of antibiotics resistance through common food items. The frequent presence of coliforms in the fresh vegetables and salads indicate suitable environmental conditions in the area for opportunistic pathogens and other harmful microorganisms to grow and dwell in the frequently consumed food items. The study highlighted the importance and need for proper cooking practices for vegetables before consumption and emphasizes the minimal use of partially cooked or uncooked vegetables*

Keywords: AMR dissemination; Antibiotics resistance; Susceptibility testing

INTRODUCTION

Vegetables such as cucumbers, spinach cabbage, mint and lettuce have been historically associated with health benefits such as positive effects on the health of diabetics and anemics etc. However contamination risks and unhealthy handlings of the vegetables may also make it a source of infection and hence may cause variety of health concerns. There is a chain of events such as production, transportation and making these vegetables to the table are prone to every possible of contamination if not taken care with properly, hence ensuring microbiological surveillance during the entire process is highly required for safe consumption (Henson and Reardon, 2005). In most of the Asian countries sewage or wastewater is added into the rivers with or without proper management in some cases. Farmers mostly rely on river water for the water demand of the crop and unstoppable flow of nutrients which is highly required to combat the ever-growing demand of food for the increasing population (Aminharati et al., 2019). This practice of using river water containing waste water is much more common in developing countries due to un availability of water treatment resources, hence rendering this supply much more risky for the potential pathogenic microbes which can certainly effect the quality of food. Variety of food borne pathogens including bacteria and viruses can be linked with this supply system and hence may lead to a food borne epidemic in a given area (Callejón et al., 2015). The Enterobateriaceae is a group of Gram-negative rod shaped bacteria (bacilli) most of which are the common commensals of humans and other animals gut also known as the normal microbiota of the gut. Members of this family are generally motile excluding *Shigella* and *Klebsiella*. Other properties of this group include the division of its members into two sub-groups such as those which can ferment lactose (lactose-fermenters) also known as coliforms and those which cannot (non-lactose fermenters) such as *Salmoenlla*, *Shigella*, *Yersinia* and *Proteus*. Coliforms can be aerobic or facultatively anaerobic and contains some of the famous foodborne pathogens such as *Enterobacter*, *Escherichia coli* (*E.coli*), *Citrobacter and Klebsiella*, all of these commonly

produce and ferment indole (Martin et al., 2016).

Many factors can contaminate the fresh produce of vegetables such as humans, soil and animals and the can contaminants can survive the processes of transportation and storage etc. "Indicator organisms" are those organisms which can reflect the general microbiological quality of the food. Coliforms are often referred as indicator organisms which can depict the general microbiological quality of a given water and food sample. They are often taken as a positive sewage or fecal contamination of any water or food sample hence highlighting the unhygienic and unsanitary processing in a given farm, field area or city (Jin et al., 2004). Recently there have been some serious findings regarding the level of detection of certain coliforms such as *Salmonella* in various food samples such as meat (Fatima et al., 2023; Tagar and Qambrani, 2023). Total coliforms are somehow abundantly present in the environment and its detection is normal however specifically the detection of *E.coli* is enough to ring the alarm bells as they are specifically associated with fecal contamination and hence can describe the poor and unhealthy microbiological quality of a given food and of vegetables in this case. Moreover the presence of *E.coli* can also be presented as an evidence of fecal contamination in routine microbiological procedures and there have been many instances of foodborne disease outbreaks involving *Salmonella* and *E.coli* after fresh vegetable produce consumption (Berger et al., 2010). Some mammals have *E.coli* other than the normal microbiota which can be diarrheagenic and hence can serve as a contaminant of fresh produce (Franz and Bruggen, 2008). Thus vegetables especially raw leafy vegetables can contain variety of containments such as *E.coli*, *Salmonella*, and other members of the family Enterobacteriaceae (Richter et al., 2019).

In this study, N= 40 samples of different fresh vegetables and salads such as cucumber, mint, coriander, cabbage, spinach, lettuce, fenugreek etc. were collected from various areas of Lahore. The samples were processed for selective isolation of coliforms followed by their, morphological and biochemical identification. The Antimicrobial resistance profiles of the isolates

was also determined by using antibiotics discs of different classes.

MATERIALS AND METHODS

Sample Collection and Microbiological Analy

The vegitable samples N=40 including coriander, cabbage, spinach, lettuce fenugreek cucumber, mint and salad were collected from various areas in Lahore and were subsequently processed from Jan 2022 to mid of April 2022. The samples were assessed for different microbiological aspects such as presence of coliforms, CFU/ml and detection of *E.coli* and *Salmonella* etc. the samples were purchased from different areas in Lahore city which included, Faisal town, Johar town, Barkat Market, Guru mangat road, Makkah colony, Muslim Town Block B, Sabzi Mandi, Kamran Block, Allama Iqbal Town, Multan Chungi, Najaf Colony Hunza block, Kashmir Block, Ghari Shahu and Zaraar Shaheed Road. These places were chosen as a part of random pool sampling and because these are some of highly populated areas of the city and hence present a better general picture of the situation. The samples were transported in air tight sterilized plastic bags and were processed with in 24 h after the transportation. The 25g of each sample was weighed which was then chopped and mixed in 225 ml of sterile peptone water (1:10). N-agar was chosen for the preliminary isolation of the bacteria as the sample mixtures were serially diluted up to 6 times and 20 µl of each dilution was spread onto N-agar plates followed by the overnight incubation at 37°C.

CFU/ml was calculated by the formula:

$$CFU/ml = (no.of\ colonies \times dilution\ factor) / volume\ of\ culture\ plate$$

Selective Isolation, Biochemical Characterization and Identification of the Isolates

Initial isolation resulted in rich primary culture having diverse microbial growth on N-agar plate. The morphologically different colonies were picked and streaked onto various selective media such as MacConkey and EMB agar followed by overnight incubation at 37°C. Following the incubation, various characteristics of colonies were taken into consideration such as color, margins, elevation, shape and size etc. Gram-staining

procedure was done for every isolate followed by biochemical characterization that included, Oxidase test, Methyl Red test, MRVP test, Indole test, Catalase test, Original VP test, Barrett's method, Citrate Utilization test, Motility test, Triple Sugar Iron test and Urease test.

Antibiotics Susceptibility Testing (AST) of the Selected Isolates

After initial identification of the selected isolates, isolates of interests were spread plated onto Muller-Hinton agar. The AST was measured using famous disc diffusion method developed by Kirby-Bauer. Discs already impregnated with antibiotics were applied onto the plates containing lawn of isolates of interests and were incubated overnight at 37 °C. Zone of inhibitions (ZOIs) were measured in accordance with CLSI standards in which all plates were incubated at 37C for 18 to 24 hrs. Results were recorded by measuring the diameter of zones of inhibition (ZOIs) in millimeters and activity was interpreted as resistant, sensitive or intermediate according to CLSI 2020 criteria for each antibiotic class used in this assay.

RESULTS AND DISCUSSION

CFU/mL Count and Colony Morphology

Cabbage and Spinach produced higher number of CFU/mL. CFU/ml for each vegetable sample was reported with Cabbage having the highest average CFU/ml of 8.51e8 following spinach, cucumber, mint, coriander and lettuce, respectively. The isolates were assessed for variety of colony features such as, elevation, texture and other features on respective selective media, such as McConkey agar and details of which are given in the table 1 and 2.

Table 1. Colony characteristics of isolates on MacConkey agar

Isolates	Shape	Margin	Texture	Color	Elevation	Size
Sa2	Circular-c	Entire-e	Smooth-s	Purple	Raised-r	Small-s
C6	Circular-c	Entire-e	Mucoid-m	Dark purple	Raised-r	Small-s
CU1	Circular-c	Entire-e	Smooth-s	Purple /dark spots	Raised-r	Small-s
CU3	Circular-c	Entire-e	Slimy	Purple /dark center	Raised-r	Small-s
M8	Circular-c	Entire-e	Slimy	Purple /green	Raised-r	Small-s

				sheen		
S4	Circular-c	Entire-e	Smooth-s	Purple /green sheen	Raised-r	Small-s
S3	Circular-c	Entire-e	Smooth-s	Yellow	Raised-r	Small-s
S1	Circular-c	Entire-e	Mucoid-m	Light purple	Raised-r	Small-s
D4E	Circular-c	Entire-e	Smooth-s	Purple	Raised-r	Small-s
Sa3	Circular-c	Entire-e	Smooth-s	Dark purple	Raised-r	Small-s
Sa1	Circular-c	Entire-e	Smooth-s	Purple	Raised-r	Pinpointed
CO13	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
D4	Circular-c	Entire-e	Smooth-s	Purple	Raised-r	Small-s
C7	Circular-c	Entire-e	Smooth-s	Purple	Raised-r	Small-s
S2	Circular-c	Entire-e	Mucoid-m	Purple	Raised-r	Small-s
L13	Circular-c	Entire-e	Smooth-s	Purple	Raised-r	Small-s
CO11	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
CO14	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
M31	Circular-c	Entire-e	Slimy	Purple	Raised-r	Small-s
M40	Circular-c	Entire-e	Smooth-s	Purple	Raised-r	Pinpointed
M2	Circular-c	Entire-e	Smooth-s	purple	Raised-r	Small-s
C1F4	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
M22	Circular-c	Entire-e	Smooth-s	Dark purple	Raised-r	Small-s
L1	Circular-c	Entire-e	Smooth-s	Light purple	Raised-r	Pinpointed
L3	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
CO22	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Pinpointed
L4	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Pinpointed
C1F1	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
CIF2	Circular-c	Entire-e	Smooth-s	Light Pink-p	Raised-r	Small-s
C1F3	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
CO21	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
CH13	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s

FG2	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
SP2	Circular-c	Entire-e	Smooth-s	colorless	Raised-r	Pinpointed
CL13	Circular-c	Entire-e	Smooth-s	Colorless	Raised-r	Small-s
SP1	Circular-c	Entire-e	Smooth-s	Light purple	Raised-r	Small-s

Table 2. Morphological details of the isolates on EMB Agar

Isolates	**Elevation**	**Color**	**Margin**	**Texture**	**Shape**	**Size**
S4	Raised-r	Pink-p	Entire-e	Smooth-s	Circular-c	Small-s
C6	Raised-r	Pink-p	Entire-e	Mucoid-m	Circular-c	Small-s
CU1	Raised-r	Pink-p	Entire-e	Smooth-s	Circular-c	Small-s
CU3	Raised-r	Pink-p	Entire-e	Smooth-s	Circular-c	Small-s
M8	Raised-r	Pink-p	Entire-e	Smooth-s	Circular-c	Pinpointed
S3	Raised-r	Reddish	Entire-e	Smooth-s	Circular-c	Small-s
S2	Raised-r	Pink-p	Entire-e	Mucoid-m	Circular-c	Small-s
D4E	Raised-r	Pink-p	Entire-e	Smooth-s	Circular-c	Small-s
S1	Raised-r	Pink-p	Entire-e	Slimy	Circular-c	Small-s
Sa3	Raised-r	Pink-p	Entire-e	Mucoid-m	Circular-c	Small-s
Sa2	Raised-r	Light Pink-p	Entire-e	Smooth-s	Circular-c	Small-s
Sa1	Raised-r	Light Pink-p	Entire-e	Smooth-s	Circular-c	Pinpointed
C7	Raised-r	Pink-p	Entire-e	Slimy	Circular-c	Small-s
L1	Raised-r	Colorless	Entire-e	Smooth-s	Circular-c	Pinpointed
M31	Raised-r	Pink-p	Entire-e	Slimy	Circular-c	Small-s
M22	Raised-r	Light Pink-p	Entire-e	Smooth-s	Circular-c	Small-s
CO11	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Small-s
CO13	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Small-s
D4	Raised-r	Pink-p	Entire-e	Smooth-s	Circular-c	Pinpointed
CO14	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Small-s
M40	Raised-r	Pink-p	Entire-e	Smooth-s	Circular-c	Small-s

L13	Raised-r	Pink-p	Entire-e	Smooth-s	Circular-c	Small-s
M2	Raised-r	Colorless	Entire-e	Smooth-s	Circular-c	Pinpointed
L3	Raised-r	Light Pink-p	Entire-e	Slimy	Circular-c	Pinpointed
CO21	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Small-s
L4	Raised-r	Light Pink-p	Entire-e	Slimy	Circular-c	Pinpointed
C1F1	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Small-s
C1F3	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Small-s
C1F4	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Small-s
CIF2	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Pinpointed
CO22	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Small-s
FG2	Raised-r	Yellow	Entire-e	Mucoid-m	Circular-c	Small-s
SP1	Raised-r	Yellow	Entire-e	Smooth-s	Circular-c	Pinpointed
SP2	Raised-r	Light Pink-p	Entire-e	Smooth-s	Circular-c	Small-s
CL13	Raised-r	Colorless	Entire-e	Mucoid-m	Circular-c	Small-s
CH13	Raised-r	Colorless	Entire-e	Mucoid-m	Circular-c	Small-s

Selective colonies were picked from N-agar and streaked on MacConkey agar. This agar is a selective and differential media at the same time as it can not only inhibit the growth of gram positive bacteria but can also differentiate between coliforms and non-coliforms, with the former give a pink color and the latter being colorless.

Colonies N-agar were streaked into EMB agar plates which serves not only as a selective media but also as a differential media between lactose fermenters and non-fermenter with lactose fermenters forming Pink-p colonies while non-lactose fermenters being colorless colonies due to increasing pH in light of deamination of proteins and Table 3.

Table 3: Biochemical Charaterstics of the Isolated Strains

Strains	Citrate	Indole	Urease	Oxidase	Catalase	VP	MR	TSI	SIM	Identified strain
CU1	+	-	-	-	+	-	+	A/A and H2S (+)	+	*Citrobacter*
C7	-	+	-	-	+	-	+	A/A and H2S -	+	*E.coli*
C6	+	-	+	-	+	+	-	A/A and H2S -	-	*Klebsiella*
S4	-	+	-	-	+	-	+	A/A and H2S -	+	*E.coli*
S3	+	-	-	-	+	-	+	A/A and H2S -	+	*Enterobacter*
Sa3	+	-	-	-	+	+	-	A/A and H2S -	+	*Enterobacter*
CU3	-	+	-	-	+	-	+	A/A and H2S (+)	+	*E.coli*
CO14	+	-	-	+	+	+	-	K/K and H2S +	+	*Pseudomonas*
S2	+	-	-	-	+	-	+	A/A and H2S -	+	*Enterobacter*
S1	+	-	-	-	+	-	+	K/A and H2S (-)	+	*Salmonella*
Sa2	+	-	-	-	+	-	+	A/A and H2S (+)	+	*Citrobacter*
CO11	+	-	+	-	+	-	+	K/A and H2S +	+	*Proteus*
M8	-	+	-	-	+	-	+	A/A and H2S -	+	*E.coli*
Sa1	+	-	-	-	+	+	-	A/A and H2S -	+	*Enterobacter*
M22	+	-	-	-	+	+	-	A/A and H2S -	+	*Enterobacter*
D4	-	+	-	-	+	-	+	A/A and H2S -	+	*E.coli*
D4E	-	+	-	-	+	-	+	A/A and H2S -	+	*E.coli*
M31	+	-	-	-	+	-	+	A/A and H2S (+)	+	*Citrobacter*
CO13	+	-	-	-	+	-	+	K/A and H2S +	+	*Salmonella*
L13	-	+	-	-	+	-	+	A/A and H2S -	+	*E.coli*
M40	-	+	-	-	+	-	+	A/A and H2S -	+	*E.coli*
L3	+	-	-	-	+	-	+	A/A and H2S (+)	+	*Salmonella*
L4	+	-	-	-	+	-	+	K/A and H2S (-)	-	*Shigella*
FG2	+	-	+	-	+	-	+	K/A and H2S +	+	*Proteus*
C1F1	+	-	-	-	+	-	+	K/A and H2S +	+	*Salmonella*
CH13	+	-	-	-	+	-	+	A/A and H2S (+)	+	*Citrobacter*
L1	+	-	-	-	+	-	+	K/A and H2S +	+	*Salmonella*
CIF2	+	-	-	-	+	-	+	K/A and H2S +	+	*Salmonella*

C1F4	+	-	-	+	+	-	-	K/K and H2S +	+	*Pseudomonas*
CO21	+	-	- a	+	+	-	-	K/K and H2S +	+	*Pseudomonas*
M2	+	-	+	-	+	-	+	K/A and H2S +	+	*Proteus*
CO22	+	-	+	-	+	-	+	K/A and H2S +	+	*Proteus*
C1F3	+	-	-	-	+	-	+	A/A and H2S -	-	*Shigella*
SP1	+	-	-	+	+	-	-	K/K and H2S -	+	*Pseudomonas*
SP2	+	-	-	-	+	-	+	K/K and H2S -	-	*Shigella*
CL13	+	-	-	-	+	-	+	A/A and H2S (+)	+	*Citrobacter*

Gram staining results are confirming typical Gram-negative pattern.

Biochemical Identification

Different biochemical tests characterized each strain on the basis of results obtained. A N= 36 strains were characterized on the basis of morphological and biochemical identifications. All biochemical tests were performed within 24 h using fresh cultures on nutrient agar plates. Overall biochemical results are shown in the following table. The isolates C7, CU3, M8, S4, S3 D4, D4E, L13 and M40 were confirmed as *E.coli*. The isolate S3, S2, Sa3, Sa1 and M22 were detected as *Enterobacter*. Similarly isolates S1, L1, L3, C1F1 and CIF2 were confirmed as *Salmonella*. The results depict a typical coliform biochemistry hence morphological identification of the strains followed by the growth on selective media were reconfirmed by the biochemical identification scheme (Table 3). In total 19.4% isolates belonged to *Salmonella* and *E.coli* respectively followed by *Citrobacter* and *Enterobacter* with 11.3 % respectively (Fig. 1).

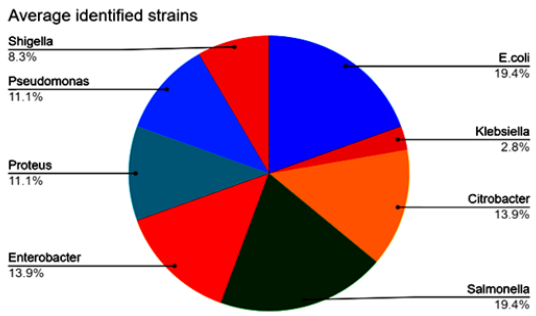

Fig. 1. Percentage of isolated strains from sample

Antibiotic Sensitivity Pattern of the Recovered Isolates

The AST shows that, among all the antibiotics applied, Cefazolin, Cefepime, Polymyxin B and Erythromycin were among the least active against the isolates showing total resistance of 97%, 100%, 97% and 100 % respectively. Alternatively it also shows the recovered isolates are

highly resistant against these antibiotics which could pose a major concern for the respective authorities dealing with food with food hygiene such as Punjab food authority. Rest of the details are given in the following table 4.

Table 4. AST results of the identified strains

Antibiotics	Intermediate	Sensitive	Resistant	Total	Resistance %
Ampicillin	6	5	25	36	69.40
Cefazolin	1	0	35	36	97.20
Streptomycin	15	14	7	36	19.40
Cefepime	0	0	36	36	100
Ceftriaxone	6	5	25	36	69.40
Imipenem	6	29	1	36	2.77
Polymyxin B	0	0	36	36	100
Ciprofloxacin	10	20	6	36	16.60
Levofloxacin	0	34	2	36	5.55
Chloramphenicol	10	15	11	36	30.50
Gentamicin	2	33	1	36	2.77
Tetracycline	0	11	25	36	69.40
Tobramycin	6	27	3	36	8.33
Amikacin	2	34	0	36	0
Azithromycin	0	28	8	36	22.20
Erythromycin	1	2	33	36	91.60

There is always a chance of disease transmission with the contaminated irrigation water as it can harbors a variety of pathogens with serious health implications. The level of contamination is linked to the depth of the water as ground water can act as a good irrigation source, in contrary surface water has poor microbiological quality and is generally not fit for the irrigation purpose (Ungureanu et al., 2020). However among all, the human waste water can be of the poorest quality and it should always be treated before it can be used for the irrigation processes (Khalid et

al., 2018). Different studies have revealed that Salmonella and E.coli etc. can easily colonize plants and may reach to the sub-stomatal cavities (Cooley et al., 2003; Deering et al., 2012; Nicholson et al., 2015). This research was aimed to highlight the potential contamination of fresh vegetables produce due to contaminated water and overall handling and storage process. N= 40 samples were assessed for their microbiological load and coliforms were targeted specifically along with the other members of Enterobateriaceae family. The polyphasic taxonomic approached applied in the identification contained growth on selective media followed by biochemical identification which showed the presence of coliforms in these vegetables at alarming level. In Nigeria and other developing countries the use of untreated wastewater used for irrigation has resulted in several outbreaks previously (Grivokostopoulos et al., 2022; Du Plessis et al., 2015; Chigor et al., 2020). The contamination rates of different vegetables were almost similar and *E.coli* was found to be present in higher numbers suggesting fecal contamination of the irrigated water. Factors such as improper handling and storage may also account for this *E.coli* presence. The use of antibiotics in animal husbandry and agriculture has resulted in the evolution of multi drug and extensively drug resistant pathogens which have now become a global concern as these pathogens are unstoppable with the majority of the current lot of antibiotics. In this study the most common coliform was found to be *E. coli* which is a major concern as its CFU/ml count was found to be 8.51 E+08. The higher resistance rates towards notable antibiotics is a grave concern as these pathogens can be a source of foodborne epidemic with some of the isolates showing 100% resistance. A study from US showed that the risk of contamination in fresh produce increases with the gradual steps followed from harvesting to storage and supply (Possas et al., 2023; Alegbeleye et al., 2018).

Many factors can contribute in overall cross contamination of the fresh produce and every factor itself can be a sole reason or a contributing factor in the entire scenario. Insufficient knowledge regrading handling, storage and supply can also be accountable for

the growing contamination reports of vegetables (El-Ramady et al., 2015; Gil et al., 2015). Another common practice among the vendors is the continuous sprinkling of water over vegetables to maintain its freshness. Poor microbiological quality of the sprinkled water can also contribute to the contamination of vegetables (Salamandane et al., 2020).

There are various reports that support the fact that the incidences of antimicrobial resistance in the food samples are on the rise and different studies have recently reported the detection of resistant pathogens from fruits (Olanbiwoninu et al., 2024; Brunn et al., 2022; Lima et al., 2019), vegetables (Kgoale et al., 2024; Jia et al., 2024; Patra et al., 2024), dairy products (Pires et al., 2024; Khalid et al., 2023) and meat (Algammal et al., 2024; Nastasijevic et al., 2024; Tenea et al., 2023; Martino et al., 2024). Contamination of raw produce with microorganisms have been reported previously in many aspects but detection of coliforms in fresh produce form the second most populated city of Pakistan should be a concern and this study highlights important aspects of vegetables handling, harvesting and storage. Each step in its own way can contribute to the contamination of the fresh produce but mainly the contaminated irrigation water which contain sewage mixing is the most crucial quality check in this regard. To achieve satisfactory levels of less microbial load as specified by FDA in produce, regular monitoring at various levels is important.

CONCLUSION

Consuming raw vegetables is a public health concern due to their unhygienic production practices and further contamination throughout the processing until brought to the tables. A proper surveillance system is essential to ensure food cleanliness, prevent cross-contamination, and manage water pollution for safe agricultural use. Since irrigation sources greatly impact crop sanitation, water treatment is vital for crop production. Regular check on vendors, determination of random microbiological quality of fresh produce and proper cooking practices are some of the highly required steps to stop dessimination of anibiotics resistance through this route

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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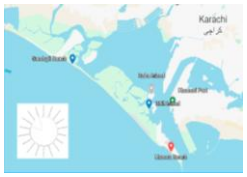

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Performance of Various Wheat (*Triticum aestivum* L.) Cultivars under Salinity Stress

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ABSTRACT: *Salinity is an abiotic stress limiting the physiological mechanisms, growth and yield in wheat. The aim of this research was to evaluate various wheat verities under different salinity stresses. The data was collected for the study using a CRD design with three replications. For the pot experiment, five different wheat types were chosen: Sarsabz, Kiran-95, T.D.-1, T.J.-83, and Moomal to find out the tolerant cultivar under four different salinity (NaCl) levels viz; 0, 4, 8, and 12 dSm^{-1}.The results further suggested that cultivar Sarsabz shows higher levels of proline (12.12 µmol g^{-1}) and glycine betaine (14.18 µmol g^{-1})when compared to the control. Furthermore, at 12 dSm^{-1}, the K^+/Na^+ ratio was higher in Sarsabz, Moomal, and Kiran-95, indicating that these cultivars were more salinity-tolerant than the others. The T.J.-83 and Kiran-95 cultivars demonstrated enhanced yield at the maximum salinity threshold of 12 dSm^{-1}. Findings from the interaction between Moomal and control (non-treated) led to the highest 1000grain weight (52.1g). The Sarsabz, Moomal, and T.J.-83 strains showed tolerance by having higher amounts of proline, glycine-betaine and cell membrane stability. Such concentration of proline, glycine-betaine and sufficient concentrations of K^+ over Na^+ in the cytoplasm can lead the plants for tolerance.*

Keywords: Cultivar; Physiological traits; Salinity; Wheat; Yield

INTRODUCTION

Modern agriculture is facing enormous challenges from a growing population and an increasing need for food. Worldwide food needs are expected to increase by at least 38% by 2025 and by as much as 50% by 2050 in response to the nutritional demands of a growing global population. Cultivating the best land available worldwide alone is not enough to meet the ever-growing demand for food. Research shows that due to the increase in tropical temperatures, causing salinity stress finally creating food shortage. It is difficult to develop practical methods to increase agricultural productivity without a thorough understanding of how plants react to abiotic stressors (Lyuben et al., 2014). Abiotic stressors cause low crop yields in modern agriculture, which presents serious issues (Kosova et al., 2013; Kausar and Gull, 2014). Many plants commonly build up proline during osmotic control in response to salinity toxicity, acting as a defense against salt damage (Wang et al., 2007). Accumulation of prolinein plants and serves as osmoregulation and an important tool for selection of salinity tolerant cultivar (Haroun, 2002; Ueda et al., 2007). Solutes like proline, glycine, betaine, and polyols build up in the cytoplasm, which helps keep the vacuole's water potential and balance Dos Reis et al., (2012). Cui et al. (2003) found that proline is essential for maintaining protein stability at the cellular and membrane levels, especially in situations where osmotic stress is elevated. Different species prefer high potassium and low sodium ratios, suggesting that the competition between potassium and sodium for binding sites leads to sodium toxicity. A greater K^+/Na^+ ratio indicates reduced sodium toxicity. Several writers (Gadallah, 1999; Haroun, 2002) have documented a reduction in the K^+/Na^+ratio under salt stress. Increased Na+ concentration decreased K^+ concentration, and decreased K^+/Na^+ratio in wheat grain are the outcomes of elevated salinity at 15 dSm^{-1}. According to Abbas et al. (2013), elevated salinity also affects other yield components such as the number of tillers per plant and grain weight per plant. There is a positive relationship between wheat grain yield and K^+, proline, and total soluble salts (TSS). On the other hand, there is a negative relationship between physiological characteristics and Na^+ concentrations when the soil is salty. According to Mehboob et al. (2017) high salinity specifically at 120 mMNaCl reduces ion buildup, overall growth and average grain production. Hussain et al. (2015) and Rehman et al. (2016) suggested that salt stress reduces

mineral nutrients, grain production, and gas exchange activities in wheat. Furthermore, Asgari et al. (2012) and Akram et al. (2002) documented a decrease in the number of spikelets per spike, the number of grains per spikelet and the 1000 grain weight per plant due to salinity. This research was conducted to examine suitable wheat cultivars under various salinity stresses.

METHODOLOGY

The seeds of high yielding wheat varieties were grown under the wire house conditions as well as four 0, 4, 8, and 12 dS/m were maintained by using (NaCl) salt. The experiment was Completely Randomized Design (CRD) arranged in three replications. Five wheat varieties viz; Kiran-95, TD-1, TJ-83, Moomal, and Sarsabz were examined.

Studied Parameters

Proline (µmol g^{-1})

One-gram fresh leaf samples were homogenized in 100 ml 3% sulfosalicylic acid and filtered. Filtrate 2ml separated, 2 ml ninhydrin solution and 2ml glaicial acetic acid added. Heated on water bath at 100°C and cool. Toluene 4ml added and placed in vertex for 30 seconds and readings noted at 520 nm on spectrophotometer by using the method of Bates et al. (1973).

Glycine-betaine (µmol g^{-1}): 0.01 g fresh leaves homogenized with 2N sulfuric acid and centrifuged at 12000×g (25°C) for 25 minutes. The residue crystals diluted in acetone and absorbance noted on spectrophotometer at 365 nmGrieve and Gratan (1983).

Cell Membrane Stability (%): Chopped leaf samples placed in distilled water at room temp for 24 hours. Electrical conductivity (EC) was noted by using EC meter (WTW, LA-530). Then placed in water bath for 15 minutes at 100°C, allowed to cool at room temperature and again EC was recorded by using EC meter.

Grain Yield [1000 grain weight (g)]:

Manually threshed main spike of wheat to take 1000 grains and then weighed in grams (g) using electronic balance.

Potassium and Sodium(mg 100 g^{-1}): Fresh ground leaf shoots were treated with 0.2 mm acetic acid (CH_3 COOH) in a water bath for 1 hour per-heated at 95ºC. The extracted solution was filtered to make suitable for dilution Na^+ and K^+ and concentrations were checked on flame photometer (Jennay, Model PFP7) by using the method of Flowers and Yeo (1986).

STATISTICAL ANALYSIS

The experiment was conducted in complete randomization design (CRD) and repeated thrice. Treatments means were compared by Least Significant Difference (LSD) test at 0.05 probability level (Steel and Torrie 1984).

RESULTS AND DISCUSSION

Proline Content (µmol g^{-1}) in Leaves of Wheat under Various Salinity Stresses.

The maximum amount of proline (14.74) in wheat leaves was determined at high salinity stage12 dSm^{-1}followed by (8.66) and (6.31) at 8 and 4 dSm^{-1} and the lower proline (5.09 µmol g^{-1}) was observed on control, respectively. The mean of varietal performance was significant, the more proline content (12.12) in variety Sarsabz followed by (10.60) in Moomal and the lowest proline (6.81 and 6.69 **µ**mol g^{-1}) values were recorded in TD-1 and Kiran-95 cultivars, although the variety T.J-83 also exhibited significant response. The interactive results indicated that the higher concentration of proline (22.87) was recorded at interaction of Sarsabz x 12 dSm^{-1} whereas the lower concentration of proline (4.95 **µ**mol g^{-1}) was observed in same variety Sarsabz x control (non-treated), respectively. This is achieved by minimizing the concentrated of salt management rise inside of solute recently acknowledge as survival strategy to salts stress in various crops (Khan et al., 2010; Ashraf and Sarwar, 2002) and proline is a major constituent in salinity tolerance mechanism (Qasim et al., 2003). The proline ratio is varied depending upon the varieties. In stress circumstances proline is a common organic osmolyte (Khan et al., 2006). Highest accumulation of proline in Sarsabz, Moomal and T.J-83 under salinity stress resembling with the observations have been recorded by (Qasim et al., 2003), an increase in proline under salinity stress in *Pancratiumm aritimum* L. Similarly (Ashraf and O'Leary, 2004) also reported an increase in proline accumulation in various grass species under salinity stresses.

Table 1: Proline Content (µmol g^{-1}) in Leaves of Wheat under Various Salinity Stresses

Salinity levels (dSm^{-1})	Varieties					Mean
	T.D-1	T.J-83	Moomal	Sarsabz	Kiran-95	
Control	5.07 kl	5.20 jkl	5.17 jkl	4.95 l	5.08 kl	5.09 d
4	5.61 i-l	5.91 h-k	6.57 gh	7.83 f	5.65 i-l	6.31 c
8	6.25 ghi	7.00 fg	11.16 d	12.83 d	6.05 hij	8.66 b
12	10.30de	11.07 d	19.49 b	22.87 a	9.98 e	14.74 a
Mean	6.81 d	7.29 c	10.60 b	12.12 a	6.69 d	
	Salinity		**Varieties**		**Salinity × Varieties**	
SE =	**0.10**		**0.12**		**0.24**	
LSD 5% =	**0.22**		**0.24**		**0.29**	

Glycine-betaine (µmolg^{-1}) in Leaves of Wheat under Various Salinity Stresses

The various concentrations of Glycine-betaine were obtained as highest**,** moderate and lowest (22.86, 10.75, 7.22 and 6.14 µmol g^{-1}) when plants were treated with 12, 8,4 and control treatments, respectively. Significantly higher glycine-betaine content (14.18) in the cultivar of Sarsabz, Secondly, Moomal cultivar shows superior amount of glycine-betaine(12.19), thirdly, T.D-1 possess greater assets of glycine-betaine (11.25µmol g^{-1}). While the lowest glycine-betaine contents 10.55 and 10.54 recorded in TJ-83 and Kiran-95 cultivars, respectively. The interaction outcome indicated that under control conditions the lowest glycine-betaine(5.64 µmol g^{-1}) was noted in Kiran-95 cultivar. Although, on 12 dSm^{-1} the Sarsabz cultivar obtained highest glycine-betaine **(**28.58 µmol g^{-1}**)**. Sarsabz and Moomal varieties showed higher glycine-betaine content than the control, T.D-1, T.J-83, and Kiran-95 cultivars at the highest salinity level of 12dSm^{-1}. According to Sairam et al. (2002), the amount of glycine-betaine was much lower in the varieties that were vulnerable, while the amounts were higher in the tolerant varieties in both the saline and control conditions. The response of both Sarsabz and Moomal cultivars was observed to have a higher increase in glycine-betaine accumulation, thereby making them tolerant cultivars. There are dissimilarities in accumulation of glycine-betainein various wheat cultivars when screened on 100 mmol L^{-1}NaCl salt stress. However, salinity tolerant wheat

cultivar showed superior glycine-betaine content (Khan et al. 2012).

Table 2: Glycine-betaine (µmolg^{-1}) in Leaves of Wheat under Various Salinity Stresses

Salinity levels (dSm^{-1})	Varieties					Mean
	T.D-1	T.J-83	Moomal	Sarsabz	Kiran-95	
Control	6.95 ghi	5.74 i	5.96 i	6.42 hi	5.64 i	6.14 d
4	8.16 g	6.48 hi	6.81 ghi	7.91 gh	6.73 ghi	7.22 c
8	11.48 f	7.83 gh	10.00 f	13.81 e	10.63 f	10.75 b
12	18.40 d	22.17 c	26.00 b	28.58 a	19.16 d	22.86 a
Mean	11.25 c	10.55 d	12.19 b	14.18 a	10.54 d	
SE =	**0.17**	**0.19**	**0.39**			
LSD 5% =	**0.36**	**0.40**	**0.80**			

Cell Membrane Stability (%) in Leaves of Wheat under Various Salinity Stresses

According to the results of cell membrane stability of different wheat varieties under different salinity levels. The lowest and highest cell membrane stability (0.57, 0.570and 32.46 %) recorded on the control 4 and 12 dSm^{-1} treatments, while on the treatment of 8dSm^{-1}the level of cell membrane stability was (4.18%). Accordingly, the T.J.-83 variety had the highest cell membrane stability of 11.80 %, followed by the Kiran-95 variety with 10.35%. The mean values for the various varieties showed a significant response. The Sarsabz variety had the lowest stability (6.16%). Conferring to the results of interaction, T.J-83 and 12 dSm^{-1} had the highest cell membrane stability (40.00%), while the interaction between Sarsabz and 4 dSm^{-1} was the lowest (0.540%). Furthermore, how stable the cell membranes are in low-quality water under 12 dSm^{-1} considering the various wheat cultivars viz; T.D-1 (83%, 194%, and 238%), TJ-83 (35%, 112%, and 135%), Moomal (127%, 173%, and 220%), Sarsabz (31%, 100%, and 156%), and Kiran-95 (68%, 121%, and 163%). Variations Kiran-95 and T.D-1 were deemed significantly susceptible up to 12 dSm^{-1} compared to the control, while variations Sarsabz, Sarsabz and Moomal showed tolerance. Similar results were found by Shafqat and Azam (2006), with the greatest injury percentage

(74.2%) at 25 dSm^{-1}, possibly due to the combined effects of Na^+ toxicity and cellular injury mediated by Na^+ and K^+ absorption. Ashraf et al. (2005) claim that high salt concentrations affect membrane stability by generating an improper ionic cell balance. Cell membrane vitality, a reliable standard assessment, has determined the degree of salt tolerance (Meloni et al., 2003; Sairam et al., 2005). According to Umrani et al. (2013), the wheat varieties T.J.-83 had better recovery of cell membrane stability and lower conductivity than the wheat varieties Mehran-89, Abadgar-93, SKD-1, Imdad-2005 and Anmol-91.

Table 3: Cell Membrane Stability (%) in Leaves of Wheat under Various Salinity Stresses

Salinity levels (dSm^{-1})	Varieties					Mean
	T.D-1	T.J-83	Moomal	Sarsabz	Kiran-95	
Control	**0.583 g**	**0.617 g**	**0.567 g**	**0.540 g**	**0.543 g**	**0.570 c**
4	0.583 g	0.617 g	0.567 g	0.540 g	0.543 g	0.570 c
8	4.333 ef	6.000 e	4.000 ef	2.583 fg	4.000 ef	4.183 b
12	35.000 b	40.000 a	30.000 c	21.000 d	36.333 b	32.467 a
Mean	10.125 bc	11.808 a	8.783 c	6.166 d	10.355 b	
SE =	**0.62**		**0.70**		**1.40**	
LSD 5%=	**1.27**		**1.42**		**2.84**	

K^+/Na^+(mg 100 g^{-1}) in Wheat under Various Salinity Stresses

The table no. 4 summarized the maximum level of K^+/Na^+ ratio (106.40mg 100 g^{-1}**)** obtained in the wheat straw when pots were watered with 12 dSm^{-1} higher concentration of NaCl salt, followed by (76.00mg 100 g^{-1}**)** on the treatment of 8 dSm^{-1}, and the lowest K^+/Na^+(61.40mg 100 g^{-1}**)** noted in the control treatment. The Moomal variety and Sarsabz cultivars possessed superior highest K^+/Na^+ratio (97.91 and 97.50 mg 100 g^{-1}**)** in straw, respectively. The T.D-1 variety listed the lowest K^+/Na^+ ratio 55.75 in straw $plant^{-1}$, whereas the Moomal and Sarsabz cultivars exhibited the ratio of 74.25. Furthermore, T.J-83 variety also exhibited a notable response. The interaction between the Moomal

variety and 12 dSm^{-1} produced the highest K^+/Na^+ratio (127.67 mg 100 g^{-1}**)** in wheat straw, while the considering the collaboration of T.D-1 cultivar and control (non-treated) having lowest K^+/Na^+ratio (39.00mg 100 g^{-1}**)**. In low water quality the wheat plants obtained (106.40 mg 100 g^{-1}**)** of K^+/Na^+ when electrical conductivity (EC) was 12 dSm^{-1}. When compared to the control, the Moomal and Sarsabz cultivars performed better, but the T.J.-83, Kiran-95and T.D.-1 cultivars showed poor response at 12 dSm^{-1}. Different wheat cultivars showed a progressive decrease in K^+ with increasing saline levels as reported by El-Bassiouny and Bekheta (2001) and Saleh et al. (2015). Similarly, the lowest K^+/Na^+ ratio was recorded in the wheat varieties of Faisalabad and Anaj-17 (0.67 and 0.50) under 10 dS/m NaCl. While the highestK^+/Na^+ratio in leaves was found in Galaxy-13 under treatment of 15 dS/m NaCl suggested by Iqra et al. (2020).

Table 4: K^+/Na^+(mg 100 g^{-1}) in Wheat under Various Salinity Stresses

Salinity levels (dSm^{-1})	Varieties					Mean
	T.D-1	T.J-83	Moomal	Sarsabz	Kiran-95	
Control	39.00 cd	41.00 c	85.00 abc	86.00abc	56.00 b	61.40 d
4	46.00 bc	50.00 bc	88.00 abc	91.00 ab	59.00 b	66.80 c
8	61.00 b	60.00 b	91.00 ab	97.00 ab	71.00 abc	76.00 b
12	77.00 abc	100.33 ab	127.67 a	116.00 a	111.00 a	106.40 a
Mean	55.75 d	62.83 c	97.91 a	97.50 a	74.25 b	
SE =	**0.68**	**0.76**		**1.52**		
LSD 5%=	**1.21**	**1.42**		**2.85**		

Effect of Salinity on Grain Yield [1000 grain weight (g)] in Various Wheat Cultivars

Result concerning grain yield depicted that highest 1000 grain weight of various cultivars was **(**45.10 g) noted on control treatment, followed by (40.27 g) on treatment 4 dSm^{-1} and the lowest 1000 grain weight **(**25.44 g) weighed on high salinity of 12 dSm^{-1}. In terms of cultivar performance, the Sarsabz cultivar showed the highest grain yield (39.03 g), followed by the T.J-83 (38.70 g) and Moomal cultivars

(38.44 g). The mean values for the various varieties showed a significant response. The variety Kiran-95 has the minimum grain yield (32.90 g). Furthermore, the results demonstrated that the T.D-1 cultivar exhibited a reasonable performance. The interaction between cultivars x treatments suggested that the cultivar Kiran-95 x 12 dSm^{-1} had the lowest grain yield (20.99 g) and cultivar Moomal x control (non-treated) had the greatest 1000 grain weight (52.20 g). Additionally, decrease in the 1000 grain weight in response to an increase in root zone salinization, although the impact varied across wheat varieties. Various other findings suggested that 7.00 dSm^{-1} is best for salinity tolerant for wheat crop and limit the 25% yield on 9.00 dSm^{-1}Kramer and Amtmann (2012). Kalhoro et al. (2016) confirmed that on high level of salt stress drastically minimize the yield in 1000 grain weight. The average grain yield of wheat on salinity status of (8 and 12dSm^{-1}) was (12.51 and 11.63g) and reduced7.13 and 13.66%, respectively compared to the control treatment which was (13.47 g) Kreet and Samira (2020).

Table 5: Effect of Salinity on Grain Yield [(1000 grain weight (g)] in Various Wheat Cultivars

Salinity levels (dSm^{-1})	**Varieties**					**Mean**
	T.D-1	**T.J-83**	**Moomal**	**Sarsabz**	**Kiran-95**	
Control	44.42 bc	39.35 ef	52.20 a	45.39 bc	44.14 bcd	45.1 a
4	39.63 ef	32.49 g	46.71 b	40.20 ef	42.33 cde	40.27 b
8	30.66 gh	28.54 j	33.74 gef	37.97 f	24.15 i	31.01 c
12	28.09 h	24.43 k	21.12 g	32.57 g	20.99 ij	25.44 d
Mean	35.70 c	26.70 b	43.44 b	39.03 a	32.90c	
SE =	**0.38**		**0.42**		**0.85**	
LSD 5% =	**0.77**		**0.86**		**1.73**	

CONCLUSION

In conclusion, brackish water and salinity provide serious obstacles to agricultural output. This is a

global issue for arid and semi-arid regions Sindh, Pakistan. Different types of cultivars like Sarsabz, Moomal, and T.J.83 performed better under the status of 12 dSm^{-1} of salinity stress compared toKiran-95 and T.D-1 based on proline, betaine and cell membrane stability as well as more potassium (K^+) than sodium (Na^+) constitute in the tissues. Molecular markers, physiological and biochemical approaches can also improve the selection of both tolerant and sensitive cultivar types.

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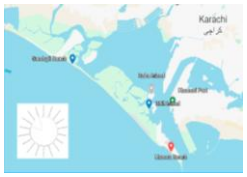

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Chemical and Biological Evaluation of *Albizia lebbeck, Psoralea corylifolia,* and *Trifolium indicum*

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ABSTRACT: *Herbal medicine, rooted in traditional practices, is increasingly recognized for its diverse therapeutic applications. Albizia lebbeck, Psoralea corylifolia, and Trifolium indicum are valued in traditional medicine for their potential health benefits, including anti-inflammatory, antioxidant, and metabolic regulatory properties. This work delves into the rich spectral fingerprint of methanolic extracts from Albizia lebbeck, Psoralea corylifolia, and Trifolium indicum, aiming to elucidate their unique biochemical composition. Employing advanced spectroscopic techniques, including Flame and Atomic Absorption Spectroscopy (AAS) and Fourier Transform Infrared Spectroscopy (FTIR), the minerals and bioactive compounds present in these herbal extracts were meticulously characterized. The study extends beyond conventional spectroscopic analysis by correlating the spectroscopic signatures with the biological potential of the extracts. This study unveiled biological evaluations including time and concentration-dependent antioxidants, anti-inflammatory, antihyperglycemic, analgesic, and anti-anxiety properties of these botanicals through rigorous in vitro and in vivo assessments. Each herbal extract was found to have characteristic chemical composition and biological potential. The present work not only contributes to the fundamental understanding of the spectral features of these herbs but also positions them as promising candidates in the realm of disease management.*

Keywords: Herbal medicines, Spectral analysis, Bioactivity profile, biological activities, Chemical composition

INTRODUCTION

Inflammation is a complex phenomenon producing pain along with redness, also termed as a non-specific internal response of the body (Leelaprakash and Dass, 2011), where infiltration of leukocytes along with capillary infiltration leads to vascular permeability and activation of fibroblast, fibrosis, infiltration, and proliferation of monocytes, macrophages, and neutrophils in acute and chronic inflammation. It is distinguished due to the longer residence of macrophages and lymphocytes, which mainly produce fibrosis and tissue necrosis (O'Byrne and Dalgleish, 2001; Wong et al., 2012). Prostaglandins (PGs), histamine and kinins are released in response to inflammation, which direct the blood flow towards affected areas by producing alterations in the structure of the membrane (Leelaprakash and Dass, 2011). Inflammation normalizes the tissue's functions by promoting the proliferation and differentiation of stem cells in the affected tissue as well as by promoting angiogenesis, the formation of new blood vessels (Gurtner et al., 2008).

A significant number of the population of the world is facing anxiety-related problems, causing stomach issues, motor sympathetic hyperactivity, stress, and fear. Exposure to these conditions for a longer period makes a man mentally and physically ill (Shri, 2010). Oxidants are produced because of different chemical reactions (outside and inside the body) and diseases like inflammation. Reactive oxygen species (ROS) are natural byproducts of cellular metabolism having unpaired electron, making them highly reactive and capable of damaging cellular components, including proteins, lipids, and deoxyribonucleic acid (DNA), while low levels of ROS are necessary for normal cellular functions. There are several types of ROS, including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and singlet oxygen ($^1O_2$) (Valko et al. 2006). ROS have been implicated in the pathogenesis of various diseases, including cancer, neurodegenerative diseases, cardiovascular disease, diabetes (Sies, 2015), inflammation, damaging blood vessels and atherosclerosis (Griendling and FitzGerald, 2003). Antioxidants counteract the harmful effects of oxidants by donating electrons to free radicals, hence neutralizing free radicals and preventing them from damaging cells (Sies, 1993). There are several types of antioxidants, including vitamins C and E, beta-carotene, selenium, and flavonoids present in a variety of foods, having ability to prevent the onset of various chronic diseases by reducing oxidative

stress (Block et al., 2002). Phytochemicals are naturally occurring compounds found in plants, having crucial role in the prevention and treatment of chronic diseases such as inflammatory, cardiovascular disease, cancer, diabetes, and neurodegenerative diseases (Liu et al., 2014). For example, resveratrol, has shown to reduce the risk of heart (Silva et al., 2013) with improved insulin sensitivity and glucose metabolism (Goh et al., 2014). They impart characteristic properties such as anti-inflammatory, anti-oxidant, analgesic, anti-anxiety, anti-bacterial, anti-hyperglycemic, anti-viral and anti-cancer to the herbal plants. (Yasmin et al., 2020b).

The growing interest in natural sources of antioxidants is driven by the need for alternative therapeutic options that are effective, affordable, and have fewer side effects compared to synthetic drugs. Herbal plants, rich in bioactive compounds, offer a promising source of such natural antioxidants (Embuscado, 2015). *Albizia lebbeck* (commonly known as Siris or Shiris), *Psoralea corylifolia* (commonly known as Babchi), and *Trifolium indicum* (commonly known as Nakhona or Indian clover) are medicinal plants with traditional uses in managing various ailments. However, a comprehensive understanding of their phytochemical composition and biological activities remains limited. Therefore, the present study was designed to evaluate methanolic extracts of *Albizia lebbeck*, *Psoralea corylifolia*, and *Trifolium indicum* for their anti-inflammatory, anti-hyperglycemic, anti-anxiety, analgesic, and antioxidant potentials with detailed phytochemical and mineral investigation to understand their chemical profiles and how these associates with their biological effects. The uniqueness of this research lies in filling existing gaps in literature along with its integrated approach, combining chemical, mineral, and biological evaluations to explore how these factors play their role to the overall effectiveness of the herbal extracts in treating conditions related to inflammation, pain, anxiety, oxidative stress and hyperglycemia.

MATERIALS AND METHODS

Collection of Herbs

Selected medicinal herbs including *Albizia lebbeck*, *Psoralea corylifolia*, and *Trifolium indicum* were obtained in dried form from Ajmal Dawa Khana located near Ichra, Lahore, Pakistan. These herbs were confirmed by the botanist from the Department of Botany, University of the Punjab, Lahore, before further processing. Then, the herbs were purified, ground to fine

powder and stored in glass vials for further experimental work.

Preparation of Herbs Extract

Herbal extracts were prepared by dissolving 10 g of each medicinal herb in powdered form with 200 mL of methanol used as solvent followed by stirring overnight using magnetic stirrer. The mixture was filtered using Whatman filter paper and the residue obtained was again mixed with 100 mL of methanol and left overnight. The mixture was then filtered on next day and the filtrate obtained was mixed with previously obtained filtrate. The extract was obtained after evaporating filtration and stored in glass vials for chemical and biological evaluation.

Chemical Profiling

Fourier Transform Infrared Spectroscopy Analysis

The methanolic extract of selected herbs was subjected to Fourier Transform Infrared Spectroscopy (FTIR) analysis in the region of (4000-650) cm^{-1} (Agilent Technologies Carry 630 FTIR) (Khalil et al., 2013).

Phytochemicals Analysis and Mineral Detection

The phytochemicals constituents including terpenoids, flavonoid, tannins, steroids, phlobatannins, anthraquinones, alkaloids, saponins, cardiac glycosides, reducing sugars and carbohydrates were evaluated in the selected medicinal herbs by the using methods published earlier (Yasmin et al., 2020b)**.**

The wet method was employed for the evaluation of mineral content in selected herbs sample. Briefly, each herb (0.5 g) in powdered form was added into the beaker followed by adding concentrated nitric acid (HNO_3). This solution was heated till the formation of clear solution. After digestion, the obtained solution was diluted with distilled water up to 50 mL. Concentrations of different minerals were estimated in prepared herbs solution by preparing 5, 10, 15, 20 and 25 parts per million (ppm) standard solutions of respective metal followed by the estimation of mineral content by Flame and Atomic Absorption Spectroscopy (AAS) (Rahayu, 2020).

***In Vitro* Biological Profiling**

Antioxidant Activity

The methanolic extract of selected medicinal herbs was subjected to three antioxidant assays for the evaluation of their antioxidant potential. The $FeCl_3$ Iron Reducing Power, Phosphomolybdenum and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) activities were performed as described previously to estimate the antioxidant potential of herbs (Tajammal et al., 2017; Yasmin et al., 2020a; Samra et al., 2022).

***In Vivo* Biological Profiling**

Experimental Animals

The Sprague Dawley (SD) rats were used for the evaluation of *in-*

vivo biological potential of methanolic extract of selected medicinal herbs. Animals were kept in a departmental animal house under controlled temperature and humidity and cared for in accordance with international instructions. The experiments were approved by the Ethical Committee, School of Chemistry, University of the Punjab, Lahore. Pakistan.

Experimental Groups and Dose Selection

Rats were fasted overnight and divided into 5 groups (Number of rats (n) =5 in each group):

Group 1: Standard Drug (Diclofenac sodium; 20 mg/kg)

Group 2: Control (Carboxymethyl Cellulose (CMC); 10 ml/kg)

Group 3: *Albizia lebbeck* (300 mg/kg)

Group 4: *Psoralea corylifolia* (300 mg/kg)

Group 5: *Trifolium indicum* (300 mg/kg)

Anti-inflammatory Effect

The carrageenan induced paw edema method in rats was used to determine anti-inflammatory potential of methanolic extract of selected herbs by producing inflammation in the rat paw as reported previously (Samra et al., 2022; Samra and Basra, 2023).

Percentage edema inhibition was calculated by using following formula

% edema inhibition = $(V_c - V_t / V_c) * 100$

Whereas V_c = stands for the mean paw volume of control group rat and V_t stands for the mean paw volume of drug treated group rat.

Anti-hyperglycemic Activity

SD rats were fasted overnight and anti-hyperglycemic effects of methanolic extract of selected medicinal herbs were evaluated by oral glucose tolerance test (OGTT) as reported previously (Tajammal et al., 2017). Rats having blood glucose level between 80-100 mg/dl were selected to check the anti-hyperglycemic activity.

Analgesic Activity

Analgesic potential of methanolic extract of selected medicinal herbs was evaluated by acetic acid-induced method as reported previously (Samra et al., 2022; Samra and Basra, 2023).

The % inhibition of writing was calculated using the following formula

% Inhibition of writhing = $(W_c - W_t/W_c) \times 100$

Whereas W_c stands for mean writhing of control group rat and W_t stands for mean writhing of drug treated group rat.

Anti-anxiety Activity

The Elevated Plus Maze (EPM) test was employed for evaluating the anti-anxiety potential of selected herbs as described previously (Samra and Basra, 2023).

The % of time spent in open arm was calculated using following formula

The percentage time spent in open arm = (T_o/T_t) x 100

Whereas T_o stands for the time spent in open arms and T_t stands for the time spent in both open and closed arms.

Statistical Analysis

In this experimental work, all the data of the *in vivo* experiments were presented in the form of Mean ± standard deviation and this statistical analysis was done using one-way ANOVA, which was done with the help of GraphPad prism (version 7.0).

RESULTS

Chemical profiling

Fourier Transform Infrared Spectroscopy and Phytochemicals Analysis

The FTIR spectra of *Psoralea corylifolia* extract revealed the absorption bands at 2923.2, 2855.6, 1609, 1449, 1120 and 1053.9 cm^{-1} showed the presence of $-CH_2$, (Alkanes), -CH stretching (Aldehydes), C=N (Imines), C=C (Aromatic ring), C-N (Aromatic amines) and C-O (Anhydrides) respectively. The methanolic extract of *Albizia lebbeck* presented the different absorption bands at 3336.0, 2924.6 and 2855.6 cm^{-1} which showed the presence of O-H, $-CH_2$ and -CH group (Alkanes) respectively. The absorption band at 1735.1 and 1617.7 cm^{-1} depicted the presence of C=O group (Aldehyde) and C-N group (primary amines) respectively. In addition to this, the absorption band at 1028.7 cm^{-1} indicated the presence of R-O group (Ethers). Moreover, the spectra of *Trifolium indicum* contained the different absorption bands at 3336.9, 1577.6, 1117.3 and 1047.4 cm^{-1} exhibited the presence of O-H group (Alcohol and phenol), C=C stretching (Aromatic), C-O group (ethers), and C-O group (primary alcohol) (Figure 1a-c, Table 1).

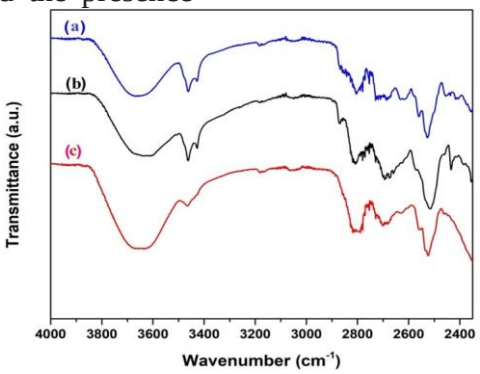

Fig. 1. FTIR Spectra of medicinal herbs. FTIR spectra of (a) *Psoralea corylifolia*, (b) *Albizia lebbeck*, and (c) *Trifolium indicum* depicting key molecular vibrations representative of their chemical characteristics

Table 1: FTIR analysis of methanolic extract of herbs

Functional Groups	Herbs Peaks (cm^{-1})		
	Albizia lebbeck	***Psoralea corylifolia***	***Trifolium indicum***
(O-H)	3336.0	3359.3	3336.9
$(-CH_2)_{alkanes}$	2924.6	2923.2	-
$(C=C)_{aromatic}$	-	-	1577.6
$(-CH)_{alkanes}$	2855.6	-	-
$(C=O)_{aldehydes}$	1735.1	-	-
$(C-N)_{amines}$	1617.7	-	-
$(-CH)_{aldehyde}$	-	2855.6	-
$(C=N)_{Imines}$	-	1609	-
$(C=C)_{aromatic\ ring}$	-	1449	-
$(C-N)_{aromatic\ amines}$	-	1120	-
$(C-O)_{anhydrides}$	-	1053.9	-
$(C-O)_{ethers}$	-	-	1117.3
$(C-O)_{alcohols}$	-	-	1047.4
$(R-O)_{ethers}$	1028.7	-	-----

Furthermore, biochemical tests revealed the presence of terpenoids, reducing sugars and carbohydrates in all herbs, while steroids and cardiac glycosides were only found in *Albizia lebbeck* and *Trifolium indicum* and alkaloids were best found in *Psoralea corylifolia*. Moreover, saponins and flavonoids were found to be effectively present in the extract of *Trifolium indicum* (Table 2).

Table 2: Phytochemical analysis of methanolic extract of herbs

Sr. No	Phytochemicals	Medicinal herbs		
		Albizia lebbeck	***Psoralea corylifolia***	***Trifolium indicum***
1	Terpenoids	+	+	+
2	Tannins	-	-	-
3	Saponins	-	-	++
4	Flavonoids	-	-	++
5	Reducing sugars	+	+	+
6	Carbohydrates	+	+	+
7	Steroids	+	-	+
8	Cardiac glycosides	+	-	+
9	Phlobatannins	-	-	-
10	Alkaloids	-	+	-
11	Anthraquinones	-	-	-

Where, (-) shows the absence, (+) shows the presence and (++) show more presence of respective phytochemical.

Mineral Detection by Flame and Atomic Absorption Spectroscopy

All the herbs were subjected to mineral detection and found to have Ni (Nickel), Fe (Iron), Mg (Magnesium), Ca (Calcium), Cu (Copper), Co (Cobalt) and K (Potassium) in different concentrations. Ni, Fe and Ca were found in maximum concentrations of 102.3, 199 and 97.9 ppm respectively in *Psoralea corylifolia* while, Mg, Co and K were present in maximum concentrations of 115.2, 167.7 and 47.5 ppm in *Albizia lebbeck* and Cu was found in maximum concentration of 28.8 ppm in *Trifolium indicum*, as represented in Table 3.

Table 3: Quantitative investigation of minerals in ppm by Flame and Atomic Absorption Spectroscopy in medicinal herbs

Sr. No	Minerals	Medicinal herbs (ppm)		
		Albizia lebbeck	***Psoralea corylifolia***	***Trifolium indicum***
1	Nickel	9.701	102.3	85.5
2	Iron	66.85	199	179.2
3	Magnesium	115.2	30	38.8
4	Calcium	26.34	97.9	31.5
5	Copper	4.263	28.6	28.8
6	Cobalt	167.7	148.5	135
7	Potassium	47.5	42.68	31.05

***In Vitro* Biological Profiling**

Antioxidant Potential

The ability of the phytochemicals presents in the herbal extracts to reduce Fe^{3+} to Fe^{2+} was evaluated by $FeCl_3$ iron reducing power activity. All herbs were found to show an increasing trend in their reducing power with decrease in their IC_{50} values with respect to time. After 30 minutes of incubation, *the Trifolium indicum* showed the lowest IC_{50} value and hence the maximum antioxidant potential among all herbs. The antioxidant potential of methanolic extracts of herbs to reduce Mo (VI) to Mo (V) was determined by Phosphomolybdenum activity. It was observed that after 30 minutes of incubation, methanolic extract of *Psoralea corylifolia* depicted the maximum antioxidant potential and lowest IC_{50} value

among all herbal extracts (Table 4).

Table 4: Antioxidant activities of methanolic extracts of medicinal herbs

Activity	Herbs Extract	IC_{50} (mg/mL) with respect to time (minutes)					
		10	15	30	45	60	120
$FeCl_3$ iron reducing power	*Albizia lebbeck*	0.063	0.063	0.063	0.062	0.059	0.053
	Trifolium indicum	0.014	0.013	0.012	0.011	0.010	0.011
	Psoralea corylifolia	0.024	0.021	0.020	0.020	0.021	0.018
	Ascorbic acid	0.006	0.006	0.006	0.005	0.006	0.005
Phosphomolybdenum	*Albizia lebbeck*	-	0.072	0.091	0.084	0.079	0.079
	Trifolium indicum	-	0.074	0.072	0.069	0.071	0.073
	Psoralea corylifolia	-	0.002	0.0029	0.003	0.005	0.006
	Ascorbic acid	-	0.001	0.001	0.001	0.0020	0.0023
2,2-diphenyl-1-picryl-hydrazyl	*Albizia lebbeck*	-	110.900	59.500	44.500	35.00	23.500
	Psoralea corylifolia	-	5.780	6.190	4.470	5.700	5.900
	Trifolium indicum	-	0.920	0.850	0.890	1.000	1.260

Furthermore, the free radical scavenging potential of methanolic extract of selected herbs was evaluated by DPPH assay and results are shown as IC_{50} values in table 4. The methanolic extract of *Trifolium indicum* showed maximum free radical scavenging potential among all herbs and lowest IC_{50} value, hence showed maximum antioxidant activity at interval of 30 minutes among other medicinal herbs. The antioxidant assays showed that the selected herbs extracts had impressive antioxidant potential, among them *Trifolium indicum* and *Psoralea corylifolia* showed significant antioxidant potential.

***In Vivo* Biological Evaluation**

Anti-inflammatory Potential

The *in vivo* anti-inflammatory potential for the methanolic extracts of selected herbs was evaluated by carrageenan-induced paw edema method in SD rats. Each herbal extract was dissolved in 0.5 % CMC solution and given orally. Negative control group received CMC (10 ml/kg) solution while positive control group was given Diclofenac sodium as standard drug at a dose of 20

mg/kg. A magnificent suppressive effect was observed for herbal extracts in comparison with control group after first hour of carrageenan induction (Fig. 3). The methanolic extract of *Albizia lebbeck* showed remarkable repression in paw volume as compared to other herbal extracts with maximum anti-inflammatory potential equal to standard medicine after third hour of carrageenan induction, while other herbal extracts also showed good anti-inflammatory potential (Fig. 2 a-e). The increasing anti-inflammatory trend was observed for all herbal medicinal extracts after third hour of carrageenan induction as shown in Fig. 3-4.

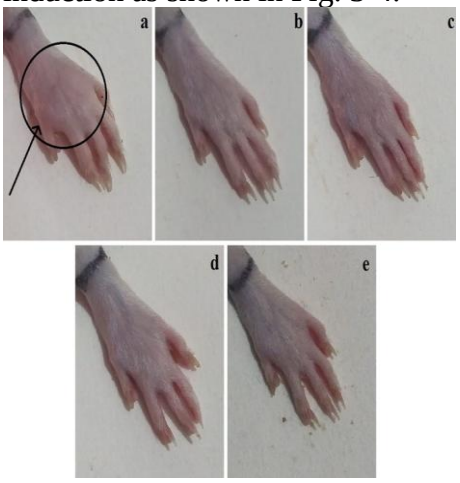

Fig. 2. Edema suppressing effect at 3 hours of carrageenan induction in rat's paw. (a) Inflamed paw (Untreated), (b) Diclofenac sodium treated paw, (c) *Albizia lebbeck* treated paw, (d) *Psoralea corylifolia* and (e) *Trifolium indicum* treated paw edema. Each image illustrates the effect of treatment by representative medicinal herbs on edema inhibition.

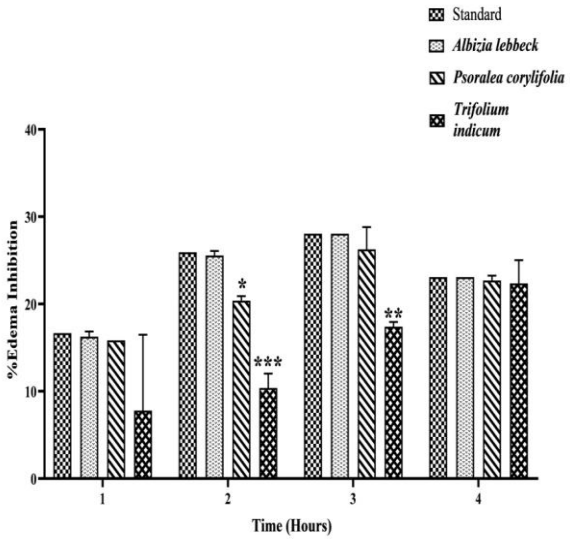

Fig. 3. The percentage of edema inhibition by herbs. Statistically significant differences are $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared with standard and all the data is represented in percentage values as Mean ± standard deviation. Diclofenac sodium was used as a standard drug.

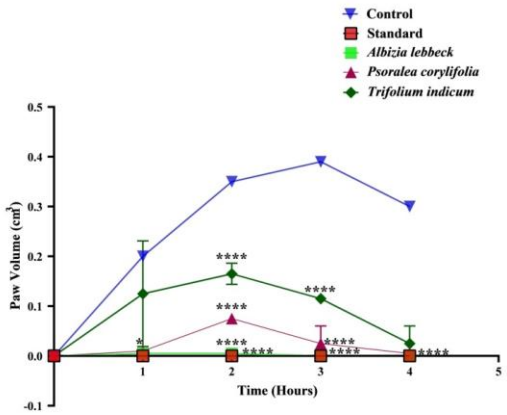

Fig. 4. Reduction in rat's paw volume with respect to time Diclofenac sodium was used as a standard drug. $^{*}P < 0.05$ and $^{****}P < 0.0001$ are the statistically significant differences when compared with the control group and all values are represented as mean ± standard deviation

Anti-hyperglycemic Activity

Among the tested herbs *Psoralea corylifolia* showed magnificent anti-hyperglycemic effect at 210 min, while *Trifolium indicum* also showed good effect on reducing glucose level in the blood as compared with Diclofenac sodium used as reference drug. The decreasing trend in anti-hyperglycemic effect observed at 210 min was *Psoralea corylifolia* > *Trifolium indicum* > Standard > Control > *Albizia lebbeck* as shown in Fig. 5.

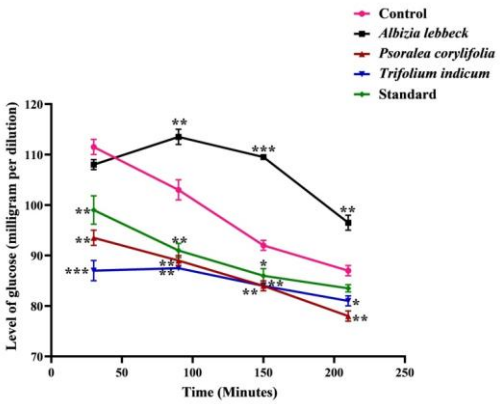

Fig. 5. Antihyperglycemic potential of herbs. Diclofenac sodium was used as a standard drug. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ are the statistically significant difference when compared with control group and all values are represented as mean ± standard deviation.

Analgesic Activity

Analgesic effect for the selected herbs to check their efficacy in injury was evaluated by acetic acid-induced writhing test. *Albizia lebbeck* showed highest inhibition in writing at a dose of 300 mg/kg as compared to reference medicine which was administered at the dose of 20 mg/kg (Fig. 6).

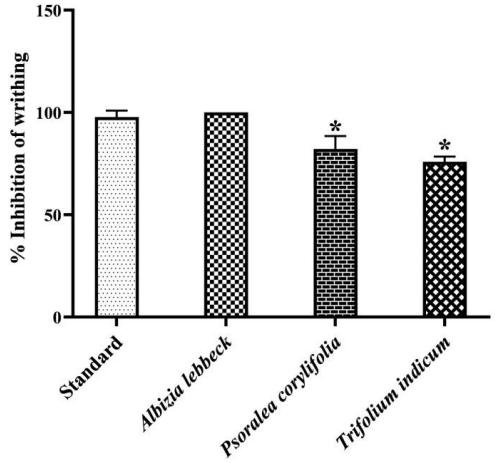

Fig. 6. Analgesic potential of herbs. Diclofenac sodium was used as standard drug while, $^{*}P < 0.05$ is the statistically significant difference when compared with standard and all values are represented as mean ± standard deviation

Anti-anxiety Potential

The results showed the rat group treated with extract of *Albizia lebbeck* and *Psoralea corylifolia* remained in the open arm for maximum period as compared to standard group, which showed that *Albizia lebbeck* had the highest *Psoralea corylifolia* showed significant anti-anxiety potential as compared with Diclofenac sodium used as standard (Table 5).

Table 5: Anti-anxiety effect of herbal plants by elevated plus maze (EPM) test

Sr. No.	Plant herb	% Time spent on open arm	No. of entries in open arm	No. of entries in closed arm
1	Control	10.25	3.00	3.50
2	Standard	22.00	3.50	3.50
3	*Albizia lebbeck*	42.30	7.00	8.00
4	*Psoralea corylifolia*	26.50	5.50	5.50
5	*Trifolium indicum*	0.50	0.50	1.50

A decreasing trend of anti-anxiety potential observed in herbs was *Albizia lebbeck* > *Psoralea corylifolia* > standard > control > *Trifolium indicum* (Fig. 7).

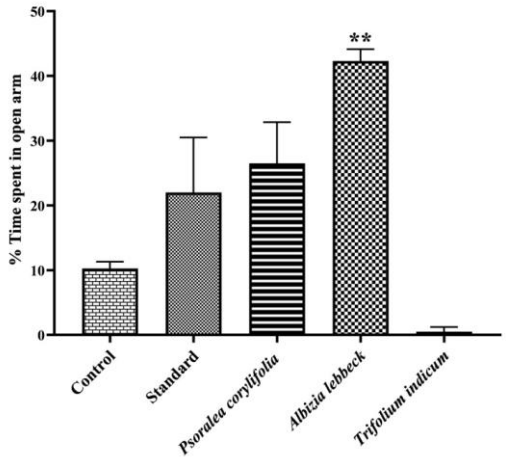

Fig. 7. Anti-anxiety effects of *Albizia lebbeck, Psoralea corylifolia, Trifolium indicum* and standard drug in terms of percentage of time spent in open arm. Diclofenac sodium was used as standard. $^{**}P < 0.01$ is the statistically significant difference when compared with control group and all data is represented as ± standard deviation.

DISCUSSION

The Fabaceae family is known to include medicinal plants with a wide range of potential benefits, including anti-inflammatory, analgesic, anxiolytic, anti-hyperglycemic, antioxidant, antibacterial, anticonvulsant, anticancer, and anti-asthma potentials (Ahmad et al., 2016). Examples include *Acacia nilotica*, *Platypodia elegans*, and *Dolichos biflorus*. These herbs are rich in phytochemicals such as alkaloids, flavonoids, phenols, terpenoids, and steroids, which are responsible for their anti-inflammatory, antioxidant, anticancer, antiviral, antibacterial, and antifungal effects (Jurenka 2009). For instance, garlic (*Allium sativum*) contains sulfur compounds known as allicin, which have notable antibacterial, antifungal, and antiviral properties (Ankri and Mirelman, 1999). Moreover, the Fabaceae family member *Glycine max* (soybean) contains genistein and daidzein, which provide anti-inflammatory potential (Yu et al., 2016). Furthermore, members of the Fabaceae family are rich in minerals such as calcium, magnesium, potassium, and iron, which play crucial roles in maintaining health (Didinger and Thompson, 2021). For example, calcium is essential for bone health, muscle movement, neurotransmitter release, and blood clotting (Heaney, 2002), while potassium helps to maintain heart health and blood pressure (Weaver, 2013). Similarly, iron contributes to oxygen-carrying ability in hemoglobin, and magnesium supports water balance and antioxidant functions (Quintaes and Diez-Garcia, 2015). Additionally, *Aegle marmelos*, with its content of niacin, enhances its anti-inflammatory

potential (Angajala et al., 2014) and cobalt has a distinct role in boosting anticancer potential (Veeralakshmi et al., 2015). The present research has conducted chemical and biological profiling of methanolic extracts from Fabaceae family herbs, including *Albizia lebbeck*, *Psoralea corylifolia*, and *Trifolium indicum*. It has successfully examined the phytochemicals and minerals in these herbs, revealing their antioxidant, anti-inflammatory, antihyperglycemic, anti-anxiety, and analgesic properties. In the present study, $FeCl_3$ Iron Reducing Power, Phosphomolybdenum and DPPH assays showed that *Trifolium indicum* had the maximum antioxidant potential among all herbs. The previously reported work on *Psoralea corylifolia* showed that its ethanolic and acetone extracts showed IC_{50} values of (115.82±0.06 and 172.27±0.04 µg/ml) respectively by DPPH method, while its ethanolic extract showed IC_{50} value of (102.81±0.05 µg/ml) by $FeCl_3$ iron reducing activity (Karale et al., 2022). The present study conducted on the methanolic extract of *Psoralea corylifolia* showed its lowest IC_{50} value of (0.0180 mg/ml or 18 µg/ml) after 120 minutes of incubation by iron reducing power activity, (0.002 mg/ml or 2 µg/ml) after 15 minutes of incubation by Phosphomolybdenum assay and maximum antioxidant potential than its previously reported work (Szakiel et al., 2011). Various members of the family Fabaceae for example *Acacia Arabica*, *Arachis hypogaea*, *Cassia tora*, *Dalbergia sissoo*, *Acacia catechu* , *Acacia nilotica* (Ahmad et al., 2016) have been found to show anti-inflammatory activity. The present study shows the maximum edema inhibition by methanolic extract of *Albizia lebbeck* (28.05%) at a dose of 300mg/kg while, *Psoralea corylifolia* showed significant result (26 %). This study, which was conducted on methanolic extract of *Psoralea corylifolia* by glucometer method showed excellent anti-hyperglycemic effect among all herbs and standard and *Trifolium indicum* showed significant anti-hyperglycemic potential at an interval of 210 min. In another study, the methanolic extract of *Albizia lebbeck* was found to have anti-hyperglycemic effect (Patel et al., 2015), while the present results of *Albizia lebbeck* also coincides with the previously reported work, showing it is good in reducing blood glucose level. Previously reported studies investigated that various members of the family Fabaceae such as *Acacia modesta* Wall, *Sutherlandia frutescens*, *Pterocarpus marsupium* Roxb, *Dalbergia sissoo* have shown analgesic effects (Ahmad et al., 2016). In a study reported, the extract prepared by the mixture of

ethyl acetate, methanol and petroleum ether of *Albizia lebbeck* had shown significant analgesic potential with 52.4% inhibition of writhing (Saha and Ahmed, 2009) and the methanolic extract of *Psoralea corylifolia* possesses significant analgesic potential (Kumar et al., 2015). This study is significant in this regard as results demonstrated that *Albizia lebbeck* showed maximum (100 %) analgesic effect in comparison with others, while *Psoralea corylifolia* and *Trifolium indicum* showed significant analgesic potential. *Albizia lebbeck* is found to have shown excellent anxiolytic properties as reported previously (Mishra et al., 2010) and *Psoralea corylifolia* contains anti-depressant potential (Mahajan et al., 2022). The results of the present investigation are significant as it showed that the rats treated by methanolic extract of *Albizia lebbeck* showed powerful anti-anxiety activity with 42.30% time spent in open arm while, *Psoralea corylifolia* was found to have significant anti-anxiety effect (26.50% time spent in open arm). The present results are different from previously reported literature due to differences in methodologies, extraction techniques and plant sources, which may affect the results. Additionally, the age and growth conditions of the plants, as well as the geographical location can influence the phytochemical and mineral composition and ultimately biological activity of the plants (Szakiel et al. 2011). Therefore, it is important to evaluate the biological potential of plant extracts using various methods and compare the results to previous studies to identify any inconsistencies.

CONCLUSION

Chemical and biological analysis of selected medicinal herbs including *Albizia lebbeck*, *Psoralea corylifolia*, and *Trifolium indicum* revealed the presence of bioactive compounds and minerals which are imparting biomedical properties. *Psoralea corylifolia* and *Trifolium indicum* exhibited impressive antioxidant potential. *Albizia lebbeck* found to contain impressive anti-inflammatory effects, while *Psoralea corylifolia* showed better anti-hyperglycemic effects. These herbs also showed pain reducing potential along with anti-anxiety effects. Keeping in view these results, these plants might be used to reduce glucose level and suppress the inflammation and pain without any anxiety, but further research work is needed to evaluate their pharmacodynamics effects at molecular level.

CONFLICT OF INTEREST

All authors have read the manuscript completely and thoroughly and take responsibility for this experimental work and

assure that all questions related to the correctness of this work have been thoroughly resolved. All individuals who contributed to the writing and conducting of this experimental work are named authors and all authors declare no conflict of interest.

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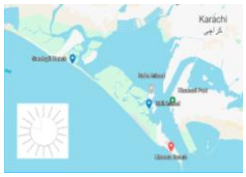

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Protective Effect of Thymoquinone Coated Zinc Oxide Nanoparticles Against Aflatoxin B_1 Induced Hepatotoxicity in Albino Rats

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ABSTRACT: *Aflatoxins are a group of mycotoxins which can cause hepatotoxicity and can eventually lead to liver cancer. The current study was designed to check the hepatoprotective effect of thymoquinone coated zinc oxide nanoparticles against aflatoxin induced hepatotoxicity in albino rats. Aflatoxin B1 (AFB1) was produced by solid state fermentation on rice using Aspergillus flavus and quantified by HPLC. TQ-coated ZnONPs were prepared by the conjugation of thymoquinone with ZnONPs and characterized by UV-Visible spectrophotometer, and Zeta potential analysis. The albino rats were fed with different diets by dividing them into five groups i.e., group A (normal diet), group B (20 ppb AFB1), group C (20 ppb AFB1 and 25 ppb ZnONPs), group D (20 ppb AFB1 and 10 ppb TQ) and group E (20 ppb AFB1 and 25 ppb TQ-ZnONPs). Blood sample and liver tissues were taken for histopathological study. Histopathological examination of liver revealed that AFB1 causes swelling, focal necrosis and decreased sinusoidal spacing. Biochemical tests like ALT (116 U/L) and AST (109 U/L) were significantly raised while there was decline in level of glucose, cholesterol, HDL and TG by AFB1. Liver function enzymes ALT (28 U/L) and AST (35 U/L) were significantly improved by treatment of TQ-ZnONPs. These changes caused by AFB1 were ameliorated by TQ-ZnONPs. It was concluded that the combined effect of TQ and ZnONPs is an effective approach towards the lethal hepatotoxic effects caused by aflatoxins present in animal feed.*

Keywords: Aflatoxin B1, Zinc oxide nanoparticles, Thymoquinone, Hepatotoxicity, Liver

INTRODUCTION

Mycotoxins are considered toxic secondary metabolites produced by fungi. There are five types of mycotoxins, i.e., aflatoxins, ochratoxins, zearalenone, deoxynivalenol and fumonisins. These toxins are present in a variety of food and feed items which badly affect human and animal body as they are readily absorbed, affecting the liver and cause metabolic disorders (Khan et al., 2024).

Aflatoxins are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* (Tola and Kebede, 2016; Sun et al., 2022). Different food commodities like spices, millet, cocoa, sesame seeds, wheat, maize, rice, fig, peanut and peanut butter are contaminated with aflatoxins (Mahato et al., 2019). Aflatoxins are difuranocoumarin derivatives in nature and have mutagenic, hepatotoxic, tetrogenic, cytotoxic, immunosuppressive and estrogenic effects in mammals (Benkerroum et al., 2020; Klvana and Bren, 2019). Aflatoxin exposure is not only linked with liver cancer but also leads to malignancies of different organs like kidney, bladder and bone (Dabuo et al., 2022).

Liver is a vital organ as it is involved in metabolism, secrete harmful materials and store useful products in the body. It is the main site of body which remove injurious chemicals, detoxify different drugs and eliminate xenobiotics. Any malfunctioning in the liver leads to liver damage which results in the disturbance of these processes. Liver disease is regarded as a serious public health concern in several parts of the world (Rishi and Subramaniam, 2017). Aflatoxin B_1 is the primary reason of liver cancer in humans and much evidence support that aflatoxins are primarily involved in hepatocellular carcinoma (Rushing and Selim, 2019).

In the light of previous observations, it is very important to develop different physical, biological and chemical approaches to detoxify various mycotoxins. The use of nanotechnology to reduce the toxic effects of aflatoxins on human and animal health is the most promising approach (Ajayi et al., 2015; Hassan et al., 2021). Nanoparticles are the smallest particles having size range from 1 to 100 nm (Irshad et al., 2020). From the past few decades, nanoparticles are extensively used in a variety of fields i.e., medicine, environment, drug discovery, therapy and biotechnology (Patra et al., 2018). Nanoparticles have gained special importance because of their antitoxin, antitumor, antimicrobial and antifungal activity (Kiruthika and Somanathan, 2014). They increase the safety and efficacy of drugs, enhance their bioavailability and

stability, allow targeted delivery and improve their effectiveness in the target tissue. A wide range of drugs can be delivered via nanoparticles to various organs like liver, brain, spleen, lungs and lymphatic system (Zahin et al., 2020). They have positive effects on the reproduction of livestock and poultry. They alter the fermentation process in rumen of animals (Swain et al., 2015). ZnONPs have been regarded as effective agents in both vivo and in vitro studies. They can easily absorb through body barriers, efficiently targeting cells and molecules in various diseases (Tang et al., 2016). ZnONPs were reported to reduce the toxic effects of AFB_1 in rabbits by scavenging free radicals. This mechanism guard liver cells from toxic effects of AFB_1 (Atef et al., 2016).

Thymoquinone is a biologically active chemical present in the seeds of *Nigella sativa*. It is commonly named kalonji, black cumin or black seed. It exhibits a variety of properties like antioxidants, anti-cancerous, antibacterial, anti-hypersensitive, immunomodulatory and anti-inflammatory (Torabi et al., 2017). It has been reported that it possesses several pharmacological characteristics including antioxidant activity and protective effects against hepatotoxins (Meydan et al., 2019). It could dock with different cancerous and apoptotic targets which make it a hepatoprotective agent. It enhances the anti-cancerous effect of different chemotherapeutics (Khan et al., 2019). According to previous research, ZnONPs when coupled with TQ show more cytotoxicity (Banupriya et al., 2020; Banupriya et al., 2022).

Thymoquinone coated zinc oxide nanoparticles can lead to enhanced anti-cancerous and hepatoprotective effects. It will be a novel approach towards reliable therapeutical intervention against aflatoxins induced hepatotoxicity. So, this study is designed to determine the hepatoprotective effect of TQ coated ZnONPs against aflatoxin B_1 in terms of biochemical and histopathological analysis.

MATERIALS AND METHODS

Synthesized and characterized ZnONPs

Synthesized and characterized ZnONPs were procured from the Biotechnology Department, Kinnaird College for Women University.

Preparation and characterization of TQ coated ZnONPs

The thymoquinone stock solution was prepared by dissolving 2 mg/mL in acetone. 10 mg ZnONPs were dissolved in 1 mL of acetone. The ZnONPs solution was supplemented with the thymoquinone solution. After 24 hours of stirring, the thymoquinone was completely

adsorbed to the surface of the zinc oxide, creating the TQ-ZnONPs. The resulting suspension was centrifuged at 6,000 rpm before being washed three times with distilled water and dried in a vacuum (Perera et al., 2020). Synthesized ZnONPs were characterized by Ultraviolet-visible spectroscopy at 280 nm and zeta potential analysis.

Production of AFB_1

AFB1 was produced using rice by solid-state fermentation. 20 g of rice was taken in flask and 5 mL of distilled water was added and autoclaving was done for 15 min at 121 °C and 15 psi. The rice was inoculated by *A. flavus* and shaken daily. The flask was incubated at 28 °C in dark for 21 days (Lai et al., 2015). 3 g fermented rice was mixed with 20 mL of acetonitrile water (70:30) and was placed in a shaking incubator for 2 hours. The mixture was filtered using Whatman filter paper (0.45 µm). The filtrate was then purified by liquid-liquid extraction using chloroform. The chloroform was evaporated by placing the filtrate in a water bath at 70 ^{o}C and the remaining crust was dissolved in 1 mL of methanol. Further analysis of toxin was done by using HPLC (Bayman et al., 2002). The level of AFB1 in 3g rice sample was calculated using formula given below:

$$\text{Concentration of sample (µg/mL)} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard}$$

Study Design

Two months old, 20 albino rats were randomly selected and divided into 5 groups (A-E) each including 4 rats. Five types of diets were designed for five different groups as A (control group with normal feed), B (20 ppb AFB_1 contaminated diet), C (20 ppb AFB_1 contaminated diet and 25 ppb ZnONPs/kg feed), D (20 ppb AFB_1 contaminated diet and 10 ppb TQ/kg feed) and E (20 ppb AFB_1 contaminated diet and 25 ppb TQ-ZnONPs/kg feed). The experiment was conducted according to the Ethical Review Committee, University of Veterinary and Animal Sciences, Lahore.

Histopathological examination

Liver tissues were collected and fixed in 10% of formalin solution for 24 hours. In the next step, dehydrating agent, ethyl alcohol is used for dehydration of tissues. Then, clearing of tissues was done by xylene. Following this, tissues were kept in a jar of molten paraffin wax, embedded in paraffin blocks, and cut into sections of 4 µm thickness. Tissue sections were stained with hematoxylin and eosin solutions and viewed through light microscope for histopathological examination (Bancroft et al., 1984).

Estimation of Serum Biochemical Parameters

At the end of biological trial, 5 mL of blood was collected through

cardiac puncture at random from each group of rats. Serum was separated and stored at -20 ºC. Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline Phosphate (ALP), cholesterol, triglycerides, blood glucose, LDL, HDL and bilirubin were analyzed from serum samples by using kits on a chemistry analyzer (Micro-lab 300; Merck).

STATISTICAL ANALYSIS

All the results were analyzed statistically by one-way ANOVA and means will be compared by Duncan's Multiple Range test using SPSS software. The P-value < 0.05 will be considered as significant.

RESULTS AND DISCUSSION

Estimation of AFB_1

The quantity of aflatoxin extracted from rice was estimated by HPLC against the standard. The calculated value of AFB_1 was 0.008 µg/mL per 3 g of rice.

Characterization of TQ Coated ZnONPs

UV-Visible Spectroscopy was done to confirm the synthesis of ZnONPs. Reduction of Zn ions to ZnONPs was observed by mixing the plant extract and zinc acetate stock solution at 1:50 ratio (pH 12) and stirring for 2 hours. Appearance of colloidal solution was the first indication for the initiation of ZnONPs synthesis. Free electrons in metal NPs exhibit an absorption band of surface plasmon resonance (SPR) due to the resonance of electron vibrations with light waves. The emergence of peaks during UV–Visible characterization reveals the specific SPR which could be a confirmation for each type of produced NPs. The UV-Vis absorption spectrum of ZnONPs, TQ and TQ-ZnONPs is shown in (Fig. 1).

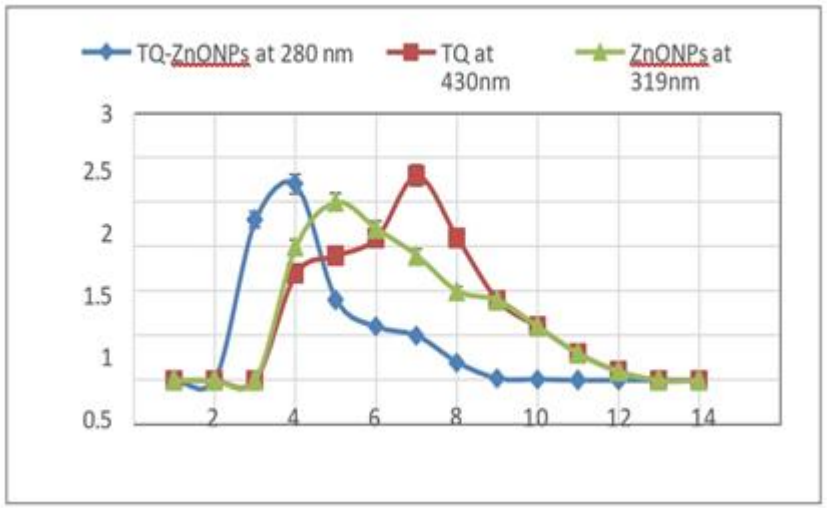

Fig. 1: UV- Visible Spectrophotometric Analysis of TQ-ZnONPs, TQ and ZnONPs

UV-Visible Spectroscopy showed the absorbance of TQ, ZnONPs and TQ-ZnONPs at 430 nm, 319 nm and 280 nm respectively. A prominent peak at 319 nm using UV-Visible Spectrophotometer shows the characteristic band of ZnONPs which is comparable with 320 nm, band of ZnONPs synthesized from Cayratia pedata leaf extract (Jayachandran et al., 2021) whereas TQ gives the absorbance at 430 nm as reported in several studies (Mohammed et al., 2019; Laskar et al., 2016).

Surface charges and stability of the synthesized ZnONPs and THQ-loaded ZnONPs were revealed through zeta potential analysis. For ZnONPs and THQ-loaded ZnONPs, strong peaks at negative potentials of -12.3 mV and -14.3 mV were detected, respectively as shown in (Fig 2). Metal oxide NPs possessing negative potential are particularly common while employing plant extract. Furthermore, the emergence of single peaks with negative potential demonstrates not only the constant distribution of surface charge but also the stability of the material in aqueous media (Jebril et al., 2020).

Histopathological examination

Liver tissue of control group (A) demonstrated normal architecture of the liver hepatocytes in cord as well as normal sinusoidal spaces between hepatocytes (Fig 3).

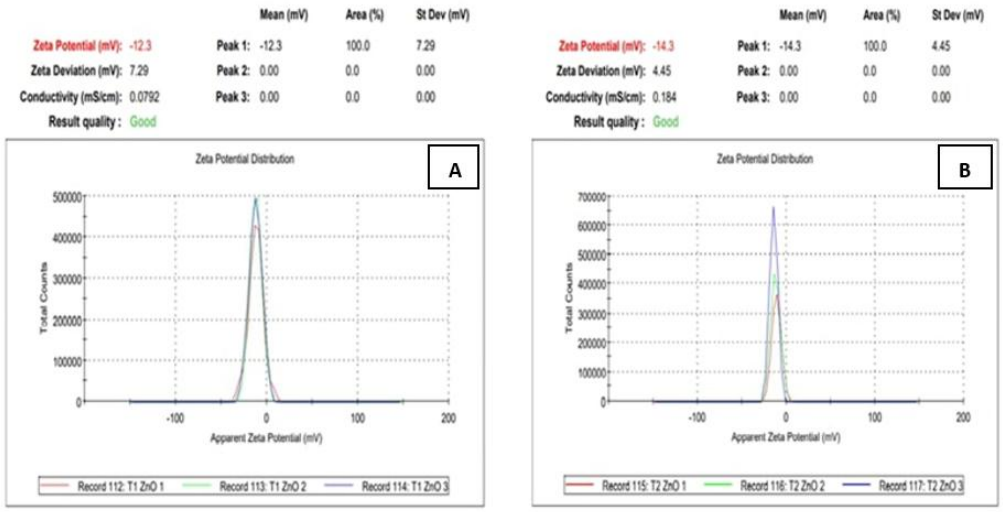

Fig. 2: Zeta Potential of (A) ZnONPs (B) THQ-loaded ZnONPs

Severe swelling represented with a black arrow appeared in the hepatocytes of rats fed with 20 ppb AFB_1 (group B). Focal necrosis was observed in some areas in this group and illustrated using a red arrow. There was a decrease in sinusoidal spacing as shown by the blue arrow. Histopathological examination of liver tissue of rats fed with AFB_1 and ZnONPs (group C) showed mild swelling of hepatocytes with distorted hepatic cords as compared to the toxin group. Normal architecture was retained with less distortion of hepatic cords in the liver of group D treated with AFB_1 and TQ as compared to groups B and C. Group E which was given TQ-ZnONPs also retained its normal appearance. According to the findings of the histopathology examination, the hepatocytes of the toxicated group B were noticeably larger than those of the control group. A disorganized pattern of hepatic fibers was seen in group B as well. Qing et al. (2022) reported some histopathological abnormalities like hepatocyte cellular damage, oedema, inflammatory cell in the portal triad, and localized hepatic necrosis followed by hepatocellular apoptosis that occurred in the livers of aflatoxicated rats. Another change observed in this study was mild swelling of hepatocytes in ZnONPs treated group which was also documented by Naqvi et al. (2023). In another study, thymoquinone showed preserved hepatocytes arrangement, sinusoids, central veins and portal areas (Abduh et al., 2023). The liver sections in TQ treated group displayed partial improvement in liver architecture. A mild degree of inflammation with moderate

congestion in the portal vein and little infilteration in inflammatory cells were seen in TQ treated group (Mohamed et al., 2021). Usual central vein, normal hepatocytes, blood sinusoids and Kupffer cells are indicated in the livers of thymoquinone nanoparticles and thymoquinone groups (Nassar et al., 2023).

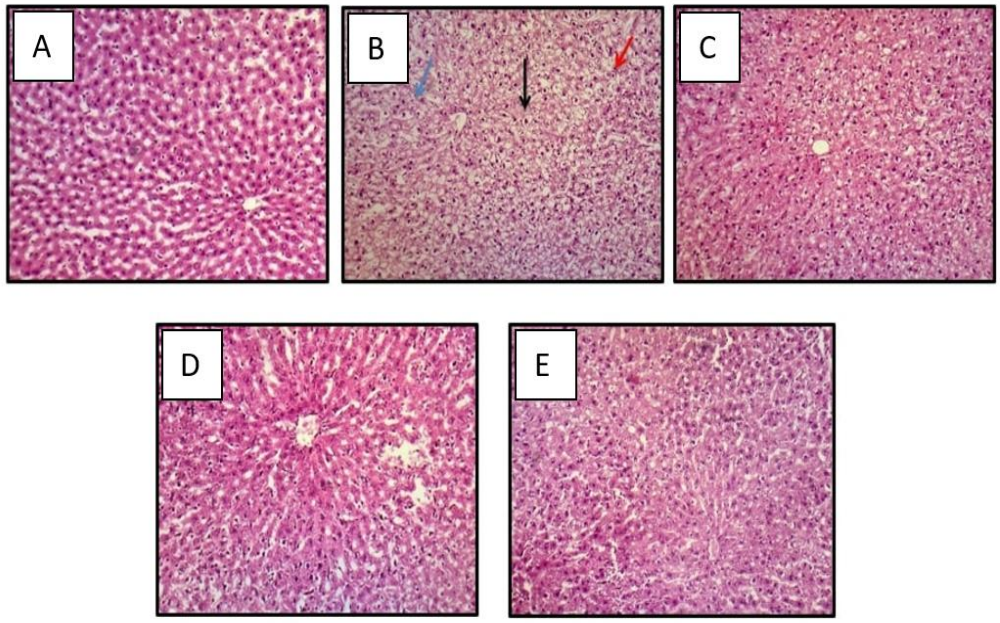

Fig. 3: Histopathological analysis of AFB1-intoxicated liver treated with ZnONPs, TQ, and TQ-ZnONPs. (A) Control liver, (B) AFB1 (20 ppb) exposure causing hepatic cord distortion, hepatocyte swelling (black arrow), focal necrosis (red arrow), and reduced sinusoidal spacing (blue arrow), (C) Mild hepatocyte swelling with distorted hepatic cords in AFB1 + ZnONPs (25 ppb) group, (D) Improved hepatic architecture in AFB1 + TQ group, (E) Enhanced hepatocyte recovery with TQ-ZnONPs. H&E staining, 40X objective lens.

Estimation of serum biochemical parameters

The results of liver function tests are given in Table 1. ALT was observed significantly highest for the toxin group while significantly lowest for the group treated with ZnONPs and thymoquinone group. The ALP values were marked significantly highest for the positive control group while there was a significant decline for the ZnONPs and thymoquinone treated group. A significant elevated level of AST was observed in groups treated with contaminated feed while there was a decrease in AST for group treated with ZnONPs and thymoquinone group. According

to the findings, liver biomarkers improved in the groups that were treated with TQ-ZnONPs. This shows the combined treatment is most effective in lowering values of ALT, ALP and AST. There was an increase in bilirubin value in groups fed with AFB_1 and the second highest value was observed for the ZnONPs treated group. The group treated with thymoquinone alone gave almost similar values of bilirubin like nanoparticles group. The most effective treatment was the combined treatment of ZnONPs and thymoquinone as the group showed the lowest bilirubin value. According to Shrestha et al. (2021), an increase in enzyme activity occurs when liver cells are injured, which also results in an increase in the permeability of the cell membranes of the liver cells releasing ALT and AST into the blood. The administration of ZnONPs significantly decreased the levels of ALT and AST in rats (Mirzaei et al., 2024). The treatment of aflatoxins with thymoquinone led to considerable changes in liver enzymes, as well as the histological appearance of liver tissue (Asgharzadeh et al., 2017). Elevated activity of bilirubin shows the liver damage as literature showed that liver's ability to process bilirubin is impaired, leading to an increase in its level in blood. Long term liver damage and biliary obstruction which is blockage in the bile ducts that carry bile from liver to small intestine can prevent the elimination of bilirubin leading to buildup in blood (Guerra Ruiz et al., 2021).

Table 1 Effects of AFB1, ZnONPs, TQ and TQ-ZnONPs on Liver function tests of albino rats (Means±SD)

Group	**Level of Liver parameters**			
	ALT (U/L)	**AST (U/L)**	**ALP (U/L)**	**TB (mg/ dL)**
A (Normal diet)	75 ± 1.00^{a}	59 ± 4.00^{b}	111 ± 1.00^{b}	0.6 ± 0.01^{a}
B (20 ppb AFB_1)	116 ± 1.00^{b}	109 ± 4.00^{a}	136 ± 3.00^{b}	0.8 ± 0.02^{a}
C (20 ppb AFB_1+ 25 ppb ZnONPs)	45 ± 1.00^{c}	59 ± 4.00^{a}	94 ± 3.00^{c}	0.7 ± 0.03^{a}
D (20 ppb AFB_1+ 10 ppb TQ)	39 ± 1.00^{d}	53 ± 3.00^{b}	116 ± 1.00^{b}	0.6 ± 0.01^{a}
E (20 ppb AFB_1+ 25 ppb TQ-ZnONPs)	28 ± 1.00^{e}	35 ± 4.00^{a}	56 ± 1.00^{a}	0.5 ± 0.02^{a}

Different superscripts written on means in a column show significant differences among groups ($p<0.05$)

Fig. 4 depicts the results of lipid profile and glucose level. Cholesterol levels were observed highest for the negative control as well as for the group treated with ZnONPs and thymoquinone. There was a decline in cholesterol level in positive control. The results showed no positive effect of the combined ZnONPs and thymoquinone treatment in lowering cholesterol levels. An elevated value of high-density lipoproteins was observed for negative control while the lowest value was observed for positive control. The combined treatment of both thymoquinone and ZnONPs gave an HDL value close to positive control. In negative control, low density lipoproteins value was significantly lowest. The highest value was observed for the ZnONPs treated group. The positive control and the group treated with combined treatment of ZnONPs and thymoquinone showed almost similar values for LDL. The lowest triglycerides value was observed for the ZnONPs treated group whereas the highest value was seen in group treated with the combined treatment of thymoquinone and ZnONPs. The negative control also had a higher value of TG as compared to positive control. All these parameters were satisfactorily normalized after treatment with TQ-ZnONPs and toxin. A significantly highest glucose level was observed in the negative control. On the contrary, there was a significant fall in the level of glucose observed in the positive control group. The group treated with ZnONPs alone also showed high values of glucose. Almost similar results were observed for the group treated with thymoquinone and that treated with the combination of ZnONPs and thymoquinone. There is a decrease in blood glucose level in group B treated with aflatoxins as compared to other groups. Gowda et al. (2008) found that AFB1 brought about a reduction in cholesterol levels. Another finding revealed an increased level of LDL and a decrease in HDL level because of liver damage (Abdel-Wahhab et al., 2010). A study showed that AFB_1 feeding results in the lowering of glucose level (Amiridumari et al., 2013).

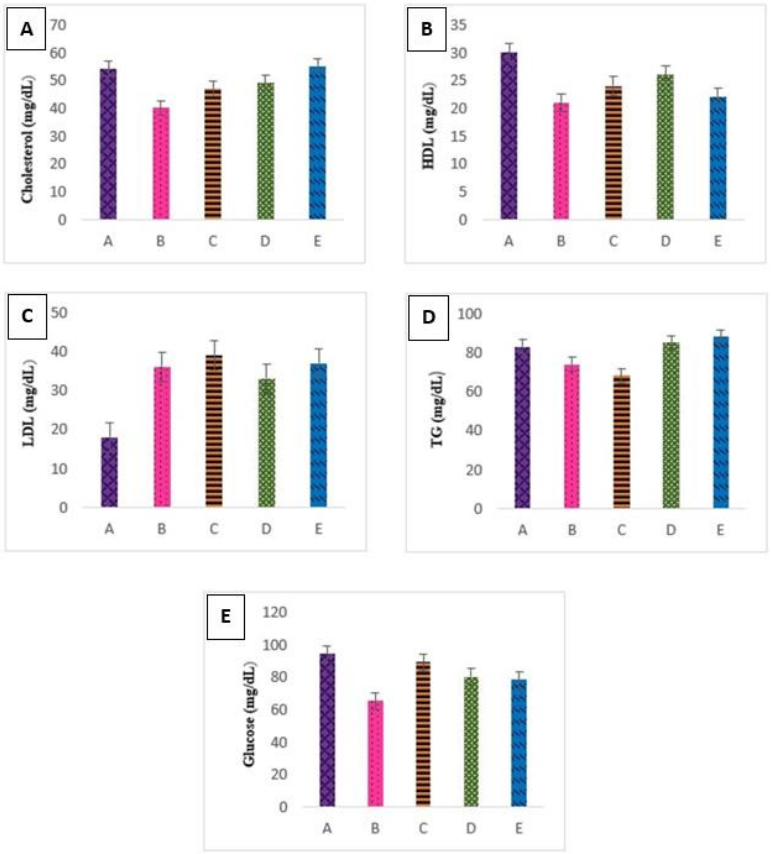

Fig. 4: Effects of AFB1, ZnONPs, TQ and TQ-ZnONPs on (A) cholesterol, (B) HDL, (C) LDL, (D) TG and (E) glucose level. Group A shows control group, group B represents toxin fed group, group C illustrates AFB1 + ZnONPs treated group, group D depicts AFB1 + TQ and group E displays AFB1 + TQ-ZnONPs

CONCLUSION

Based on the findings of current study, it was revealed that AFB_1 had toxic effects on liver in rats. TQ coated ZnONPs have shown promising results in ameliorating the toxic effects of AFB_1 in terms of improvement in histopathological profile and biochemical tests. The combination of TQ and ZnONPs can enhance the hepatoprotective effect, providing an excellent platform for developing safe and efficient therapy against liver damage caused by AFB_1.

ETHICS APPROVAL

All the experimental work related to animal handling and sampling was performed according to the guidelines of the Ethical Review Committee at University of

Veterinary and Animal Sciences, Lahore.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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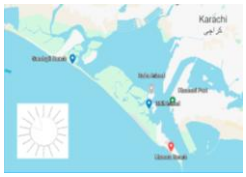

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Reproductive Behavioral Observations of Two Isopsera Species (Orthoptera: Tettigoniidae: Phaneropterinae) under Laboratory Conditions

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ABSTRACT: *Observations on the reproductive behavior of Isopsera spinosa Ingrisch 1990 and I. pedunculata Brunner von Wattenwyl 1878 were made in the laboratory. Specimens were collected from different districts of Sindh, including Matiari, Mirpurkhas, Tando Allah Yar, Hyderabad, and Badin, from various habitats such as wheat fields, mango orchards, guava farms, and grassy areas. A total of sixty adult katydids were taken from the wild, paired, and raised in the laboratory (25°23'N, 68°24'E) under relative humidity ranging from 26% to 61% and temperature fluctuations between 28 ± 2°C and 39 ± 2°C. These conditions closely resembled the field environment. The insects were provided with grass and moistened feeding pellets (comproids). Eggs were collected daily from oviposition trays and transferred to Petri dishes placed on the chamber floor. Fresh food and water were supplied regularly. The optimal time for copulation in both species was similar, occurring in the morning. The duration of oviposition was 33 min for Isopsera spinosa and 53 min for I. pedunculata. The eggs' size, shape, color, and texture were analyzed. For Isopsera spinosa, egg measurements were 5.17–5.22 mm in length and 1.3–1.5 mm in width, while for I. pedunculata, the dimensions were 5.3–5.46 mm in length and 1.4–1.6 mm in width. Differences in the reproductive behaviors of Isopsera spinosa and I. pedunculata were also noted. The exploration of reproductive activities will be beneficial for implementing control measures at appropriate times.*

Keywords: *Isopsera*, Tettigoniidae: Phaneropterinae, Copulation, Oviposition

INTRODUCTION

Sindh province has its agricultural land and cultivates crops like wheat, maize, and other cash crops (Sultana and Wagan, 2015). Fruit farms and vegetable farms are also part of this land. Sindh is also called the land of Sufis. Due to the

restricted mobility and exposed nature of the larval stage, oviposition sites have a significant role in determining the success of the larval stage for most ground beetles and many other holometabolous insects (Thiele 1977), (Lovei and Sunderland, 1996). The Orthopteran family Tettigoniidae includes long-horned grasshoppers. A wide range of grassland insects are found in this group. The term "katydid" is sometimes used to refer to members of the Tettigoniidae family. The order Orthoptera is one of the most destructive insect orders; it comprises cockroaches, crickets, and grasshoppers among other insects. The large number of species and diverse ecosystems that must be taken into consideration make the problem extremely difficult for Pakistan to resolve. Orthoptera are a subject of much interest and research because of their size, conspicuous behaviors, and loud noises. It has been shown by scientists that ten mature grasshoppers in each square yard will wipe out the entire area. There are some predatory grasshoppers. During the present study, insects were reared in cages, and all cages were provided with sand dishes for oviposition. Females deposited eggs in these beakers. In the laboratory, oviposition was rarely seen because it typically happened at night. It was found out at night when the patient came back to the lab. She was uncomfortable being photographed, so she did not finish her pod, but she retracted her abdomen and rested for thirty minutes before leaving the oviposition site. Globally, about 8,361 species of Tettigoniidae, representing 1,365 genera, have been found (Cigliano et al., 2024). Just 159 species, spread across 72 genera, have been identified as being indigenous to India (Shishodia et al., 2010). The following are some significant research on the Tettigoniidae's distribution and classification in India, (Barman and Srivastava, 1976), (Shishodia, 2000), (Shishodia and Tandon, 2000), (Shishodia et al., 2003). Many earlier co-authors have carried out research on the taxonomic status and DNA barcode of Orthoptera (Sultana et al., 2014, 2015), (Samejo and Sultana, 2019), (Ashfaq et al., 2022). However, there is very deficient data on reproductive behavior, except for studies on *Hieroglyphus* by

(Sultana and Wagan, 2007, 2008, 2009a, 2009b, 2010a, 2010b, 2010c), (Shah and Sultana, 2024). No study has been conducted on the reproductive activities of (Tettigoniidae: Phaneropterinae). Therefore, this attempt is being made.

Several authors have contributed to various aspects of Orthoptera, including (Bhanger et al., 2024), (Kumar et al., 2022), (Sultana et al., 2021), (Samejo et al., 2021), (Sanam et al., 2021), (Soomro and Sultana, 2024), (Chandio and Sultana, 2024), and (Memon et al., 2024). However, there is limited focus on the Tettigoniidae: Phaneropterinae, highlighting a gap in the current research.

MATERIALS AND METHODS

Study area

Specimens were collected from different districts of Sindh, from various crops such as wheat, mango fruit farms, guava farms, and grassy areas, as shown in Fig. 1. The specimens were collected by hand, net and by hand during the years 2021-2022. The material was examined and reared using the standardized entomological method described by (Sultana and Wagan, 2015).

Rearing of insects

In 2021, sixty adult katydids of both sexes that were collected in the field were released. Sixty adult crickets that were taken from the wild were coupled and raised in a laboratory at 25° 23'N, 68° 24'E, with a temperature variation of 28±2°C to 39±2°C and a relative humidity of 26% to 61%. These regimes of relative humidity and temperature are comparable to those found in the field. In addition to grass, the crickets were given soaking rat comproids, or food pellets. Every day, petri dishes on the chamber floor were filled with fresh food and water, and the eggs were taken out of the oviposition trays. Soon after being deposited, the eggs were taken out of the oviposition trays, carefully cleaned with tap water, and put on moist filter paper in petri plates. Four little wooden cages with fine-wire mesh coverings held the petri dishes (1 mm) Fig. 2. Similarly, five pairs were selected to observe mating, they were kept isolated, and the duration of copulation was noted.

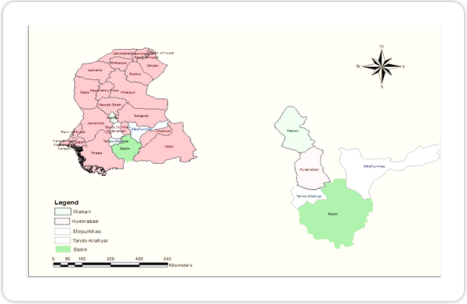

Fig. 1: Map of surveyed districts of Sindh

RESULTS AND DISCUSSION

Copulation Behavior

Though there are certain exceptions, knowledge of how insects behave during copulation includes the use of their genitalia—is essential. Reproductive isolation and the development of intraspecific compatibility and interspecific incompatibility serve as the main evolutionary conditions for modifications in genital conformation and copulatory behaviors. The males and females of *Isopsera spinosa* Ingrisch, 1990 and *I. pedunculata* Brunner von Wattenwyl, 1878 are dimorphic and have distinct sizes; they are not like one another. When sexually mature, the males, who are smaller than the females, call out to conspecific females with a low, species-specific call. If the female is responsive, she uses her antennae to touch the male.

The male backs up to the female along her sides when he finds her to be receptive. After grabbing hold of her with his cerci, the male flips into the mating posture, which involves engaging the female with his cerci, turning 180 degrees to face away from her, and hanging from her in an upright position. The female also hangs by clasping a branch or other support to keep her forelegs in place. Between two and five minutes after copulation, the male begins a series of abdominal contractions to release the spermatophores. After ten to fifteen minutes of copulation, the male continues to compress his abdomen forcefully backward and forward for another 9 to 10 min. Both male and female then stay motionless for a duration of 25–43 min (Fig. 2 and 3). Over 75% of the spermatophylax a gelatinous bolus that some male insects release during copulation along with spermatophores is consumed by the female during this time. Males and females may engage in combat over who gets to eat more spermatophylax, but to preserve the spermatophore, females only let males eat 10–15% of it. After that, the female goes

into a refractory phase, where she jumps away to ward off overtures from other males. According to observations, the male would kick with his rear legs to reject the female if he is not sexually mature, and vice versa. The series of occurrences illustrating *Isopsera pedunculata* Brunner von Wattenwyl, 1878, and *Isopsera spinosa* Ingrisch, 1990, mating behavior is presented. The cage tests yielded important results. Comprehending habitats is crucial for elucidating seasonality and provides insights into the local and regional dispersion of katydid fauna. According to the cage study, katydids are oligophagous, with adults demonstrating a clear preference for soft fruit pods and flower components. These Phaneropterinae of late spring and early summer are probably extinct because of the seasonal passing of late spring and early summer flowering vegetation. For these katydids, changes in the floral component translate into changes in the faunal makeup. This could provide a brief explanation for the delayed seasonal emergence and survival of species such as *huasteca* and *grallator* until mid-July. The study suggested that these insects, unlike the flower-feeding Phaneropterinae, are less reliant on seasonal variations.

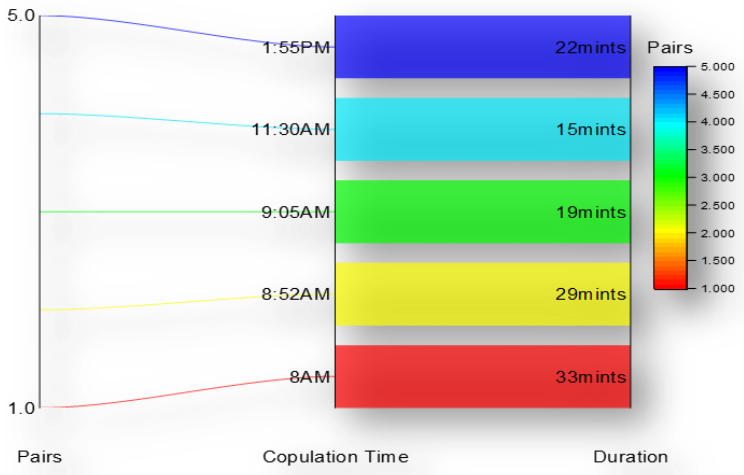

Fig. 2: Duration and copulation starting time of *Isopsera spinosa.*

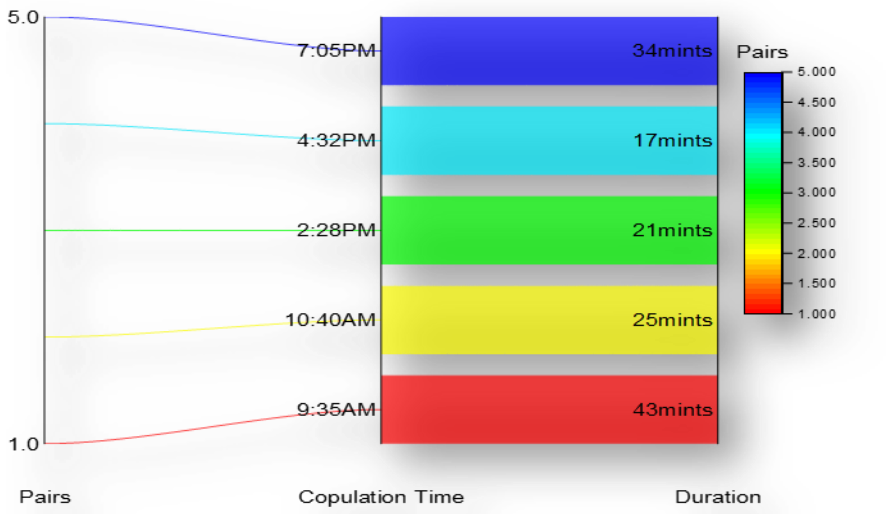

Fig. 3: Duration and copulation starting time of *I. pedunculata.*

Oviposition Behavior

During the present study, insects were reared in cages, and all cages were provided with sand dishes for oviposition. Females deposited eggs in these beakers. In the laboratory, oviposition was rarely seen because it typically happened at night. When the patient returned to the lab at night. She did not finish her pod because the photographing bothered her, but she did withdraw her abdomen and rest for half an hour before departing the oviposition spot. During the present investigation, it was observed that the egg-pod is full of eggs without any empty space. When the freshly deposited eggs were exposed to air and sunlight, their color faded. Their hue was altered over time. Most females released their eggs in clusters. Frothy fluid was discharged from the start of egg laying until the last egg was placed, encircling and holding the eggs in a batch together. Fig. 5 and 6. Secretions and the egg itself comprise an egg. Conversely, a few eggs were collected in the field. All of these were found just below the surface of the earth on tiny, desolate terraces. By excavating the dirt in these terraces, eggs were found as shown in (Fig. 4).

Fig. 4: Rearing of *I. pedunculata.* under lab conditions and eggs of *I. pedunculata*

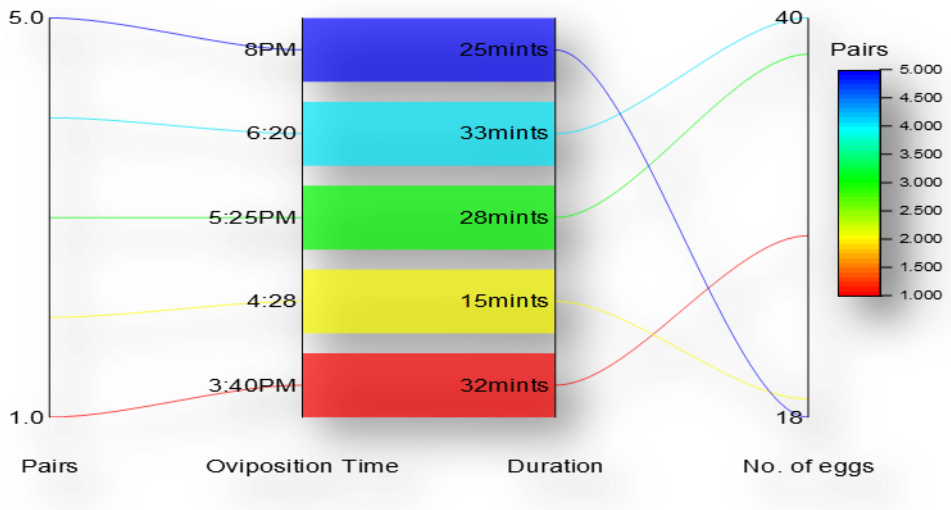

Fig. 5: Oviposition timing and total duration of Isopsera spinosa

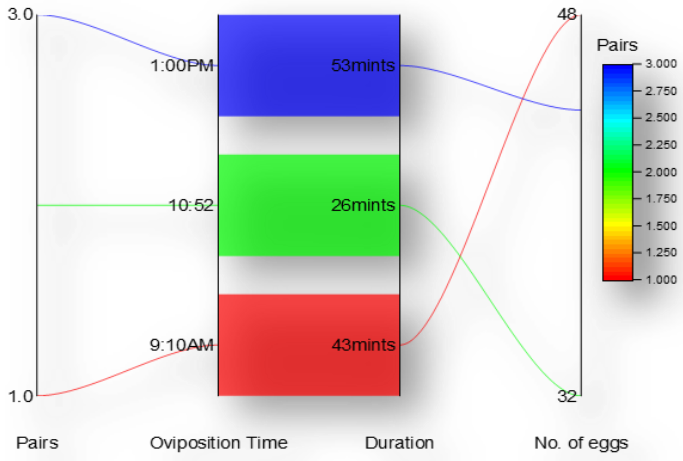

Fig. 6: Oviposition timing and total duration of *I. pedunculata*

Morphology and Morphometry of eggs

Depending on the species, Tettigoniidae eggs can range in size from 1 to 5 millimeters Table 1. They typically have an oval or long shape and appear slightly flattened. These eggs range significantly in color from pale green, white, or cream to dark brown or black. The eggs' exterior shell is smooth, firm, and typically has a hint of shine Figure 4. Tettigoniidae eggs are placed individually in certain species, and in clusters adhering to a surface like a twig or leaf in others.

Table 1: Morphometry of *Isopsera* eggs

Species	Parameters			
	Length of Egg		Width of Egg	
	Range	Mean ± SD	Range	Mean ± SD
Isopsera spinosa	5.17-5.22	5.19±25.95	1.3-1.5	1.4±7
I.pedunculata	5.46-5.3	5.4±27.02	1.4-1.6	1.52±7.6

In this study, specimens were gathered from different districts of Sindh and classified into two species: *Isopsera spinosa* Ingrisch, 1990, and *I. pedunculata*. Although they might rarely seem dark or grey with translucent veins on the tegmina, species in the

Phaneropterinae family are typically green in color. These five Sindhi districts were visited on a regular basis and many different species were collected from them. The subfamily Phaneropterinae includes the leaf crickets, bush crickets, and leaf katydids (false katydids). Our results add to the growing body of knowledge on the reproductive biology and ecology of Tettigoniidea, highlighting the significance of considering a variety of variables while attempting to comprehend the evolution of insect copulation behavior. We propose that the copulation behavior of Tettigoniidea may be significantly influenced by physiological restrictions such genital anatomy, as well as ecological factors like mate choice and male-male competition. In addition to examining the possible effects of environmental variables like humidity and temperature on copulation behavior, future research could examine the connection between copulation behavior and fitness results. Sindh's study of Tettigoniidea biology is extremely important, especially considering the species' reproductive habits. Numerous Tettigoniidea species display unusual mating behaviors, such as promiscuity, polygamy, or monogamy. The development of insect reproductive behavior and the variables affecting it can be better understood by examining these mating systems. The ecological function of Tettigoniidea is another important facet of their biology in Sindh. As many Tettigoniidea species are herbivores, plant ecosystems can be greatly impacted by their eating patterns. Furthermore, a variety of predators, such as birds and mammals, use Tettigoniidea as significant prey, which enhances ecosystems' overall biodiversity. This study highlights reproductive activities of Phaneropterinae. We believe that the current findings presented here will serve as a valuable resource for future entomologists, ecologists, and researchers interested in exploring pest management strategies. By addressing the ecological impact and behavioral characteristics of these species, this research contributes to a comprehensive understanding of insect dynamics in agricultural and natural ecosystems, facilitating informed conservation and management practices.

CONCLUSION

In conclusion, research on the biology and diversity of Tettigoniidea in Sindh is crucial since it can provide insight into the ecological and evolutionary mechanisms influencing the variety of these insects. The vast diversity of Tettigoniidea species found in Sindh is a result of the region's varied habitats and location near the southern border of the Himalayas. Gaining knowledge of the reproductive strategies and ecological functions of Tettigoniidea in Sindh can improve our comprehension of the wider patterns of variation and development within this class of insects.

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DATA AVAILABILITY STATEMENT

The data used to support the outcomes of this study is available from the corresponding author on request.

AUTHORS' CONTRIBUTION

MSD surveyed the area and reared the species; RS designed the study and analysed the data.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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