LGU JOURNAL OF LIFE SCIENCES
Volume 8(1) JANUARY-MARCH (2024)

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801319
Paper Submission: 26th Dec, 2023; Paper Acceptance: 13th Feb 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

A Morphometric Analysis of Suid Remains of the genus *Conohyus* from the Siwalik Beds of District Jhelum, Punjab, Pakistan

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ABSTRACT: *The current fresh Suid dental material was gathered from Pakistan's Siwalik hills, which are located in Punjab and stretch into Northern Pakistan. There have been reports of fossils being found at sites belonging to this genus in the Hasnot hamlet, which is situated in the Punjab province of Pakistan in the district Jhelum region. These particular examples are representatives of a small suid genus called Conohyus, which is commonly found in the Siwalik Mountains. The material that has been analyzed has provided a significant amount of insight into the underlying dentition that is typical of this genus. The teeth of the genus Conohyus are a little more advanced than the condition found in the genus Palaeochoerus.*

Keyword: Artiodactyla, *Conohyus*, Hasnot, Siwalik hills, Suidae

INTRODUCTION

The Siwalik region in Northern Punjab, as well as other locations within these hills, is home to a large population of suids, which are categorized as artiodactyl mammals. Numerous researchers began excavating several different areas on the hills of the subcontinent in the nineteenth century and continued doing so to the present day. During their excavations, they discovered a significant quantity of fossils. For example, among the well-known paleontological researchers who had undertaken research in this region include Falconer and Murchison (1868),

Lydekker (1884), Stehlin (1899), Pilgrim (1910, 1926), Colbert (1935, 1980), Pickford (1988), Made (1996, 1998), Pickford and Morales (2003), and Batool et al. (2015). All of these researchers have conducted their studies in this region. Despite this, there have been a significant number of other paleontological experts who have carried out research in this part of the world, including Draz et al. (2020), Waseem et al. (2021), Raza et al. (2022), Samiullah et al. (2022), Nadeem (2023, 2023a, 2023b). Thousands of years ago, the family Suidae was highly prevalent in the Siwalik hills of the Indo-Pakistan region. At that time, many genera were present as well. This was the result of the family being able to generate a large progeny (Pickford and Obada, 2016; Spassov et al., 2018; Mors et al., 2019).

On the other hand, while numerous species from that era have been repeatedly found in fossil records, certain species have a considerably lower number of representative fossils. As a consequence of this fact, the excavation of any new material of these old species is supposed to be of the utmost importance for the purpose of acquiring adequate knowledge of the role that they performed in evolutionary history and what is their specific position in the course of the development of this genus. The vicinity of Hasnot encompasses five distinct constituent formations, each of which may be discerned from the rest. The Kamlial, Chinji, Nagri, and Dhok Pathan formations are also present, alongside the Soan Formation (Ghaffar and Akhtar, 2012). The Siwaliks in the region of Jhelum were going to be the focus of an investigation that was going to be carried out in order to locate some fossils and identify them to the level of genus or species.

The current exploration work targeted the Nagri formation of the area. The Siwalik beds were divided into three levels, i.e., the Lower Siwaliks (Kamlial and Chinji Formations), the Middle Siwaliks (Nagri and Dhok Pathan Formations), and the Upper Siwaliks (Pinjor, Boulder Conglomerates and Tatrot Formations), by Pilgrim (1913). The targeted Nagri Formation (8-10 million years of age) belonged to the Middle Siwaliks (approximately 1800 meters thick), and it is Miocene in age (Pilgrim, 1910, 1910a, 1913; Jacobs, 1978). Jacobs (1978) also correlates the Siwalik's Nagri Formation with the Valesian Formation of Europe. The Nagri Formation is composed of red clay with included nodules. The Nagri outcrops exhibit cyclic alternation of

sandstone with subordinate clay and conglomerates. The sandstone is grey, greenish-grey, light grey, and medium to coarse-grained. In some places, this sandstone is bluish-grey, calcareous, and poorly cemented. Subordinate clay is sandy, silty, brown, chocolate brown, reddish grey, or pale orange in appearance, while the Conglomerates consist of pebbles of igneous rocks and Eocene limestone.

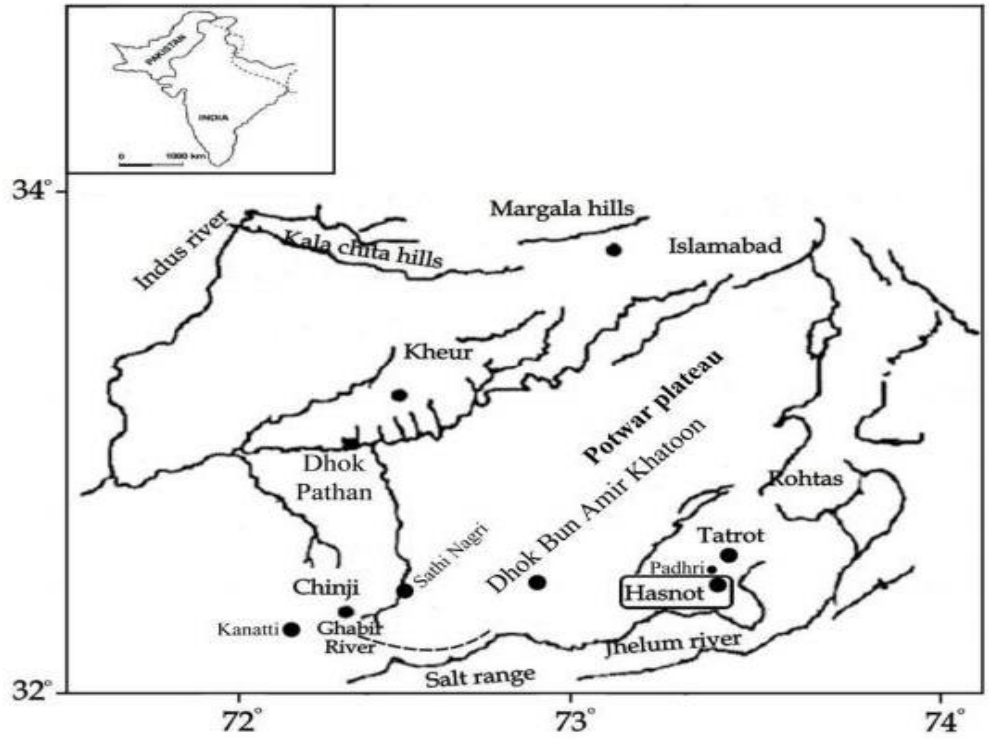

Fig. 1. A map of the Potwar Plateau in Punjab and northern Pakistan shows the research region (Barry et al., 2002)

MATERIALS AND METHODS

The material under study was collected from the Nagri Formation located in Punjab, Pakistan, in the general location of the Hasnot (Lat. 32.824167 and Long. 73.131111) hamlet of district Jhelum. It was found partially exposed and hence recovered carefully with the help of paleontological tools, including a light hammer, chisels, sharp needles, brushes, etc. Currently, the collected material is deposited in the Department of Zoology's paleontological collections at Government College, now known as GC University, situated in Lahore, Pakistan. Millimeters (mm) were used as the unit of measurement for the measurements that were taken with a

Vernier caliper from the metric system. An examination is made of the morphometric characteristics of the specimen that is being investigated. The specimens have a serial catalog number, the denominator of which is the year of collection and the numerator of which is the specimen's serial number. For example, A Government College Palaeontological Collection Number 377/2001 is also abbreviated as G.C.P.C. No. 377/2001. Both the terminology and the techniques of the measurement of recovered material are based on a reference to Pickford (1988), which serves as the foundation for both of these aspects. Different specimens present in the palaeontological collection of the University of the Punjab, Lahore, were also examined along with the literature review of the previously described specimens like the "Transactions of the American Philosophical Society by Colbert (1935)".

Abbreviations used: The following abbreviations are used in the manuscript;

G.C.P.C. Government College Palaeontological Collection.

P.U.P.C. Punjab University Palaeontological Collection.

Ind. Mus. Indian Museum, Calcutta.

RESULTS

Systematic Account The systematic palaeontological account of the excavated sample is as under;

Order Artiodactyla Owen, 1848

Family Suidae Gray, 1821

Genus *Conohyus* Pilgrim, 1926

Species *C. sindiensis* (Lydekker, 1884) Pilgrim, 1926

The specimen under investigation

G.C.P.C. No. 377/2001, is an isolated upper second molar of the right side recovered from Hasnot, located in District Jhelum of Punjab, Pakistan.

DESCRIPTION

The morphometric details of the recovered specimen under study are as follows:

Specimen 377/2001 (Second Molar) (Fig. 2).

The isolated right upper second molar G.C.P.C. 377/2001 was taken from Hasnot, district Jhelum, Punjab, Pakistan. It may be a second molar because there is a noticeable pressure mark on both the front and back sides of it. The specimen is virtually square with a low-crowned structure (Table 1). The specimen appears rough from its lingual surface, which may be the result of the weathering process; the tooth is well preserved and covered with a thick layer

of corrugated enamel. The enamel layer is shining all around the tooth, with the exception of the lingual side. Additionally, there is a delicate layer of cement that can be found on the posterolingual sides of the tooth. The ratio of its height to width index indicates that it is a type of tooth that is classified as a bunodont-brachyodont relationship. On the anterior as well as posterior sides of the tooth, a robust multi tuberculated cingulum is present. On the lingual side, it is just slightly weak, and on the labial side, it is virtually entirely no longer there. The crown of the tooth is relatively thin or narrow.

Crown view **Labial view** **Lingual view**

Fig. 2. Right upper molar of *Conohyus sindiensis* recovered from Hasnot

Every single one of the four major cusps is clearly defined. In terms of vertical height, the lingual cones, which are referred to as hypocone and protocone, are lower than the labial cones, which are recognized as paracone and metacone. The front portion of the tooth's protocone has been worn down to a significant degree, and as a result, a small landmass has emerged on the front side of the protocone, revealing the dentine. The suid grooves on the front, back, and middle are just starting to form. The anterior accessory conule and the protocone are linked through a narrow channel that connects the two structures. The tooth's paracone has a significant amount of wear and tear. There is a little dentinal channel that is present, and it is connected to the anterior accessory conule. It has a very thin coating of cement that lies inside of it. The three characteristic suid grooves, which are the anterior, the posterior, and the middle or median suid groove, are present and clearly observable. On the anterior aspect of the main anterior cusps, is where you will find the anterior auxiliary conule. This conule is largely worn out and has almost reached the same level as the anterior cingulum. In the middle of the crown, there is a

median accessory conule that has become flattened lingually and labially. This occurs because the crown has been worn down to a significant degree. An incipient dentine is exposed in the midst of the conule, which results in the formation of a tiny dentinal islet.

Additionally, the hypocone (a posterior cone) of the excavated tooth is largely worn out and has a vertically low height. Clearly discernible are the three characteristic suid grooves that are distinctive of the suid. A dentinal islet can be found on the upper side of the hypocone, and it is within this islet that a little dentinal valley is produced. Additionally, there is a small cover of cement that is observable lingually. A significant amount of wear and tear has also been placed on the tooth's metacone, and the labial side of the tooth is primarily injured. Notable suid grooves consist of three distinct grooves that are immediately identifiable.

At the foundation of the two principal cones, i.e., para and metacone, the substantial basal pillars on the labial side serve as a marker for the entrance of the transverse valley through the tooth. Additionally, there is a sturdy foundational structure on the inner side of the tooth, positioned near the beginning of the transverse valley. In comparison to the labial side, the lingual side of the tooth has a valley that is generally more expansive. We can also make out a valley that runs longitudinally. There is also a posterior auxiliary conule located on the back side of the tooth, which is connected to the posterior cingulum. This connects the two structures. As a result of prolonged wear, it has nearly become flattened.

Table 1: Measurements of upper dentition of *Conohyus sindiensis* (Lydekker) and its comparison with the already recovered material.

Specimen No.	Position	Length (mm)	Width (mm)	W/L Index
G.C.P.C. No. 377/2001	M^2	18.0	15.0	83.3
P.U.P.C. No. 443/69	M^2	17.0***	16.2***	95
Ind. Mus. B. 536	M^2	16.5*	16.7*	101
Ind. Mus. B. 102	M^2	16.8**	18.8**	112
Ind. Mus. B. 101	M^2	16.2**	15.4**	95

* Taken from illustrations made by Pilgrim (1926)
** Taken from Lydekker (1884)
***Taken from Ahmad (1995)

DISCUSSION

The specimen is collected from the middle Siwalik beds of the outskirts of Hasnot, located in the district Jhelum of the Punjab province, Pakistan. Since it has been collected from the middle siwaliks where prototheres and metatheres are absent as fossils. Lydekker (1876, 1884) identified a number of solitary molars and maxillary fragments as belonging to the genus *Hyotherium*. Lydekker, op. cit., pl. XII, figures 7-8 and 10-11 demonstrate that some of these specimens have primary cusps that are rounded and simple, and they have incipient grooves and median accessory tubercles. All of these characteristics characterize the molar teeth that belong to the genus Conohyus. *Conohyus sindiensis* was initially given the name *Hyotherium sindiensis* by Lydekker (1884). However, Pilgrim (1926) later changed the name of the species to *Conohyus sindiensis* and transferred it to the appropriate genus. Therefore, in this manner, Pilgrim (1926) establishes a new genus of organisms belonging to the family Suidae.

It is the isolated teeth, remnants of maxilla, and mandibles that are the most common way that *Conohyus sindiensis* is recognized. The differentiation was established based on the morphology of the summit of the 4th maxillary premolar (P^4), which is conical in shape in the *Conohyus* genus but has two cusps in other genera. In the genus *Conohyus*, the upper third and fourth premolars (P^3 and P^4) are larger relative to the molars. However, in other species of the same subfamily, these premolars are of average size.

An indication that the tooth in question does not belong to any carnivorous species but rather to a herbivorous mammal is the rounded shape of the cones that are present in the specimen in question. It is determined that the tooth belongs to the family Suidae of Gray (1821) because of the dense clustering of conelets. Five subfamilies fall under the umbrella of the Suidae family, as stated by Simpson (1945). Hyotheriinae, Listriodontinae, Tetraconodontinae, Sanitheriinae, and Suinae are the subfamilies that fall under this category. Due to the fact that the characteristics of the tooth belonging to the specimen under investigation are more closely related to the Tetraconodontinae subfamily, the specimen has been classified as belonging to this subfamily. Within the Tetracocodontinae subfamily, three genera can be found: *Tetraconodon, Sivachoerus,* and *Conohyus*. In order to make a comparison with the specimen that is

being investigated, the first two genera are too large.

The *Conohyus sindiensis*, the *Conohyus chinjiensis*, and the *Conohyus indicus* are the three species that are currently classified as belonging to this genus. *Conohyus sindiensis* and *Conohyus indicus* are the two species that make up the majority of the Siwalik material that belongs to the genus *Conohyus*. *Conohyus sindiensis* is a tiny species that belongs to the genus and has a cingulum that develops on a weekly basis. There is a massive species of the genus known as *Conohyus indicus*, which Lydekker (1884) described as spanning from the Nagri to the Dhok Pathan formation. *Hyaenodon indicum* was the name that Lydekker called it when it was first described. It was initially identified by Pilgrim (1910) as a tooth belonging to the *Hyotherium*; however, in 1926, he moved the species to the *Conohyus* genus due to his discovery. Although there is a substantial similarity between the dental material of *Conohyus sindiensis* and that of *Conohyus chinjiensis*, it is incredibly challenging to differentiate between the two species.

According to Colbert (1935), the Chinji Formation is home to a species of the genus known as *Conohyus chinjiensis*, which is a relatively small species. It is discovered that it is identical to the species *Conohyus sindiensis*. When it comes to distinguishing between *Conohyus sindiensis* and *Conohyus chinjiensis*, the characteristics that Pilgrim (1926) cites as distinguishing characteristics are questionable. According to Pilgrim (1926), the talon of the third molar is quite variable in every species of the suid. Therefore, in this specimen, the talon is relatively short, while the breadth of the tooth is relatively large.

CONCLUSION

The tooth under study is from Hasnot, which is included in the Middle Siwaliks and mainly comprised of Nagri and Dhokpatan Formations. It is quite a short, low-crowned tooth with a simple median accessory conule. All these are the typical characteristics of the genus *Conohyus sindiensis.* On the basis of comparisons and other details of the excavated material with the previously described material, it is concluded that the material belongs to the species *Conohyus sindiensis*.

AUTHORS' CONTRIBUTION

Amir Nadeem Conceived the idea, conducted field surveys, identified the excavated material, wrote the original manuscript, and reviewed and edited it for final submission.

CONFLICT OF INTEREST

The author declares no conflict of interest.

FUNDING

No one funded for the publication of this article.

ACKNOWLEDGEMENT

The author would like to thank the Chairperson of the Zoology Department, Government College, Lahore, Pakistan, for providing all the necessary facilities during this research project.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801320
Paper Submission: 6th Dec, 2023; Paper Acceptance: 25th Feb 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Illegal Hunting is a Major Threat to Important Wildlife Species of Mangla Dam Freshwater Reservoir

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ABSTRACT: *Illegal hunting at Mangla Dam Wetland is the focus of the present investigation, which aims to determine its extent and root causes. Field surveys, primary and secondary data collection, and scheduled meetings with relevant departments, communities, and hunters made up the research. Over a decade, from 2011 to 2020, five different areas of Mangla Dam were monitored for instances of hunting birds, reptiles, and mammals. In 2013, there were 793 recorded cases of hunting, and in 2019 and 2020, there was a dramatic fall in hunting (COVID-19). Pangolins, migratory birds, wild boars, jungle hares, and scorpions were among the most sought-after and illegally hunted animals. During the research, 6416 instances of illegal hunting were documented. In 2013, the hunting index reached its peak of 22 during the past decade. Game hunting, the ease of obtaining firearms (12 bore and repeater), the desire to make money by locals and animal dealers in response to poverty, poor law enforcement, and a general lack of knowledge were the primary drivers of illicit wildlife hunting. This prompted the government to take decisive action in safeguarding animals through the implementation of stringent regulatory frameworks and conservation initiatives.*

Keyword: Illegal Hunting, Migratory birds, Pangolin, Hunting Index

INTRODUCTION

The urgent necessity for a worldwide concerted effort to address the declining world's biodiversity has been highlighted by Lindsey et al. (2020). Loss of habitat and poaching pose major dangers to wildlife in Asian countries (Ullah et al., 2020). When it comes to managing and protecting animal species, studies on wildlife commerce and harm are crucial as said by Prakash et al. 2020. The conservation goals can be attained by the use of wildlife law, among other instruments (Ostrom, 1999). As a result of illegal hunting for game, meat, and trafficking, the populations of many bird and animal species were declining. Hunting wild animals was formerly a common practice in Pakistan. Challenges in protecting biodiversity included inefficient administrative processes, a high illiteracy rate, and a lack of cooperation among relevant government agencies. Poverty and rapid population expansion both contribute to the unsustainable use of natural resources to meet basic human needs, which in turn reduces biodiversity in Pakistan (GOP, 1999). There are currently several statutes in Pakistan that aim to preserve the country's forests, animals, and fisheries. However, reports continue to indicate a rapid decrease in Pakistan's biodiversity. The survival of animals in their native habitats depends on the passage of new legislation and the revision of current ones (GOP, 1999). Illegal hunting, dread, and illicit commerce accounted for the vast majority of wildlife crimes in Pakistan. While reptiles were mostly targeted due to fear, birds were the most common victims of illicit hunting. Yet, according to Haq et al. (2023), all three groups of animals were being negatively impacted by the illicit trade.

Many plant and animal species call wetland areas home. A plethora of rare, endangered, and indigenous species grace their land. Wetlands were crucial to the survival of six fish species, five mammalian species, nine bird species, six reptile species, and seven hundred and twenty-seven fish species in Pakistan (Ali, 2009). The abundance of food and shelter in wetlands makes them ideal habitats for many different kinds of wild animals (Ali and Ripley, 1983). According to Baig and AL-Subaiee, (2011), unlawful hunting and habitat destruction caused a decline in Pakistani wildlife. Basic drivers to disturb the wildlife included things like a growing human population, low incomes, and lax law enforcement.

Many indirect hazards to wildlife were reported near Mangla Dam (Ali et al., 2011). These included the exploitation of the ecosystem for firewood, livestock grazing, and encroachment, which led to habitat deterioration. The inhabitants of Mangla Dam often resort to using fighting or hunting dogs to slaughter animals. Additionally, for amusement, they unlawfully pursued foxes, jackals, and wolves.

Many Asian countries have documented the negative effects of hunting on wetland bird populations, and the current harvest level is unsustainable (Gallo-Cajiao et al., 2020).

In 1967, construction began on the Mangla Dam, which is now the ninth-biggest dam in the world. As a lacustrine (permanent freshwater lake) wetland, Mangla Dam is a type of freshwater wetland (Ali, 2006). Proper management ensures the preservation and protection of wildlife in Bund Khushdil Khan, Haleji Lake, Hub, and Terbella Dams, among other wetlands of a similar type. Despite it is one of the important wintering grounds for migratory birds and habitat ecologically important wildlife.

The present research work has been undertaken to assess the hunting pressure and causes of illegal hunting of important wildlife causes threats to wildlife at the globally important but neglected freshwater reservoir the Mangla Dam.

MATERIALS AND METHODS

Study Site and Duration

The study was conducted at five pockets of the Mangla dam freshwater reservoir (Fig. 1), which include Kanshi Pocket (Khadamabad and Siakh), Khad Pocket (Chakswari and Islamghar), Jeri Pocket (Jeri and Kakra), Poonch Pocket (Dadyal and Palak), and Mangla Pocket (Main Dam). The data on illegal hunting was collected for ten years, from 2011 to 2020 (Ali et al., 2011).

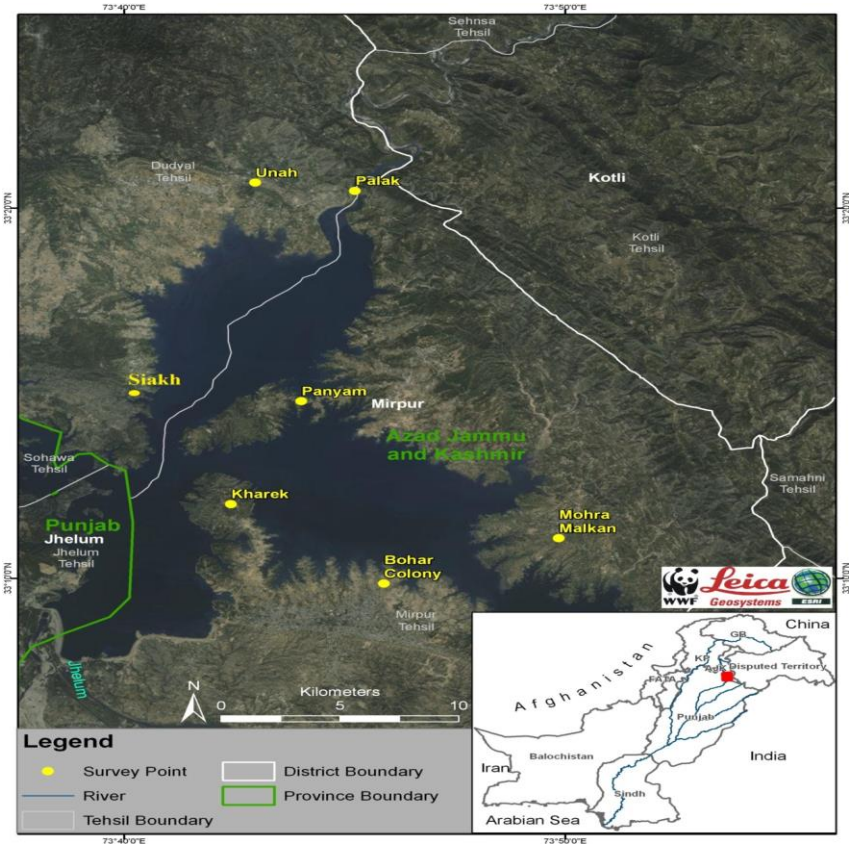


Fig. 1. Mangla Dam (Credit: WWF-Pakistan)

Data Collection

Direct Observation

The data on illegal hunting and its causes were collected as under:-

Field Surveys

The field surveys of Mangla Dam were conducted for direct observation of hunting at sites with high hunting pressure on proximities of the dam.

Boat survey

The reservoir has been divided into five pockets which were surveyed by motor and paddle boats to measure the wildlife and birds' hunting and threats to their existence.

Night surveys

The nocturnal birds and animals were also killed at night to record hunting at that time night surveys were also conducted.

Indirect Observation

Indirect observations of hunting pressure and illegal trade were collected from the local community, fishermen hunters and concerned government

departments through informal meetings with the help of a questionnaire-based study.

Field Guides

Identification of birds and mammals during surveys was carried out by using different field guides and information from experienced field professionals. The field diaries and data sheets were developed to maintain the field record. The field guides by different authors (Roberts, 1991 and 1992 and 1997; Grimmett *et al.,* 1998 and 2008;) were used during surveys.

Hunting index

During the study reports of illegal hunting were also collected from villagers, by personal observations, hunters and the Wildlife and Fisheries Department, AJK. These reports were used to determine the Hunting Index (Ali, 2009) as under:

$$\text{Hunting Index} = \frac{\text{Hunting incidents reported at Mangla Dam}}{\text{Total Number of Surveys of Mangla Dam}}$$

RESULTS

According to the current study, results are indicating that 20 species of birds, 02 species of reptiles, 04 species of mammals, and 01 species of insect. are victims of hunting. Among 27 animals of different categories, Black partridge*(Melanoperdix niger)* was Vulnerable,Indian Rock Python *(Python molurus)* was Threatened, Pangolin *(Manis crassicaudata)* was Endangered, and the remaining 24 were Least Concern.

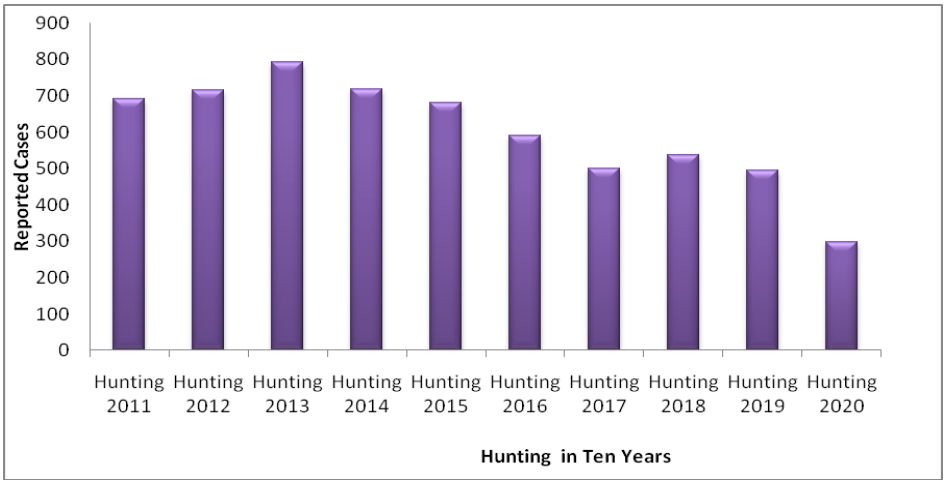


Fig. 2. Reported Illegal Hunting Cases of Wildlife Species from 2011-2020

Department of Wildlife and Fisheries AJK adopted The Punjab Wildlife (Protection, Preservation, Conservation, and Management) Act, 1974 till 2013 for the conservation and protection of wildlife. In 2014, the Department of Wildlife and Fisheries AJK fully opted Azad Jammu and Kashmir Wildlife (Protection, Preservation, Conservation and Management) Act 2014. The Fig. 2 showed the reported hunting cases of different wildlife species, indicating that the highest hunting report was collected from 2012 to 2014. In 2019 and 2020, the hunting records were 495 and 297, respectively.

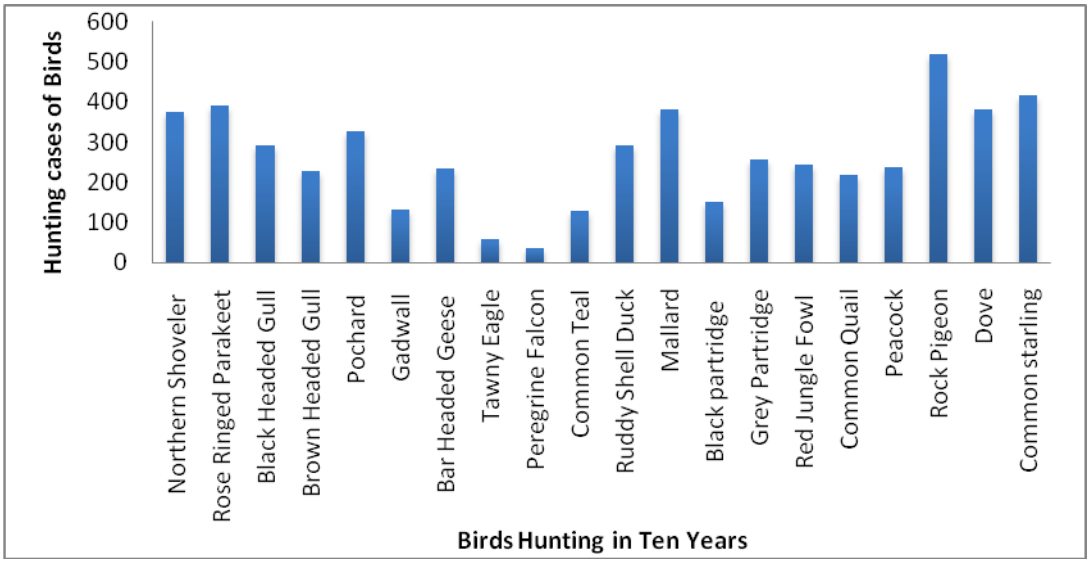
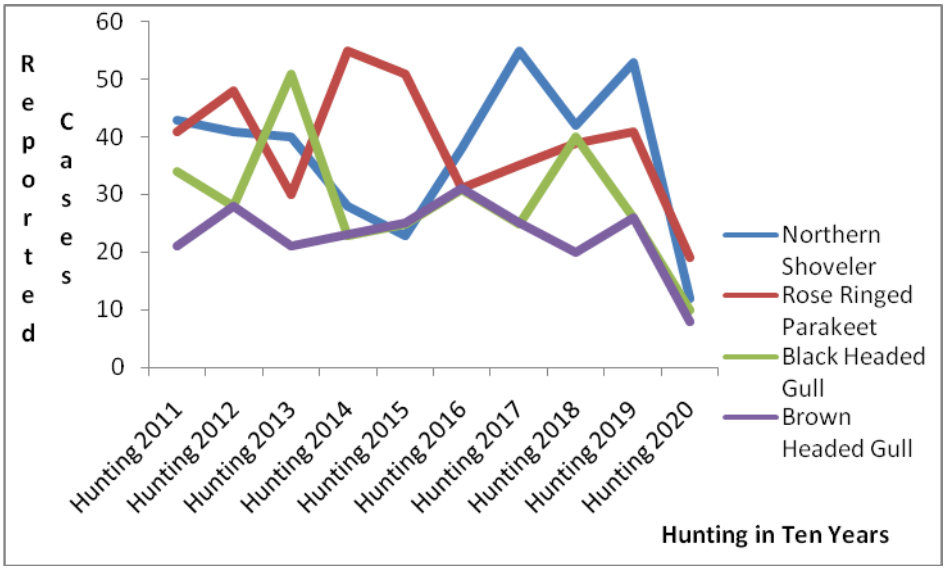
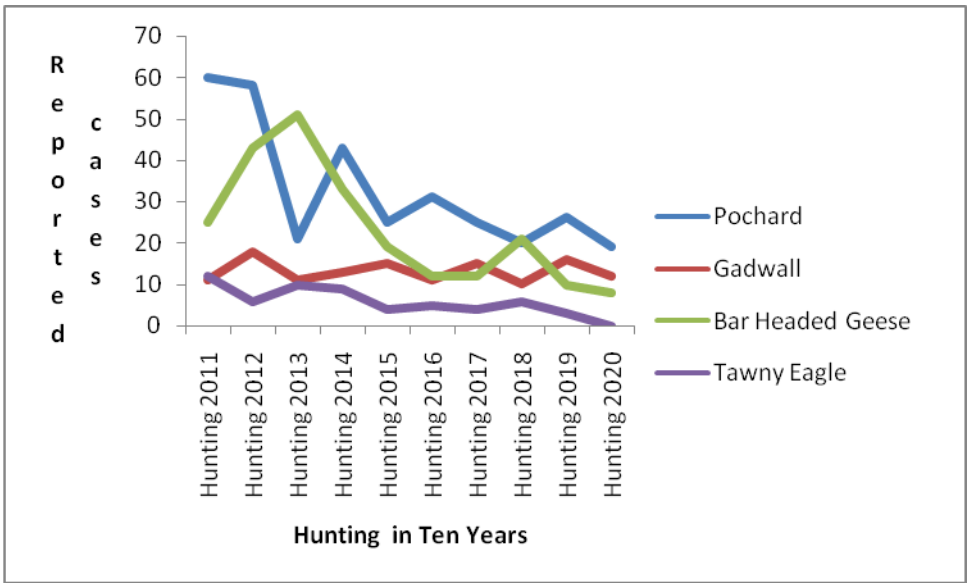


Fig. 3. Birds Illegal Hunting Cases Recorded in the Last Ten Years (2011-2020)

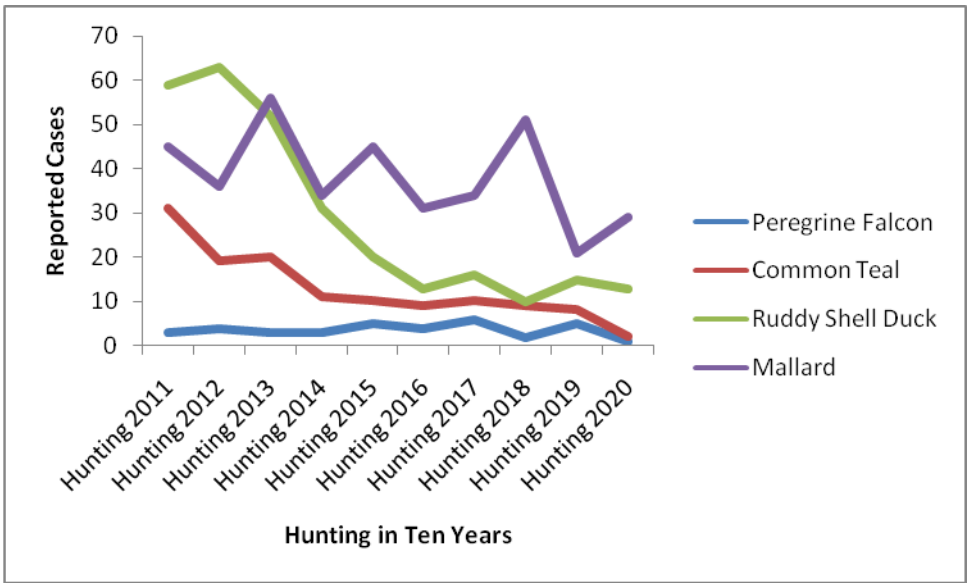
The area around the dam was rich in biodiversity and natural resources. People were involved in illegal wildlife trade and earned millions if they got a chance to sell any most demanding species. They made money by wildlife trapping, trading, and collecting. The most vulnerable hunting species at the dam were migratory birds (Fig. 3).


Reported Cases
60
50
40
30
20
10
0
Hunting 2011
Hunting 2012
Hunting 2013
Hunting 2014
Hunting 2015
Hunting 2016
Hunting 2017
Hunting 2018
Hunting 2019
Hunting 2020
Hunting in Ten Years
Northern Shoveler
Rose Ringed Parakeet
Black Headed Gull
Brown Headed Gull

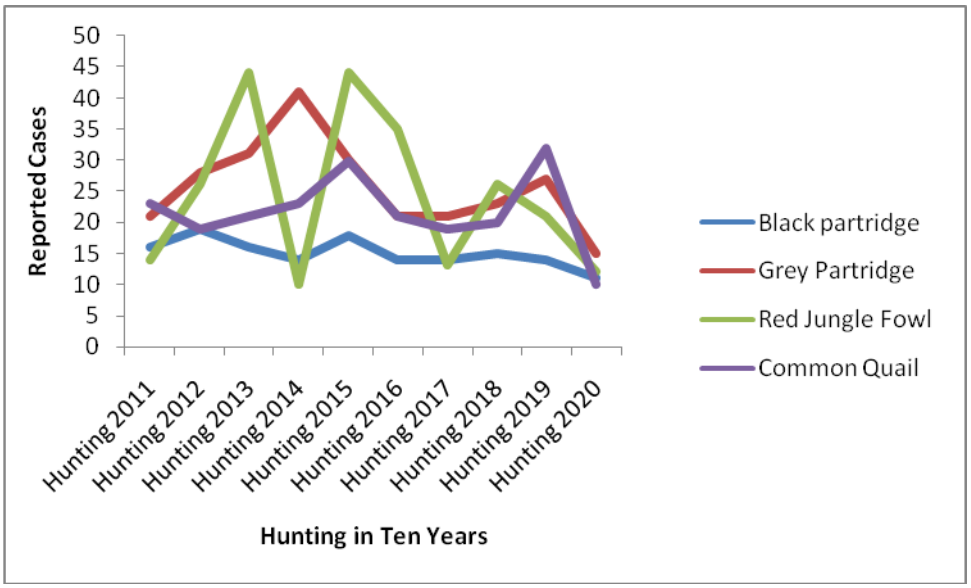
(3A)


Reported cases
70
60
50
40
30
20
10
0
Hunting 2011
Hunting 2012
Hunting 2013
Hunting 2014
Hunting 2015
Hunting 2016
Hunting 2017
Hunting 2018
Hunting 2019
Hunting 2020
Hunting in Ten Years
Pochard
Gadwall
Bar Headed Geese
Tawny Eagle

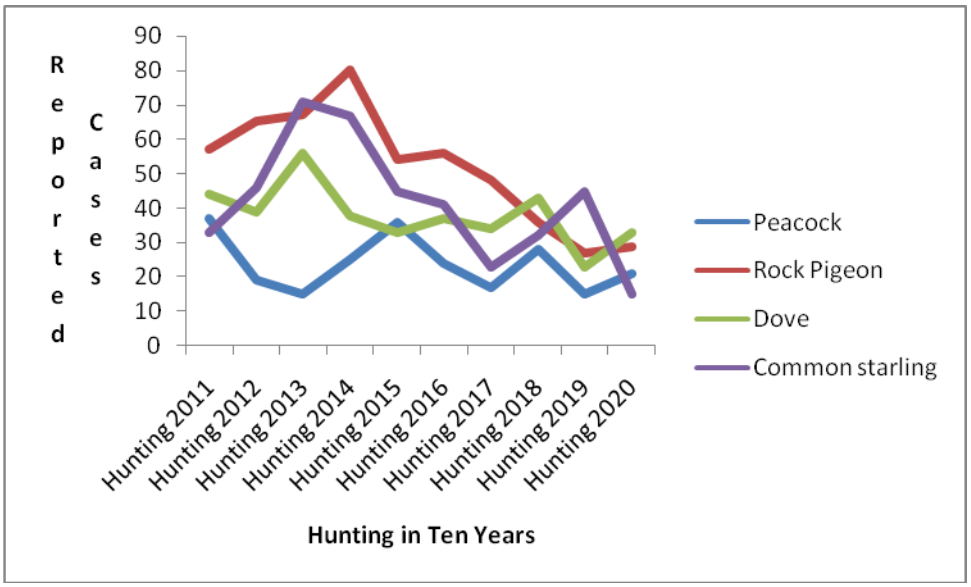
(3B)


70
60
50
40
30
20
10
0
Reported Cases
Hunting 2011
Hunting 2012
Hunting 2013
Hunting 2014
Hunting 2015
Hunting 2016
Hunting 2017
Hunting 2018
Hunting 2019
Hunting 2020
Hunting in Ten Years
Peregrine Falcon
Common Teal
Ruddy Shell Duck
Mallard

(3C)


50
45
40
35
30
25
20
15
10
5
0
Reported Cases
Hunting 2011
Hunting 2012
Hunting 2013
Hunting 2014
Hunting 2015
Hunting 2016
Hunting 2017
Hunting 2018
Hunting 2019
Hunting 2020
Hunting in Ten Years
Black partridge
Grey Partridge
Red Jungle Fowl
Common Quail

(3D)



(3E)

Fig. 3A to 3E. Recorded Illegal Hunting Among Avian Species (2011-2020)

In Fig. 3A there is an increase in the hunting of Northern Shoveler (*Spatula clypeata*), Rose Ringed Parakeet (*Psittacula krameri*), Black Headed Gull (*Chroicocephalus ridibundus*), and Brown Headed Gull (*Chroicocephalus brunnicephalus*) was noticed and a decline due to pandemic is also very clear.

According to Fig. 3B, the reported cases of illegal hunting of Tawny Eagle (*Aquila rapax*) and Gadwall (*Mareca strepera*) are very few. But hunting of Pochard (Aythya ferina) and Bar Headed Geese (*Anser indicus*) was significantly high during 2011-2014 and also declined in 2019-2020 (COVID-19).

The hunting of Common Teal (*Anas crecca*), Ruddy Shell Duck (Tadorna ferruginea), and Mallard (Anas platyrhynchos) was high from 2011 to 2013. While illegal hunting of Peregrine Falcon (*Falco peregrinus*) was recorded less within 10 years (Fig. 3C)

Black partridge (*Melanoperdix niger*) hunting cases were less as compared to Grey Partridge (*Perdix perdix*), Red Jungle Fowl (*Gallus gallus*), and Common Quail (*Coturnix coturnix*) because of their high demand in restaurants (Fig. 3D).

The Indian Peacock (*Pavo Cristatus*) trapping was less observed, while the Rock Pigeon (*Columba livia*) Eurasian collared dove (*Streptopelia decaocto)*, and Common starling (*Sturnus vulgaris*)

were hunted and trapped at a significant level during 2011-2013 (Fig. 3E)

During the study, the hunting among 20 species of birds was recorded. The migratory birds were hunted at most like Bar Headed Geese (234), Northern Shoveler (375), Pochard (328), Ruddy Shell Duck (292), Common Teal (129), and Mallard (382) Among other birds species Rock Pigeon (519), Common Starling (418) Black partridge (151) and Grey Partridge (258), Eurasian Collared Dove **(**380), and Common Quail (218) (Fig: 3A-E).

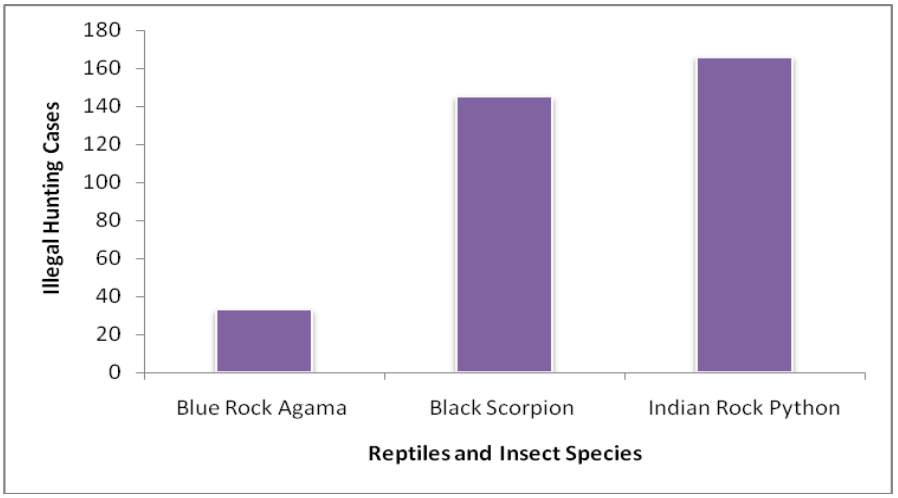


Fig. 4. Illegal Hunting Case of Reptiles and Insect (2011-2020)

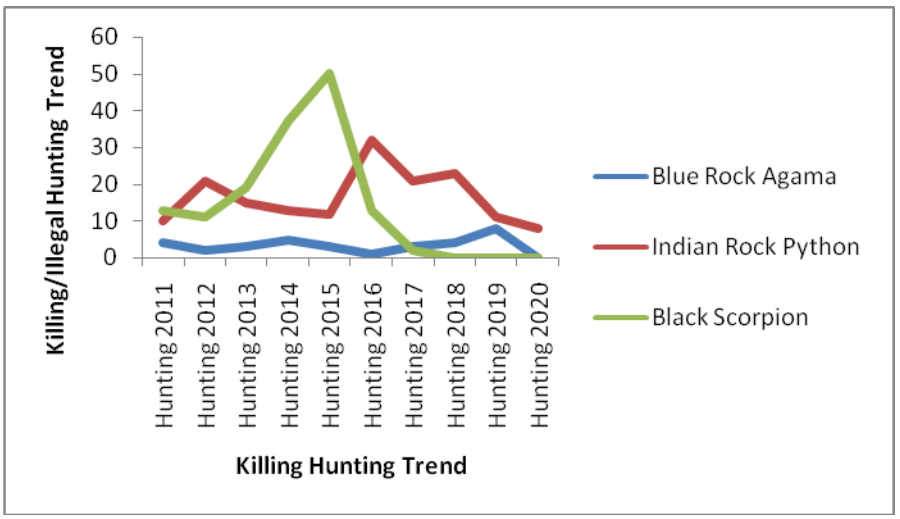


Fig. 5. Trend of Killing and Illegal Hunting (2011-2020)

As per the illegal hunting/killing cases in reptiles, two species were of great concern Blue Rock Agama (*Laudakia tuberculata*) and Indian Rock Python

(*Python molurus*). The *Python molurus* is Threatened and facing great pressure of hunting and killing. Among Insects Black Scorpion (*Androctonus bicolour*) was of great importance in illegal trade. The cases of illegal hunting of Blue Rock Agama were less but killing/illegal hunting of Indian Rock Python was very high during ten years of study. The population of Black Scorpions fell victim to illegal hunting/trade from 2012 to 2017 due to the high demand for its venom in cancer treatment (Fig. 4 and 5).

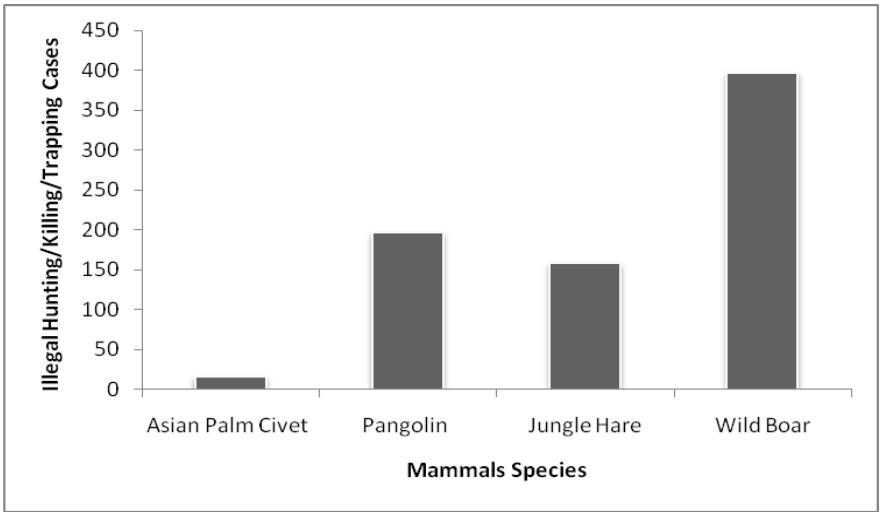


Fig. 6. Illegal Hunting/Killing/Trapping Cases of Mammal (2011-2020)

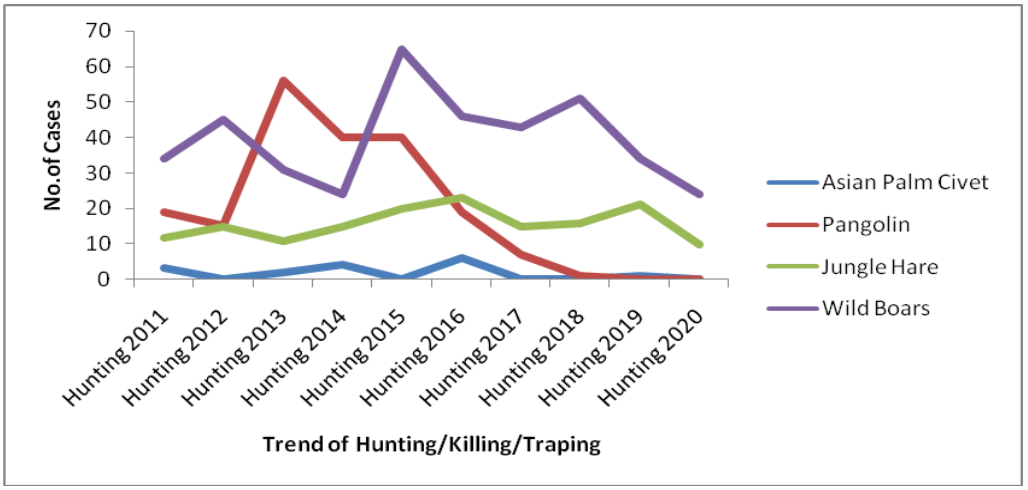


Fig. 7. Trend of Hunting/Killing/Traping (2011-2020)

The cases of illegal hunting of 04 mammal species were recorded (figures 6 and 7). The Wild Boar (*Sus scrofa*) and Pangolin (*Manis crassicaudata*)

were the main victims of these illegal activities. The Wild boars were killed/hunted due to dislikeness or for game hunting the trend lines indicate that it was on a continuous scale a small decline was observed in 2019-2020 (Pandemic). The other important mammal that was trapped or killed on a large scale was Pangolin. In 2011 and 2012, the cases were very low, but a great intensity of illegal activities was noticed from 2013 to 2016. The Pangolin is an endangered animal. Its declining population altered the conservation department, and the rate of illegal hunting and trapping was controlled from 2016 to onward due strict protection and conservation. The others suffering from illegal activities of hunting and killing were the least concerned Asian Palm Civet (*Paradoxurus hermaphroditus*) and Jungle Hare (*Lepus nigricollis*).

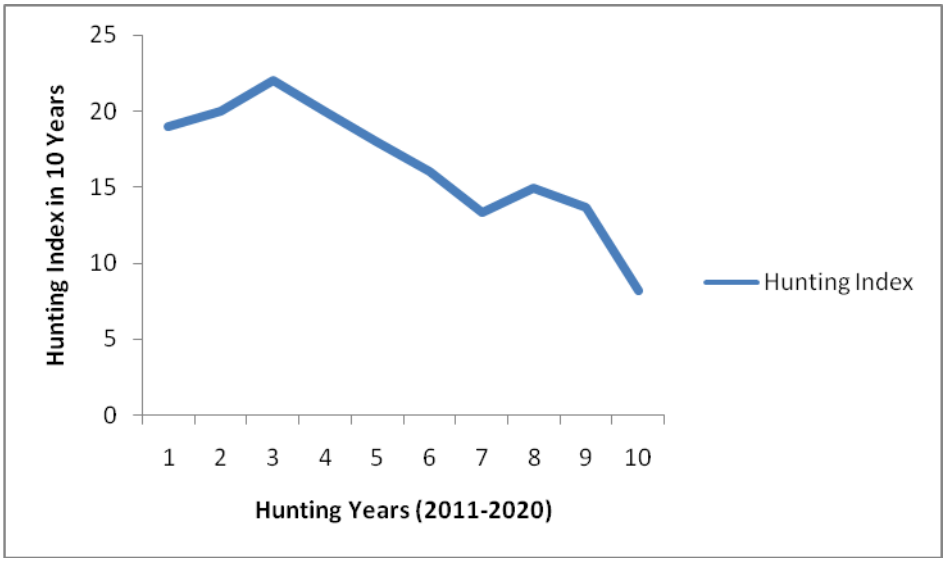


Fig. 8: Hunting Index (2011-2020)

Figure 8 indicates the trendline of the hunting index. The highest Hunting Index was recorded in 2013 (22) and it gradually declined to 13.3 in 2017. During 2020 the lowest hunting index was recorded which was 8.2.

DISCUSSION

Hunting licenses were issued by the Wildlife and Fisheries Department of AJK for Rs. 1500 per year until 2013. Following that, the practice was outlawed. This hunting prohibition can explain why there has been a drop in hunting-related incidents since 2015.

The loss of biodiversity is significantly accelerated by hunting, as stated by Benítez-López et al., 2017. In addition, the pandemic is also a reason for the decline in hunting-related reports in 2019 and 2020. The pandemic lockdown has positively affected the wildlife population, as fewer human activities have occurred in vital areas. Similarly, Behera et al., 2022 reported the same thing.

Primarily for subsistence and to supplement their income by trading, collecting, and trapping wildlife, the local community engaged in illegal hunting. The migrating birds were the most vulnerable species to shooting at the dam. The local animal dealers gained 5,000 to 10,000 rupees for a single bar-headed geese (*Anser indicus*). Even though many locals had nothing to do with it, they did mark the spot for hunters and help in hunting. They managed to capture a wide variety of birds, including Peafowl (*Pavo cristatus*), Rock Pigeon (*Columba livia*), Francolin (*Francolinus pondicerianus*), Common Quail (*Coturnix coturnix*), Rose Ringed Parakeets (*Psittacula krameri*), and Asian Palm Civet (Paradoxurus hermaphrodites). While there, hunters also sought out Common Quail (*Coturnix coturnix*), Grey Francolin (*Francolinus pondicerianus*), and Rose Ringed Parakeets (*Psittacula krameri*). For as little as Rs.1500 to Rs.5,000, the trapper would sell as many as 30–50 birds in each trap to people interested in animals or on the animal market. Ramachandran et al., 2017 state that illicit bird killing and hunting severely threaten bird conservation efforts worldwide. Unsustainable bird hunting in wetland habitats, particularly along important flyways, has contributed to avifauna defaunation and species endangerment. Since wetlands are primary habitats for migratory birds and are associated with anthropogenic landscapes, a comprehensive understanding of bird harvesting and its drivers is fundamental to reducing threats to current avifauna.

A possible decrease in the Common Starling population may have resulted from the study site's record-high number of hunting incidents (418). According to Mahmood et al. (2013), the Common Starling population has dropped dramatically during the last 6-8 years. Since starling meat dishes are promoted through local restaurants, this decline could be caused by unregulated hunting for commercial reasons. One of the greatest dangers to Common Starling populations in Pakistan during the winter is the unregulated and illegal shooting of the birds. Wildlife

regulations must be strictly enforced. The impact of fungicides and pesticides on Common Starling populations in their wintering grounds requires additional in-depth research.

As mentioned earlier, illegal hunting and trapping of wild animals was normal at the Mangla Dam and its periphery. While certain animals were targeted year-round, others were targeted seasonally based on their location within the property. In the Northern Section of Bardia National Park, Nepal, hunters utilized various tools and methods, including traps, cages, hunting dogs, motor/paddle boats, rifles, and weaponry (Repeater and 12 Bore guns). This practice was documented by Bhattarai et al. (2017) as well. For hunting and trapping, they also used the recorded sounds of birds and parrots and utilized various models of mimicking species of birds and animals. From Mangla Dam and the surrounding forest, migratory birds were the most sought-after wintertime prey. Basudine and carbo-furan granules were used to poison grazing waterfowl, including Ruddy Shell Ducks, Wigeons, and Bar-headed Geese. The diving ducks belong to Family Anatidae e.g. Red-crested pochard and common pochard were entangled and died in fishing nets. The accused were fined after court procedures e.g. Rs.5000 for Rose Ringed Parakeet, Rs.10,000 per bird for Bar Headed Geese, and Rs.10000 per bird for Tawny Eagle. However, after heavy fines were imposed, illegal hunting was not under control, and stricter legislation was demanded. The Datta, 2022 also found Illegal wildlife hunting, especially birds, is a primary global conservation concern for many dwindling species in Bangladesh.

Pythons and other snake species decline because people are naturally terrified of them. Snake charmers poaching snakes for their meat and venom, which they then use in various medicinal preparations, contribute to the declining population of snakes. Additionally, the disappearance of the python's natural habitat due to tree cutting for timber and firewood was a significant threat to this nonpoisonous snake. During the study, 11 killed snakes were found along the tracks of the dam, in forest trails, and in human dwellings. A total of 6 dead and live pythons were brought to the Office of Wildlife and Fisheries, AJK, that were captured from human dwellings and cattle yards. Haering (2015) claims that many members of society encounter animals regularly. Occasionally, these interactions are pleasant and provide numerous opportunities to enjoy and appreciate natural resources. But

sometimes, these connections between the community and wildlife are unpleasant and may cause severe conflict, which causes hazards to human lives or assets and creates financial hardship. The community also expects that the concerned department should offer quick solutions to these issues but in the present case, the AJK wildlife department was not in this position to address the community wildlife conflicts due to a shortage of trained field staff, funds, and poor implementation of the Wildlife Protection Act. The community also expects the relevant department to solve these issues promptly. Mangla Dam was also the site of unlawful hunting and trapping of the black scorpion, which had a reputation for healing cancer (Christensen, 2014; Gutman, 2013; Iacurci, 2015). The AJK Wildlife Department asserted in 2014 that they confiscated approximately 6 kg of black scorpions from Dadyal, which were involved in the unlawful trade. Under the law, the department fined hunters and merchants between Rs.10000 and Rs. 15000 for each black scorpion and 1500 rupees for each blue rock agama.

The location was mentioned as a location of extensive hunting for wild boar (*Sus scrofa*) and jungle hare (*Lepus nigricollis*). Deceased Asian Palm Civets (*Lepus capensis*) were discovered on the roadside due to road kill. But it was the Indian pangolin that was in danger; it was on the IUCN Red List for 2014 as an endangered species (Baillie et al., 2014). The pangolin species was downgraded from lesser risk/near threatened in 1996 to near threatened in 2008. There were 197 reports of pangolin poaching reported to the AJK Department of Wildlife and Fisheries between 2011 and 2020. The illegal trade of Indian pangolins, primarily for their meat and scales, posed the greatest threat to these animals. Many Vietnamese and Chinese dishes make use of their meat. The scales were found to be used in pharmaceuticals and protective vests. Even though it is banned by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), its illicit trafficking continues. In Dadyal, Dadyal, Barnala, and Mirpur, there were numerous reports of hunting. According to Waseem et al. (2020), a single pangolin might get the poacher Rs. 80,000/- in no time. This way, they can become filthy rich in a day with just one trap. The relevant department did what it could to save this endangered species, but it will be a while before their efforts bear fruit. Despite declining hunting incidents in 2016, the goal of

achieving zero hunting still needed to be met. The trappers used trained sniffer dogs to track pangolins, making the hunting process incredibly easy. When threatened, pangolins would roll up into a ball, which the poachers would pluck and put in bags. Poachers who didn't have access to sniffer dogs would use pills with a solid odour to make the pangolin roll up. A pound of its meat sells for 600 USD on the global market. To protect the critically essential creature for the environment, the Department of Wildlife and Fisheries fined the perpetrators between Rs. 10,000 and Rs.15,000 Indian rupees for each pangolin.

The wild hares and boars were hunted throughout the year (Fig. 6 and 7) from jungle cover. Throughout the year, Indian pangolins, originating from sandy soil/grassland/cultivation land, seek refuge in areas with trees and plants. Various things were made from the animals that were hunted or captured; for example, feathers from birds were used for making clothes, decorations, and even medications. Many things were made from pangolins, including medications, protective coats, and food. In the summer, the snakes were plucked from the rocks, woods, and plants surrounding the dam. Treating medical conditions with snake and black scorpion venom was common practice. The natives also utilized other animal parts for black magic, such as snake skin, pangolin scales, red fox hair, and barking deer hoofs. To generate revenue, the local community and fisherman would advise hunters on the best times and places to go hunting in the area. The natives would often spend a few days hunting or assisting the hunters, but they would spend weeks or even months if the work or deal were vital.

Mangla Dam was the site of multiple animal hazards. Several factors contribute to water pollution in dams, including farmers' pesticide use, which significantly impacts insectivorous birds, and oil leaks from diesel pumps used to collect water. Wildlife, including fish and birds, faces threats from agricultural runoff. Heavy metal emissions from the dam could be caused by rock weathering in the Pir Panjal Range and the River Jhelum catchments. Silt builds up in the river due to deforestation, which degrades habitats. One of the leading causes of the dwindling wildlife populations in AJK was the increase in hunting and trapping. A fig. 8 showed that the hunting index of sites reached 22, indicating a substantial level of hunting pressure. Illegal bird hunting using nets,

traps, and toxic substances is common in migratory and watery regions, according to Htay et al. (2023).

The availability of firearms and ammunition, a lack of education and entertainment options, poverty, hunting by the well-off, and a failure to adequately enforce the Wildlife Protection Law were the primary causes. Finally, we suggest to spread the word among the general population and school-aged children. More people will be aware of the needs of wildlife, and the services they give to ecosystems, as a result, hunting will be reduced in intensity. Finally, when these suggested conservation conditions are met, the Mangla Dam Freshwater Reservoir conservation efforts aimed at reducing wildlife hunting pressures will be effective. Furthermore, in the present conditions, wildlife conservation requires immense efforts in legislation, political initiatives, public awareness, goal prediction, moral and ethical considerations, and most momentously, partnerships between governmental and non-governmental organizations.

CONCLUSION

The results showed that there was a lot of illegal hunting, trapping, and killing of wildlife in the Mangla Dam Freshwater Reservoir and that places with a lot of hunting pressure should be the ones where conservation efforts are concentrated. One pertinent strategy is concentrating enforcement patrols in dam regions since hunting is more prevalent in nearby parts of Mangla Pockets and the surrounding forest. The local community, farmers, and fishers should be the focus of future conservation efforts aimed at reducing these recorded hunting pressures. There are a lot of other factors that contribute to wildlife killings, including negative interactions between humans and wildlife (such as crop damage), poverty, ignorance, an inadequate workforce, outdated or nonexistent transportation, and lax enforcement of the law. A local population should be provided with alternative sources of income because making a quick buck from illegal hunting is crucial. Because Mangal Dam is home to numerous endangered species and a crucial resting place for migrating birds where the Indus Flyway crosses, the current Department of Wildlife and Fisheries in AJK needs to be bolstered, and new legislation passed to end the illegal killing of wildlife there.

ACKNOWLEDGMENT

The authors are highly indebted to the Department of Wildlife and Fisheries,

AJK. The local community of Mirpur for their cooperation in the collection of data.

CONFLICT OF INTEREST

No conflicts of interest were reported by the authors.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801321

Paper Submission: 4th Nov, 2023; Paper Acceptance: 25th Feb 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Phytochemical and in Vitro Biological Profiling of *Portulaca grandiflora* Whole Plant Extracts

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ABSTRACT: *This study aimed to determine the phytochemicals and to examine the in vitro antibacterial, antifungal, anticancer and antioxidant potential of P. grandiflora. Crude methanolic, ethanolic and n-Hexane extracts of whole plant were used for this study. Antimicrobial properties were estimated through disk diffusion assay, antioxidant activity was determined by using 2,2-diphenyl-1-picryl-hydrazyl scavenging (DPPH), total antioxidant capability and reducing power assays. It was found that methanolic extract of P. grandiflora showed the highest zone of inhibition against Pseudomonas aeruginosa and Candida albicans i.e. 36 ±1mm and 18 ±1 mm respectively with MIC of 25 µg/ml. The highest amount of gallic acid equivalent phenolic and quercetin equivalent flavonoid content were found in methanolic extract of P. grandiflora i.e. 77 ± 0.68 µg GAE/mg and 63 ± 1.08 µg QE/mg extract respectiely. The significant DPPH scavenging activity (P<0.05) was recorded for methanolic extract of P. grandiflora (87%). Considerably greater antioxidant capacity (10 ± 1.18 µg AAE/mg) and reducing power (4.06 ± 0.18 µg AAE/mg) was observed in methanolic extract. MTT cell viability assay methanolic extract of P. grandiflora showed the IC_{50} of 42.70 µg/ml at 72h. It was concluded this plant extracts exhibit strong pharmacological properties.*

Keyword: Phenolic, Flavonoid, Antioxidant, DPPH, antimicrobial

INTRODUCTION

Researchers acknowledge the role that traditional medical systems and herbal remedies play in mitigating the growing global health care crisis. History reveals that different species of plants have been utilized for health maintenance in different cultures as well as the derivation of most medicinal preparations (Gadir, 2012). In a tropical climate *Portulaca Grandiflora* is well growing plant. This herb mainly considered a problematic. The researchers encourage by this fact and others potencies that could be utilized from purslane herbs. To check the efficiencies on disease several studies have been conducted, and antibacterial activity is one of these efficacies. The antibacterial activity of purslane herbs has the potential to hasten the healing process and to be developed into topical medicines for the treatment of wound (Budiawan, 2023). Using DPPH and FRAP assays, the antioxidant effects of *Portulaca grandiflora* extracts were evaluated in order to pinpoint possible sources of compounds that might be helpful in thwarting the effects of free radicals.

Medicinal plants continue to be important in the fight against disease and are a viable source of medications, particularly in underdeveloped nations (Mothana et al., 2019). Nearly all of the plant species in the world have outstanding antioxidant potential, and it is thought that two thirds of them have medicinal significance (Krishnaiah et al., 2011). There are about 3.4 billion herbal medicine users worldwide. The essential components of the antiquated conventional medical system were natural materials. The World Health Organization (WHO) defines a medicinal plant as any plant that has ingredients that are used for medical purposes (Asif et al., 2019; Shareef et al., 2016).

The free radical DPPH creates a violet solution in ethanol and is stable at room temperature. The foundation of the DPPH test is the stable DPPH free radical's capacity to interact with hydrogen donors. There is an intense UV-VIS absorption spectrum shown by the DPPH• radical. In this test, a radical solution is reduced with an antioxidant (AH) or a radical (R•) according to the following protocol, which results in decolorization of the solution: DPPH• + AH → DPPH• -H + A•, DPPH• + R• → DPPH• - R (Bangbog, 2022). With a total phenolic content of 0.7346 mg GAE/g FW, the treatment using 100 kg/ha of NPK fertilizer had the greatest value. The extracts treated

with 100 kg/ha (FRAP, CUPRAC) and 200 kg/ha (DPPH, ABTS) with NPK fertilizer showed the largest increase in antioxidant activity. As a result, purslane's plant growth, total phenolic content, and antioxidant activity can all be increased by using NPK fertilizer at recommended dosages. Based on the research, 100 kg/ha was indicated as the dose that produced the maximum antioxidant activity (FRAP, CUPRAC) and total phenolic (Putra, 2023).

Fig. 1. A plant of *Portulaca grandiflora*

P. grandiflora is utilized for detoxification, as well as the treatment of skin rashes and sore throats. It is a purported immunostimulant that causes the immune system to become non-specifically activated, resulting in cytotoxic, antibacterial, and antitumor action. The effectiveness of *P. grandiflora* on a surface antigen of the hepatitis B virus has been documented. Furthermore, it has been observed that this plant has an antimutagenic impact on the mutations caused by cyclophosphamide and aflatoxin B1, two human carcinogens, in mice. It is important to note that *P. grandiflora* aqueous extracts are safe to use; studies conducted on participants suggested a daily intake of 500 mg.

Various well-known medicines have been derived from different plants, such as morphine, aspirin, quinine, benzoin, vincristine, and vinblastine from *Papaver somniferum*, *Filipendula ulmavia*, *Cinchona pubescens*, *Slyrax tonkinensis*, and *Catharanthus roseus* (Michael et al., 1956) respectively.

Plants naturally yield a vast range of chemical constituents that have a strong medicinal value and therefore are used in pharmaceutical industries. The existence of such bioactive metabolites is also responsible for several other properties to plant particular order due

to the terpenoids; flower color is because of the presence of betalains, quinones, and tannins, and chilies have a flavor due to the presence of terpenoid capsaicin (Gülcin, 2012). Latterly the hunt for effective antibacterial representatives has been moved to herbs. The traditional evaluation recommends that 10% of all the flowering plants have been in use for at least one time by the native population but only 1% were noticed by world scientists for medicinal purposes (Kunin, 1993). More than one hundred human diseases including arthritis, CNS injury, AIDS, cancer, atherosclerosis, ischemia, and gastritis are the consequence presence of free radicals in the human body (Cook and Samman, 1996; Kumpulainen and Salonen, 1999). Numerous factors are responsible for the production of free radicals including pollution, chemicals, radiation, toxins, spicy food, and also physical stress which then weakened the immune system of the body, mutation in genes that changes their expression, and also produces unusual proteins. Reduction in the natural antioxidant in the immune system consumption of antioxidants and free radicals might be important (Halliwell, 1994; Kuhnau, 1976; Kumpulainen and Salonen, 1999; Younes and Siegers, 1981).

P. grandiflora is a small herbaceous annual herb from Portulacaceous family (Fig. 1). Sun plant, Rose Moss and Moss Rose are the common names of this plant (Brickell, 2003). A few years ago, the aqueous extract of *P. grandiflora* was used to examine its toxic effects on anti-adenoviruses activities, in vitro anti-herpes simplex viruses (Chiang et al., 2003), and Wistar rats (Chavalittumrong et al., 2004). Additionally, the water formulation of *P. grandiflora* enhances the proliferation of lymphocytes in vitro, suggesting a part in the modulation of the immune system (Sriwanthana et al., 2007). Compared to its close relative *P. oleracea* (Dkhil et al., 2011; Lim and Quah, 2007; Sanja et al., 2009; Uddin et al., 2012), comprehensive studies on the characterization, as well as health benefits of *P. grandiflora* limited. Perhaps, the anticancer activity of *P. grandiflora*is still not reported.

The use of natural antioxidants is gaining importance due to unhealthy and negative impacts of artificial antioxidants like butylated hydroxy anisole (BHA), gallic acid esters, etc., (Barlow, 1990; Branen, 1975).The presence of natural antioxidants in the diet decreases the threat of heart disorders, cancer, as well as aging diseases (Kritchevsky, 1999; Lauro and

Francis, 2000; Pszczola, 1998; Rao and Agarwal, 1999). Numerous human disorders including cancer have been treated by natural medicinal products e.g., etoposide, Taxol, paclitaxel, vincristine, and irinotecan are the drugs obtained for plants (Da Rocha et al., 2001). The study aimed to determine the phytochemicals and to examine the *in vitro* antifungal, antibacterial, antioxidant and anticancer potential of *Portulaca grandiflora*.

MATERIAL AND METHODS

Plant Collection

P. grandiflora was collected in the summer season from the home garden. Identification of the plant was done by Prof Dr. Mir Ajab Khan, Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan.

Reagents and Chemicals

Analytical grade solvents have been used for the present study, Ethanol, Methanol, and *n*-hexane (Sigma USA). Merck provided all the reagents used for the study including gallic acid, quercetin, sodium carbonate, DPPH, aluminum chloride, potassium ferricyanide, ferric cyanide, Folin-Ciocaltau (F-C) reagent, ammonium molybdate, sodium phosphate, trichloroacetic acid, sulfuric acid, ascorbic acid and potassium acetate (Merck KGaA, Germany).

Extract Formation

P. grandiflora was rinsed scrupulously with simple tap water then air dried and crushed to ultra-fine particles. Process of maceration was used to make extracts using 3 solvents *n*-hexane (NH) Ethanol (EL), and methanol (ML)with different polarity levels from non-polar to polar solvents respectively. 50grams of powdered material was soaked in 500ml of extraction solvents. All solvent extraction was performed thrice. Subsequently, filter paper (Whatman filter paper No.1) was used to filter all the mixtures. The rotatory evaporator was used at decreased pressure and 45^{o}C to vaporize the mixtures for thickening (Buchi, Switzerland).

Table 1: *P. grandiflora* extraction in different organic nonpolar to polar solvents with percent yield

Serial No.	Sample code	Solvent	% Yield (g)
1	NH	*n*-hexane	1
2	EL	Ethanol	3
3	ML	Methanol	3.5

Phytochemical screening

Determination of total phenolic contents (TPC)

The determination of total phenolic content in samples was done by the previously defined procedure (Clarke et al., 2013; Qader et al., 2011; Zhang et al., 2010). Immediately the stock solutions (4 mg/ml) of test samples were concocted in DMSO. Each extract (20µl) was moved to all wells of 96 well plate.90 µL of F-C reagent diluted with double distilled water was added to each well for 5 minutes. A 7.5 % of 90 µl of Na_2CO_3 solution was also added to the reaction mixture. After five minutes, the whole plate was incubated for 60 minutes and the microplate reader (Bioteck) was used to measure the absorbance at 650nm. The gallic acid in DMSO as well as blank DMSO as standard were run concurrently. The resulting TPC was calculated as µg GAE/mg extract.

Determination of total flavonoid content (TFC*)*

Because medicinal plants can scavenge free radicals, there has been a favorable correlation found between their antioxidant activity and overall phenolic contents (Geethalakshmi et al., 2013).

20 µl (4 mg/mL DMSO) of test samples and 10 µl of 10 % $AlCl_3$ as well as10 µl of 1M CH_3CO_2K were mixed to determine the total flavonoid content of different extracts. Distilled water was mixed with the different extracts to attain a total volume of 200 µl. The obtained solution was incubated for 30 minutes (Incubator IC83 Yomato, Japan). Absorbance was calculated at 415nm at 37^oC by microplate reader. Quercetin was used as the standard and results were articulated as µg QE/mg extract (Clarke et al., 2013).

Biological assessment

DPPH free radical scavenging assay

DPPH reagent was used to evaluate the free radical scavenging property of the collected samples. Concisely, standard solutions of 4 mg/mL of experimental samples were set ready in DMSO (Clarke et al., 2013; Qader et al., 2011; Zhang et al., 2010). A 10 µl of each sample was added in 190 µL of 0.004% DPPH in ML and incubated for 1 hr. The absorbance was taken at 515 nm with the help of a microplate reader. Ascorbic acid as well as DMSO were used ascontrol. Each sample was calculated at final concentration of 200 µg/ml. A sample that shows strong quenching activity (≥ 50%) was again

used with a lower concentration to calculate $IC_{50.}$ By following the formula percent inhibition was measured:

Percent inhibition of the test sample = % scavenging activity = $(1 - Ab_s / Ab_{c)} *100$

Where

Ab_s= Absorbance of DPPH solution with the sample,

$Ab_{c=}$ Absorbance of negative control (containing the reagent except for the sample).

Version 4of Table Curve software was used to evaluate the IC_{50}.

Determination of total antioxidant capacity (TAC*)*

The TAC of test samples was calculated by mixing 900 µl of a reagent comprising 0.6 M H_2SO_4, 4 mM $(NH_4)_2MoO_4$, and 28 mM $NaPO_4$ with 100 µl of stock solution (4mg/ml in DMSO) and incubated for one and half hour at 95^{o}C. Solution Mixtures were kept to cool at 37^{o}C and with the help of a microplate reader, the OD was calculated at 695 nm. DMSO (100 µl) was used as a blank. Different concentrations of ascorbic acid were used along with DMSO for a standard curve. The attained TAC was measured as µg AAE/mg extract (Aliyu et al., 2009).

Reducing power assay

The determination of reduction potential was done by the already defined method (Aliyu et al., 2009). 100 µl of all test samples prepared as 4 mg/ml extract in DMSO were added with 250 µl of 1% $C_6N_6FeK_3$ as well as 200 µl of 0.2 M phosphate buffer (pH 6.6). The incubation of the resulting solution was done at 50^{o}C for 20 minutes. After the incubation period, 200 µl of TCA (10%) was used to acidify the reaction mixture and centrifuged for ten min at 3000 rpm. 150 µl of supernatant was collected and added to 50 µl of 0.1% $FeCl_3$. Afterward, 200 µl of reaction mixtures were moved to the respective wells of the 96-well plate. The absorbance was evaluated at 630 nm with the help of a microplate reader. As ascorbic acid was a positive control, the results were calculated as µg AAE/mg extract.

Antibacterial Activity

Susceptibility of all samples was verified against 6multidrug-resistant bacterial strains i.e., 2-gram+ve (Methicillin-resistant Staphylococcus aureus and Enterococcus. spp) and 4-gram-ve (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Serratia marcescens*). The bacterial strains were cultured in N-broth and after culturing the strains were swabbed

onto the nutrient agar plates. Filter paper discs were soaked in five microliters (20mg/mL DMSO) of samples were put down on nutrient agar plates and incubated for 1 day. After incubation, a clear area of the zone of inhibition appeared around the discs which were measured in millimetre sand noted (Haq et al., 2012). MIC of samples showing an inhibition zone of ≥ 10.0 mm was further verified at 100, 75, 50, and 25 µg/ml by Microtiter Plate Broth Dilution Method.

Antifungal activity

The antifungal property of all samples under investigation was tested against the given fungal cultures (*Aspergillus niger* FCBP# 0198, *Mucor* specie FCBP# 0300, *Fusarium solani* FCBP# 0291, *Aspergillus flavus* FCBP# 0064 and *Candida albicans* FCBP-478) grown on SDA medium (Sabouraud Dextrose Agar; Merck Germany). Before the determination of sensitivity, fungal spores were cultured in 0.02% Tween 20 solution.

The turbidity of fungal spores was modified based on McFarland 0.5 turbidity standard. 100 µl inoculum was used to dab the plates having SDA medium. 5µlfrom each sample was used to infuse the filter-paper discs and 2.5 microliters (4mg/ml) of standard antifungal terbinafine was employed as standard discs, and incubated for 1 day at 28ºC. By using Vernier caliper clear area as the zone of inhibition was measured and noted after a given time (Ul-Haq et al., 2012).

Anticancer /Cytotoxic Activity Assay

MTT Assay

The antiproliferative effects of polar and non-polar extracts of *P. grandiflora* were evaluated through an MTT cell viability assay as described previously (Batool et al., 2017).

The % cell viability was calculated through the following formula:

% Cell viability = (OD of treated cell/ OD of control) × 100

Where OD = Optical Density

Statistical analysis

All the trials were performed in triplicate. The data were expressed as mean± standard deviation (SD). Graphpad prism version 6.01 was used to test IC_{50}. The graphical presentation was done by Origin 8.5 software.

RESULTS

A total of 3 extracts of *P. grandiflora* whole plant were prepared in three different solvents. Table 1 described the sample code as well as the % yield. The highest extraction production was gained with the methanol (ML) while n-

hexane (NH) extract was obtained in the least quantity among others.

1. Phytochemical evaluation

Total phenolic and flavonoid content (TPC: TFC)

Among all phytochemically analyzed solvent extracts, the maximum quantity of gallic acid corresponding TPC was examined in ML (77 ± 0.68 µg/mg) and EL extracts (70 ± 0.58 µg /mg) while the minimum amount of TPC was examined in n-hexane NH (15 ± 0.23µg GAE/mg) (Fig. 2).The quercetin equivalent (QE) total flavonoid content was found to be varied greatly from 6.08-63 µg/mg. The maximum amount was found in ML (63 ± 1.08 µg/mg), EL (56.45 ± 2.15 µg/mg), and a lower value was shown by NH (6.08 ± 3.08) (Fig. 2).

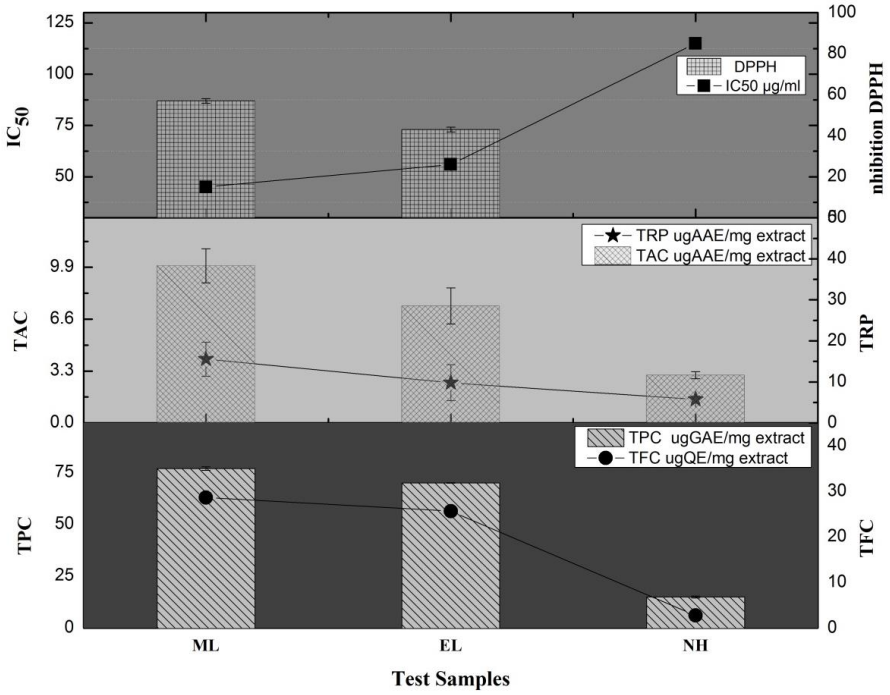

Fig. 2. Total flavonoids and phenolic content and antioxidant property of various non-polar as well as polar solvent extracts of *P. grandiflora*

2. Biological evaluation

DPPH radical scavenging activity

The % DPPH scavenging property of all samples lie between 24 to 87% with highly significant percentage activity ($p < 0.05$) being examined for ML (87%: IC_{50}= 45µg/ml), EL extracts (73%: IC_{50}= 56 µg/ml). The lowest scavenging activity of 24% scavenging potential was found in the NH extract (Fig. 2).

Phosphomolybdenum based total antioxidant capacity (TAC)

The maximum antioxidant capacity, when measured in comparison with ascorbic acid, was found for ML (10 ± 1.18µg AAE/mg). However, EL showed (7.44 ± 1.54µg AAE/mg) while NH extract (3.04 ± 1.69 µg AAE/mg) showed the least TAC.

Reduction potential

Reducing the power of test samples depend upon the color of extracts that deviates to either blue or green. The higher the absorbance greater will be reducing power. Among all analyzed samples, Methanol (ML) and ethanol (EL) soluble extracts were shown to exhibit higher reduction potential when expressed as equivalent of ascorbic i.e.,4.06 ± 0.18 and 2.56 ± 0.29 µg AAE/mg respectively. NH extract (1.5 ± 0.10 µg AAE/mg) showed the lowest reducing power.

Antimicrobial activity

The sensitivity of samples against different bacterial as well as fungal strains was evaluated by the well diffusion method. According to our findings, ML showed the highest activity against *MRSA and Pseudomonas aeruginosa* with 29 ±1.6 (MIC: 25 µg/ml) and 36 ±1 (MIC: 25 µg/ml) of the zone of inhibition followed by EL and NH shown in Table 1, minimum inhibition. Against different fungal strains ML extract was observed to exhibit the highest sensitivity against *Candida albicans* with 18 ±1 mm growth inhibition zone with MIC 25µg/ml. While the lowest activity was shown by NH against *Fusarium Solani* and *Mucor* specie (Table 2). The absence of a clear zone confirmed that DMSO did not showed any toxic effect.

Table 1: Antibacterial activity of *Portulaca grandiflora* extracts against MDR bacterial strains (Data Values are presented as mean +SD (n=3))

PG	**S. aureus**	**MIC µg/ml**	**S. marcescens**	**MIC µg/ml**	**K. pneumonia**	**MIC µg/ml**	**E. coli**	**MIC µg/ml**	**P. aeruginosa**	**MIC µg/ml**	**E. aerogenes**	**MIC µg/ml**
MtOH	29 ±1.6	25	14 ±1	75	14.5±0.71	75	18 ±1	50	36 ±1	25	32 ±2	25
EtOH	28.7±1.5	25.0	0	0	0	0	16 ±1.7	50	28.7 ±1.5	25	0	….
N-Hexane	29±1	25.0	14.7±2.5	75	13.5 ±3.5	75	18 ±1	50	35±1.7	25	0	….

Table 2: Antifungal activity of *Portulaca grandiflora* extracts against 5 fungal strains (values are expressed as Negative control = DMSO (no zone of inhibition), Positive control = Terbinafine, mean +SD (n=3)

PG	F. solani	MIC ug/ml	A. flavus	MIC ug/ml	A. niger	MIC ug/ml	M. specie	MIC ug/ml	C. albicans	MIC ug/ml
MtOH	8.3 ±0.19	0	11.67 ±0.19	75	13.00±1	50	11 ±0.58	75	18 ±1	25
EtOH	10±0.58	75	9.33±0.51	0	9.67±0.51	75	12 ±0.58	75	11.67 ±0.51	75
N-Hexane	9±0.65	0	10.7±1.5	75	11.5 ±1.7	75	9 ±0.71	0	13±0.95	75
Terbinafine	22±0.806		12±0.455		11 ±0.56		15± 0.77		10 ± 0.706	

MTT/Cytotoxicity assay

It was analyzed the effects of ML, EL, and NH extracts of *Portulaca grandiflora* on the viability of Hep2 cells and also on normal human corneal epithelial cell line (HCEC) through MTT assay. The cell line was treated to 0, 50, 100, 200, and 400 µg/ml of ML, EL, and NH extracts of *Portulaca grandiflora*, for 24, 48, and 72 h proceeded by calculation of cellular viability by the colorimetric-based MTT assay. The data shown in Fig. 3A to 3C indicated that ML extract has better cytotoxic activity against Hep 2 cell lines, with the lower IC_{50} (inhibitory concentration at which cell growth is 50% reduced) of 42.70 µg/ml as compared to the other extracts, after 72 h.

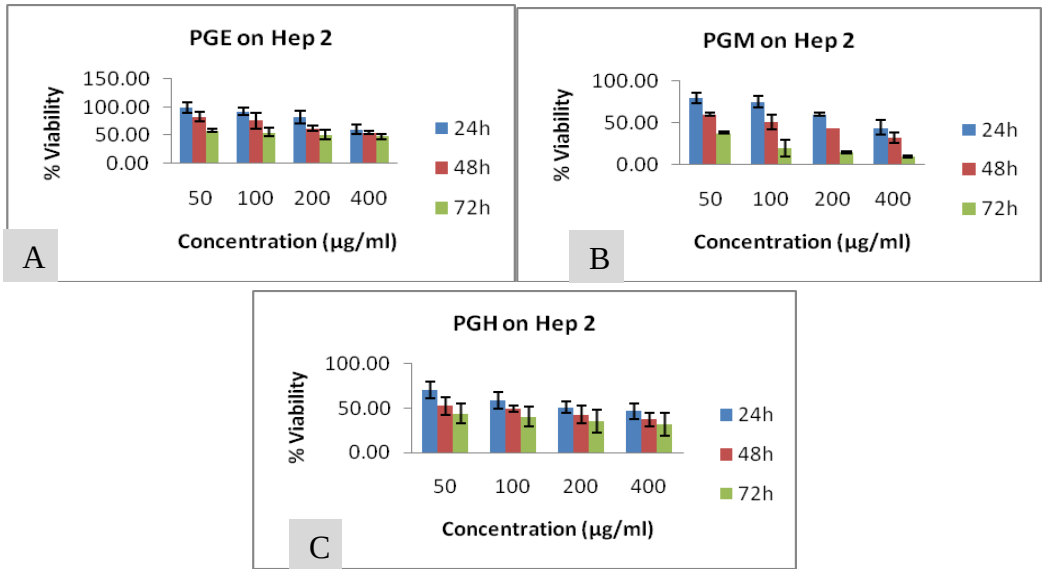

Fig. 3. Anti-proliferative effect of three different extracts of *P. grandiflora* in Hep 2 cells as shown in graph A, B, and C

DISCUSSION

In the trial of Metabolite Profiling Analysis and the Correlation with Biological Activity of Betalain-Rich *P. grandiflora,* Sporna Kucab etal found antioxidant properties against gram negative bacteria of compounds extracted from *P. grandiflora*. In the present study, *P. grandiflora* showed high zone of inhibition that showed its effectiveness against *Pseudomonas aeruginosa* and *Candida albicans* also showed high MIC and results are in agreement with Spórna-Kucab et al. (2022).

This result showed that this plant can be used in pharmaceuticals industries with some others solvents. Secondary metabolites or phytochemicals of plants are contributing actively to the treatment of several diseases, nowadays they are considered the major part of both modern as well as traditional systems of medicine. In the present study, polarity-based extract efficacy disparity has been estimated which indicates the effect of the nature of the solvent on the phytochemicals extracted, possibly due to the occurrence of delocalized electrons that make the majority of the plant constituents extremely polarisable (Lim and Quah, 2007). A battery of assays to determine the antioxidant activity was employed in this study because a single test cannot completely assess the rationality behind the activity. In this study, a number of antioxidant assays like total reducing power as well as DPPH free radical scavenging assay were used to evaluate the antioxidant property of various extracts. A useful association between the TFC, TPC, and DPPH hunting potential of ethanolic as well as methanolic extracts of *P. grandiflora* has been established which is consistent with the already reported positive correlation between TFC, TPC, as well as quenching potential. The outcomes of this study firmly correlate with the literature where maximum antioxidant activities have been observed in polar extracts such as ethanol as well as methanol. Therefore, it can be recommended that the compounds of this plant have antioxidant activity and are quite polar. These results also revealed that *P. grandiflora* extracts had comparable free radical scavenging activity in *P. oleracea* [IC50 = 0.89 ± 0.07 mg/ml] as reported by Lim and Quah (Lim and Quah, 2007).

Phenolic compounds have several biological implications such as antioxidant activities, antibacterial, antitumor, and antimutagenic, and they have a hydroxyl group present on their aromatic hydrocarbon ring (Kolář et al.,

2002; Shui and Leong, 2002). Flavonoids and phenolics have been described have being related to antioxidant activity in natural systems. High concentration of phenolics as well as flavonoids contents in methanolic as well as ethanolic extracts of *Portulaca grandiflora* respectively also described by the previous study of *P. grandiflora* (Lim et al., 2014), and their connection with antioxidant capability show our interest in determining the antioxidant potential of the given test samples. The overall antioxidant property of the extracts can be credited to the total flavonoids as well as phenoliccontents (Cai et al., 2004; Hendra et al., 2011).

In the given study numbers of bacterial and fungal strains were used to check the antimicrobial potential of *P. grandiflora*. Results were comparable with the earlier research where the highest zone of inhibition was shown against *Pseudomonas aeruginosa* and *Candida albicans* similar to our results (Shinde et al., 2014). Reactive oxygen species (ROS) are generated by other metabolic reactions as well as the electron transport chain of mitochondria. These free radicals also affect the deoxyribosyl backbone of DNA along nucleic acid bases causing genotoxicity and ultimately mutations. Mutations are the major contributor to carcinogenesis and tumor formation (Valko et al., 2004). Polysaccharides scavenge the buildup of free radicals as well as restrain immunity functions also (YouGuo et al., 2009), Polysaccharides from *Portulaca oleracea* a very close relative of *P. grandiflora* exhibit many biological properties, like anti inflammation, antioxidant, anticancer, and immunity-enhancing assets (Chen et al., 2010; Liu et al., 2009; Yang et al., 2008; Zhu and Wu, 2009). As there is no such information reported previously about the anticancer activity of *P. grandiflora*, based on studies regarding its counterpart *Portulaca oleracea* it is assumed that *P. grandiflora* also has anticancer activity due to the presence of polysaccharides.

CONCLUSION

It was concluded that *Portulaca grandiflora* is of great importance as it possesses several pharmacological properties by displaying strong antimicrobial, antioxidant, and anticancer potential associated with its diverse chemical constituents.

ACKNOWLEDGEMENT

Dr. Muhammad Ismail is acknowledged for his guidance and support in this research work.

CONFLICT OF INTEREST

Authors declare there is no conflict of interest.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801322
Paper Submission: 13th June, 2023; Paper Acceptance: 10th March 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Antibacterial Activity of Fruit Juices on Methicillin Resistance *Staphylococcus aureus*

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ABSTRACT: *The drug-resistance behavior of Methicillin-resistant Staphylococcus aureus (MRSA) has made it difficult to treat. This study aimed to discover some fruit juice's anti-MRSA potential and the presence of enterotoxin genesin MRSA. MRSA strains were confirmed phenotypically by the disc diffusion method. The well-diffusion method and Minimum inhibitory concentration (MIC) were used to determine the antibacterial effects of fruit juices. Congo red test and ring tests were performed to analyze the biofilm-forming ability. PCR detected staphylococcal enterotoxin genes Sea and Seg. A total of 90 strains, of which 26 non-clinical and 64 clinical samples were processed. Ciprofloxacin (CIP) was highly resistant to MRSA in both groups. In clinical isolates, Citrus reticulate has shown the maximum antibacterial activity against 67.1 % MRSA, whereas, Punicagranatum was most effective for 46.1% strains isolated from non-clinical sources. Punicagranatum and Citrus reticulate were inhibiting the growth of MRSA at the concentration of >64 µl/ml in both groups. Both Congo red and ring tests determined the biofilm-forming ability in 57.60 % and 65.30 % of non-clinical strains, respectively. Enterotoxin-producing gene Sea was detected in 8% of MRSA in the non-clinical group and only 2 % in the clinical group. Seg and the co-existence of both genes were found in the same ratio in both groups. This study showed that fruit juices, especially pomelo, orange, and pomegranate, have high antibacterial activity. Enterotoxin genes play a role in the spread of infections caused by MRSA.*

Keyword: Staphylococcus, MRSA, antibiotic resistance, medicinal plants, enterotoxins

INTRODUCTION

Methicillin resistance *Staphylococcus aureus* (MRSA) has become one of the most widespread pathogens in healthcare-associated settings. Due to the extensive usage of antibiotics, it has become a community-acquired deadly pathogen (Dadashi et al., 2018). Throughout the world, MRSA threatens livestock (Sharma et al., 2019). The primary causes behind the spread of pathogenic MRSA are the hospital staff and infected patients (Chukwunoso et al., 2018). *S. aureus* has caused severe outbreaks (Bennett and Monday, 2003; Fanoy et al., 2009; Bennett et al., 2013). Penicillin was used to cure infections caused by Staphylococcus species (Siddiqui and Koirala, 2020). But in no time, they become resistant to penicillin (Vestergaard et al., 2019). The horizontal gene transfer by staphylococcal cassette chromosome mecC caused resistance to methicillin. The mobile genetic element consists of genes like *mecA*or *mecC,* translated into proteins and pathogenicity (Lee et al., 2018). Alteration of the structure of penicillin-binding sites means no β lactam antibiotic can bind with them (Fetsch and Johler, 2018; Veslasco et al., 2018; Alsharif et al., 2021). As a result, food borne MRSA shows resistance against penicillin and cefoxitin. At the same time, hospital-acquired pathogenic MRSA has become multidrug-resistant (Velasco et al., 2018; Algammal et al., 2020). People with weak immune systems are highly vulnerable to hospital-acquired MRSA infections (HA-MRSA) (Chukwunonso et al., 2018). HA-MRSA infections are common in patients with extended hospital stays after surgery (Barcudi et al., 2020). These strains carry SCC *mec* types I, II, or II. They resist antimicrobial agents like macrolides, aminoglycosides, fluoroquinolones, lincosamides, etc. (Klein et al., 2019). Community-acquired MRSA (CA-MRSA) is transmitted mainly from a person who was recently discharged from hospital or community members. From the nasal cavity to the skin, CA-MRSA is present everywhere. During food preparation, the food handlers shed *S. aureus* into food and transfer it to other individuals. So sneezing and improper food handling are causes of CA-MRSA (Fooladvand et al., 2019). Different virulence factors determine the infection-producing capacity of MRSA; they include toxins, adhesins, Panton-Valentine Leukocidins (PVL), hemolysins, etc. (Karmakar et al., 2016). These virulent factors are involved in causing tissue damage, beating the host's immune response, and eventually

rising in number (Tang et al., 2019). Staphylococcal enterotoxins are involved in causing stomach infections and enhancing resistance to different chemicals (Tarisse et al., 2021). These food poisoning agents are divided into 22 subtypes, from (SE) A-E, G-I, K-T, and Y (Etter et al., 2020). Among all the serotypes SEA, SEE, and SEG are dominant. SEA and SEG are responsible for causing 95% of cases of food poisoning (Guidi et al., 2018). The primary source of toxins is improperly cooked and stored meat sources. Sea and seg work independently and with other toxins, making conditions even worse (Manyi-Loh et al., 2023). In 2017, the World Health Organization (WHO) issued a list of organisms based on their resistance to the available antibiotics. MRSA is one of the twelve pathogens that seriously impact human health (Asokan et al., 2019). Many phenotypic and molecular studies are conducted in Pakistan to check the prevalence of MRSA. The prevalence rate of HA-MRSA infections in Pakistan is 51% lower than in European countries and the United States of America (Rafay et al., 2020).Scientists are investigating medicinal plants to find new therapeutic options for MRSA's drug resistance. Some plants like lime, pomelo, orange, and tangerine have antibacterial properties, helping to cure several infections and diseases. Rhizomes of ginger, leaves of oak, and pomegranate are used as sources of antibacterial agents against MRSA (Okwu et al., 2019). Citrus plant's flavonoids, limonoids, and phenolic compounds are also good antibacterial agents. Some non-citrus fruits like pomegranate and apple also show bactericidal properties. It is reported that apples have antimicrobial activity against *E. coli, P. aeruginosa, S. serovar, S. aureus,* and *B. cereus* (Chen et al., 2018). Extensive research has been carried out on HA MRSA, but less attention has been paid to CA-MRSA, such as food handlers. Very little has been known about MRSA's carriage frequency, biofilm-forming capacity, and antimicrobial resistance profiles isolated from community settings. The current study emphasized the above-mentioned gaps and the antibacterial activity of citrus and non-citrus fruit juices. The study aimed to determine the prevalence of MRSA isolates in health and community settings. Moreover, to characterize their antimicrobial resistance profiles by disc diffusion or agar dilution techniques. We determined the presence of *Sea* and *Seg*genes. The biofilm-forming abilities of isolates were also determined. The study was done to assess the

antimicrobial activity of citrus and non-citrus fruits against MRSA isolated from three different settings (environment, food handlers, and non-food handlers).

MATERIALS AND METHODS

This cross-sectional study was conducted at the Institute of Microbiology and Molecular Genetics, University of Punjab, Lahore, from September 15, 2020, to March 31, 2021.

Sample collection

A total of 160 non-clinical samples were collected from the food handlers of different cafes, hotels, markets, non-food handlers, and the environment(basin, tabs, and locks). All the samples were proceeded according to bacteriology protocols. Out of which,26 MRSA were used for further analysis. To compare the characteristics of non-clinical samples with clinical ones, 64 isolated clinical strains of MRSA were obtained from Citi Lab and Research Centre, Lahore. The clinical strains were isolated from wounds, skin, pus, ear swabs, urine, CSF, and tracheal swabs. The current study was done on 90 isolated MRSA from clinical and non-clinical settings.

Antimicrobial susceptibility testing (AST)

AST was performed on Muller Hinton agar plates according to the Clinical Laboratory Standard Institute (CLSI) guidelines. Eleven antibiotics, Tetracycline (TE), Ciprofloxacin (CIP), Cefoxitin (FOX), Cephalexin (CN), Fusidic Acid (FA), Triglycine (TGC), Co-trimoxazole (SXT), Linezolid (LZN), Clindamycin (DA), Erythromycin (E), and Chloramphenicol(C), were applied to see the resistance of MRSA against those antibiotics. Results were noted by measuring the zone of inhibition to see whether the strains were susceptible or resistant to applied antibiotics according to CLSI 2019 guidelines.

Juice extract preparation

Citrus fruits, *Citrus maxima (pomelo)*, *Citrus reticulate* (orange), *Citrus aurantifolia* (lime), and non-citrus fruits, *Malus domestica* (apple), *Punicagranatum* (pomegranate) were chosen and collected. Fruits with no cuts or bruises were selected for sampling. Juices were extracted by grinding them into a pestle, and motor and Coarse filtration was done. All the process is carried on under sterilized conditions.

Agar well diffusion assay

Muller Hinton plates were used for agar diffusion assay. A 100 µl of 24-hourculturein broth equal to 0.5 McFarland's standard solution was spread over the plates with a cotton swab to make an even lawn. The Pasteur pipette was used to make wells an equal distance. In the respective well, 100 µl of each fruit was loaded, and plates were incubated for 24 hours at 37°C. Clear zones against the selected strain were recorded for MIC to show antibacterial activity.

Minimum inhibitory concentration (MIC)

MIC was performed to measure the minimum amount of juice inhibiting bacterial growth. Bacteria were grown in TSB, and density was adjusted with the McFarland Standard. Juices of different concentrations were prepared. For MIC, a 96-wellmicrotiter plate was used. In this, 1st column was used as blank. In the second column, only autoclaved broth was added to check sterility. From the 4th to 12th column, 100µl broth was added. Then 100 µl juices were added to 1st well. To make dilutions of 2 µl/ml, 8 µl/ml, 32 µl/ml, 128 µl/ml, and 512 µl/ml concentrations, from 1st well of the column, transfer 100µl into 2nd well and so on. 20µl of test strains were added. In 2nd last row, bacterial cultures were used as the positive control. Then 20µl of bacterial strain was added to each well. Plates were incubated at 37°C for 24 hours. The next day, the reading was taken at 600nm by an ELISA plate reader.

Biofilm formation tests

Two phenotypic tests were performed to indicate whether the strains were biofilm producers.

1. Congo Red Agar test

Strains were streaked on Congo red agar plates and incubated for 24 hours at 37°C. After 24 hours, black colonies were selected as biofilm producers, and pink were indicated as non-biofilm formers.

2. Ring test

A ring test was performed to check the ability of bacteria for biofilm production. Strains were inoculated in Tryptic soy broth and incubated for 24 hours at 37°C. The next day, without mixing, the content of the tube, which was not attached to the tube, was transferred to the falcon tubes. Falcons were centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded and the pellet was treated with 100 µl of crystal violet for 5 minutes. The solution was centrifuged again, and the pellet was treated with 200 µl of 0.85% NaCl

three times. The liquid solution was discarded. Glacial acetic acid was added to remove the leftover strain and assimilation of the pellet. At this stage, a clear ring was seen in the biofilm former strain's falcon. Optical density was taken at 523 nm by spectrophotometer. Then, the tube in which cells were attached tightly was tested. Normal saline was added to those tubes and centrifuged. All the other steps were the same, except we added 100 µl of crystal violet. Again, optical density was noted (Crabbé et al, 2019).

Polymerase chain reaction (PCR)

For the PCR reaction of *Sea* and *Seg* genes, 2µl of DNA extracted by heat lysis method (Kim et al., 2020) was added into the PCR tube, and 13µl of master mix containing $MgCl_2$, dNTPs, PCR buffer, forward and reverse primers, and Taq polymerase. The primer details and PCR conditions are described in Table 1. Gel electrophoresis was done for genomic DNA and PCR products. 2% gel was used for PCR products.

Table 1: Primer sequence for *Sea* and *Seg*genes with annealing temperature

Sr. No.	Genes		Nucleotide sequence	Product size	Annealing temp.	Ref.
1.	*Sea*	Forward	5'-GCAGGGAACAGCTTTAGGC-3'	512	47°C	(Løvseth et al., 2004)
		Reverse	5'GTTCTGTAGAAGTATGAAACACG-3'			
2.	*Seg*	Forward	5'-CGTCTCCACCTGTTGAAGG-3'	328	47°C	(Løvseth et al., 2004)
		reverse	5'-CCAAGTGATTGTCTATTGTCG-3'			

RESULTS

Antibiotic Susceptibility Testing (AST)

Out of 90 MRSA strains, CIP was highly resistant in n=10 (38.4%) non-clinical and n=63 (98.4%) clinical isolates. MRSA from the non-clinical group showed maximum resistance for TE n=8 (30.7%), FOXn=7 (26.9), and CN n=5 (19.2%). While in the clinical group, the maximum resistance was towards SXT and FOX n=57 (89.0%), FA n=56(87.5%) as given in Table. 2.

Table 2: Antibiotic Susceptibility Testing of MRSA isolated from Non-clinical and clinical samples

Antibiotics	MRSA in Non-clinical N=26n (%)	MRSA in clinical N=64n (%)
DA	0	3 (4. 6)
E	2 (7.6)	13 (20.3)
CN	5 (19.2)	52 (81.2)
SXT	2 (7.6)	57 (89.0)
TGC	1 (3.8)	9 (14.0)
LZD	1 (3.8)	4 (6.2)
TE	8 (30.7)	20 (31.2)
FA	3 (11.5)	56 (87.5)
C	1 (3.8)	9 (14.0)
CIP	10 (38.4)	63 (98.4)
FOX	7 (26.9)	57 (89.0)

Well diffusion Assay

in the non-clinical group, n=23 (88%) showed resistance toward *Citrus reticulate*, and n=24 (92.3%) resisted *Malus domestica*. However, in the clinical group, the maximum MRSA n=39 (61%) showed resistance to *Citrus aurantifolia*, and n=41 (64%) resisted *Malus domestica*. In the non-clinical group, maximum sensitivity was demonstrated for *Citrus maxima* n=5 (19%) and *Punicagranatum* n=14 (46%). Most MRSA was sensitive to *Citrus maxima* n=39 (61%), *Citrus reticulate* n=43 (67%), and *Punicagranatum* n=31(48%) in the clinical group as given in Table 3.

Table 3: Antibacterial activity of fruit juices by well diffusion assay

Citrus fruits	Non-clinical N=26n (%)		Clinical N=64n (%)	
	Resistant	**Sensitive**	**Resistant**	**Sensitive**
Citrus maxima (Pomelo)	21 (80.7)	5 (19.2)	25 (39.0)	39 (60.9)
Citrus *aurantifolia* (Lime)	22 (84.6)	4 (15.3)	39 (60.9)	25 (39.0)
Citrus reticulate (Orange)	23 (88.4)	3 (11.5)	21 (32.8)	43 (67.2)
Non-citrus fruits				

Punicagranatum (Pomegranate)	14 (53.8)	12 (46.1)	33 (56.2)	31 (48.4)
Malus domestica (Apple)	24 (92.3)	2 (7.6)	41 (64.0)	23 (35.9)

Minimum Inhibitory Concentration

MIC was performed to check the minimum inhibitory concentrations of fruit juices to kill MRSA. In the non-clinical group, *Citrus maxima* inhibited the n=18 (69.2%) strains at >64µg/ml concentration. And n=4 (15.3%) was only sensitive to non-citrus fruit *Punicagranatum.* While in the clinical group, *Citrus maxima* were inhibiting n=5 (7.8%) of strains at the 32µg/ml concentration. And n=4 (6.2%) strains were sensitive to *Citrus reticulate* at >64µg/ml concentration as in Table 4.

Table 4: Antibacterial activity of fruit juices at two different concentrations

Fruits	**32 µl/ml**	**≥64 µl/ml**
Clinical group		
Citrus maxima	5	3
Citrus reticulate	0	4
Citrus aurantifolia	0	2
Punicagranatum	0	3
Malus domestica	2	1
Non-clinical group		
Citrus maxima	1	18
Citrus reticulate	2	12
Citrus aurantifolia	1	0
Punicagranatum	3	4
Malus domestica	2	1

Biofilm production Tests

Non-clinical groups have a higher ratio of biofilm formers than clinical groups. From the non-clinical group, 57.60% (n=15) of isolates have shown moderate biofilm-forming ability in the Congo Red test. While among the clinical group, the percentage was a little low. Only 51.10% (n=32) of isolates were biofilm formers.

For the Ring Test, the percentages of biofilm formers in the non-clinical group were again higher than in the clinical group. In the non-clinical group, 65.30% (n=17) showed positive results for the Ring test. Only 48.40% (n=31) were positive for the Ring test in the

clinical group. However, 51% (n=33) of strains were non-biofilm former in the clinical group, and 35% (n=9) were non-biofilm formers in the non-clinical (Table. 5).

Table. 5: Frequency of biofilm producers and non-producers by Congo Red and Ring Test

	Non-Clinical N=26		**Clinical N=64**	
	Producer n (%)	**Non-producer n (%)**	**Producer n (%)**	**Non producer n (%)**
Congo Red	15 (57. 6)	11 (42.3)	32 (51.1)	32 (50)
Ring Test	17 (65.3)	9 (34.6)	31 (48.4)	33 (51.5)

Genetic Analysis of *Sea* and *Seg* Genes

Both genes' percentages (independent and combined) were confirmed in all clinical and non-clinical isolates. *Sea* gene was found to be in higher percentage in non-clinical isolates n=2 (8%).However, in clinical isolates, it was present in only n=1(2%) isolate. The *Seg* gene was found to be in nearly the same ratio in both groups, n=13 (50%) in the non-clinical and n=31 (49%) in the clinical group. When the co-existence of *Sea* and *Seg* was checked. It was determined that 20% of strains of both (n=5 non-clinical, n=13 clinical) groups have these enterotoxin genes in co-existence. Percentages are shown in Table. 6.

Table 6: Genetic analysis of Sea and Seg genes

Genes	Non-Clinical N = 26n (%)	Clinical N=64n (%)
Sea	2 (8)	1 (2)
Seg	13 (50)	31 (49)
Co-existence	5 (20)	13 (20)

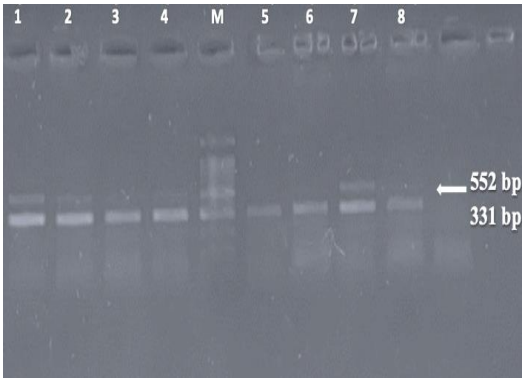

Fig. 1. *sea* (552bp) in lanes 1, 2, 4, 7 and *seg* (331 bp) in lanes 1, 2, 3, 4, 5, 6, 7, 8

DISCUSSION

MRSA is responsible for high antibiotic resistance. In this study, MRSA of both clinical and non-clinical setups, 38.4% and 98.4%,showed maximum resistance towards Ciprofloxacin (CIP), respectively. Antibiotic resistance was recorded as high in the clinical group, as 89% of Strains were resistant to co-trimoxazole (SXT) and Cefoxitin (FOX). However, 95.3% of strains of the clinical group showed sensitivity to clindamycin and, 93.7% were sensitive to Linezolid, 85.9% were susceptible to Triglycine and chloramphenicol. This study's results were different from Manandhar et al., study. He showed in his research that 100% of clinical samples were sensitive to cefoxitin, and 77% were susceptible to ciprofloxacin (Manandhar et al., 2018). It showed that infections caused by MRSA in our region can no longer be treated with the last resort of antibiotics because resistance is relatively high in this region.

However, the non-clinical samples showed less resistance to antibiotics than clinical ones. Most of the strains were sensitive to applied antibiotics. In another study in Pakistan, clinically isolated MRSA strains were susceptible to clindamycin, 84.6%, linezolid 96.7%,

Chloramphenicol83.7%, fusidic acid 70.6%, gentamicin 67.7% and tetracycline 56.8%. The resistance was shown by strains against norfloxacin 91.2%, levofloxacin 87.1%, ciprofloxacin 83.9%, azithromycin 78.6%, erythromycin 77.4%, moxifloxacin 69.8% and sulfamethoxazole/trimethoprim 54.9% (Idrees et al., 2023). In this study, the well diffusion and microdilution methods were implicated in investigating the antibacterial activity of fruit juices. The highest antibacterial activity was shown by non-citrus fruit, *Punicagranatum*, 46.1 %, and citrus fruit, *Citrus maxima,*19.2% in non-clinical samples. In clinical samples, 67.2% of MRSA showed sensitivity towards *Citrus reticulate,* and 48.4% were sensitive to *Punicagranatum*. All of this is an indication that extracts of fruit juices can be used for the treatment of infections caused by MRSA. Both *Citrus maxima* and *Punicagranatum* have shown good antibacterial activity. Using natural remedies for medicinal use has no side effects as well.

The biofilm-forming capacity of *S. aureus* imparts its pathogenicity. The two tests performed in this study to see the biofilm-forming ability of MRSA showed that 57. 6% MRSA of the non-clinical group and 51.1% MRSA of the clinical group are biofilm producers in the Congo ring test. And 65.3% and 48.4% MRSA gave positive results for ring tests in non-clinical and clinical groups. It was previously reported in a study in Nepal that 27% of clinical MRSA are moderate biofilm producers. And 11% of non-clinical strains of MRSA also can form biofilms (Manandhar et al., 2018; Mama et al., 2018). However, in this study, we observed that non-clinical strains have more biofilm-forming ability than clinical ones. It indicates that the strains of this study have robust protective mechanisms against antibiotics and host cells. This biofilm production ability of MRSA is responsible for the MDR mechanisms. HA MRSA infections are responsible for CA MRSA infections and the spread of drug resistance in CA MRSA. *S. aureus* is present on the hands, skin, and other body parts. It sheds off while handling food items. There's a need to be careful and maintain a social distance from the patients discharged from hospitals as they are a hub of microorganisms and passive transfer of germs in the community. A study in Iran has also proved that 52.9% of MRSA are strong Biofilm producers.

MRSA is responsible for severe food poisoning as it produces a variety of

toxins. Two important enterotoxins which are considered to be responsible for this are Sea and Seg as the production of toxins is dependent on the presence of genes. When the *Sea* and *Seg* genes were investigated in the clinical and non-clinical samples, it was found that the non-clinical group had a high prevalence of these genes. *Sea* was found in 8% of strains, and *Seg* was present in 51% of strains of the non-clinical group. While in the clinical group, only 2% of strains had the *Sea* gene, and 49% had the *Seg* gene. The percentage of the *Seg* gene was higher in both groups than that of the *Sea* gene. Udo et al reported that in Kuwait, the Seg gene's prevalence was also high, 24%, and *Sea* was present only in 11% of strains (Udo et al., 2009). Yimam et al also found out that in Brazilian MRSA, the *Seg* was present in 29.3% of strains (Yimam et al., 2020). The results of this study's enterotoxin genes were nearly similar to previous studies where it has been shown that the prevalence of enterotoxin *Seg*gene is higher in the MRSA than in the *Sea*. However, in comparing the clinical and non-clinical groups, our results were contrary to the other studies where the prevalence of enterotoxins was higher in the clinical group than in the non-clinical group. Al Jazirah showed that in Sudan, non-clinical samples have 34% of enterotoxin and the clinical group has 40.5% of enterotoxin genes (Ahmed, 2020).

CONCLUSION

In conclusion, enterotoxin genes are disseminated into CA-MRSA and are involved in causing infections difficult to treat. The medicinal plants used in this study have shown successful antibacterial activity results. Hence, they can be sued for combating drug resistance. Moreover, there's a need to initiate regional MRSA surveillance programs to keep track of emerging clones and to reduce the MRSA infection rate.

CONFLICT OF INTEREST

This study is part of the MS thesis of Ms. Hafiza Iqra Abdul Rasheed. All authors declare no conflict of interest.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801323
Paper Submission: 18th Oct, 2023; Paper Acceptance: 10th March 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Abattoir Effluents' Impacts on the Badri Nullah (SWABI) Stream's Water Quality

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ABSTRACT: *This investigation focuses on the Badri Nullah stream's water quality in the Swabi region. The study was conducted concerning effluents released from a nearby Abattoir (slaughterhouse). A total of five samples were taken, two were upstream and two were downstream and one was in the midstream near to slaughterhouse. Different physiochemical parameters were analyzed to find the water quality in both upstream and downstream locations. The results showed that the electrical conductivity, alkalinity, chloride, and sulfide slightly increased in concentration in downstream locations. The highest values for the above four were, E.C (1320 µS/cm),440 mg/l of alkalinity, sulfur 2 mg/l, and 48.05 mg/l of chloride for downstream samples. All the parameters were then compared with the WHO, NEQS, and many other international standards, in which the majority of them were within permissible limits, while some were exceeding the permissible limits. Which may cause health and other environmental concerns.*

Keyword: Abattoir wastewater, Impact, Badri nullah stream, Effluent, Water quality

INTRODUCTION

Globally, surface water and groundwater systems interact when groundwater is replenished by surface water, exfiltrated onto the soil surface, or discharged as return flow along stream beds (Safeeq and Fares, 2016). This may result in water contamination or an enhancement in the environment for the life forms that live in these

habitats. It is essential to comprehend groundwater-surface water interaction (GSI) to preserve good water quality and quantity as well as the health of the ecosystem. Utilizing this information can help manage water supplies and protect the environment and the ecosystem. It is crucial for creating effective policies and improving the management of water resources (Khan and Khan, 2019).

All types of water derived from various paths have their unique chemistry imparted by the processes. (Eludoyin and Ijisesan, 2020). Rainwater, overland flow, soil water, and groundwater are all combined to form stream flow in a river channel. The stream flow chemistry will be determined by the relative contributions from each of these channels (Omogbehin and Oluwatimilehin, 2022).

There are certain pollution issues associated with the ongoing endeavor to enhance meat production to meet the needs of the ever-increasing global population for protein (Akinro et al., 2009). Safety procedures are hardly ever taken into account when transporting animals to the abattoir when they are being slaughtered, and when dressing hides and flesh (Singh and Sachan 2011). For hygienic reasons, abattoirs process animals with a lot of water (slaughtering and washing), which produces a lot of effluent. The greatest harm to the environment comes from the effluent from an abattoir because of the quantity of suspended particles, liquid waste, and odor generation (Gauri, 2006). Additionally, the fact that fat, blood, dung, meat tissues, urine, and other debris are lost to sewer streams during the processing of animals at abattoirs has demonstrated the contaminating nature of slaughterhouse effluent (Bello and Oyedemi, 2009). These abattoir wastes are highly contaminated with organic materials, suspended particles, and other pollutants (Eze et al., 2013). These contaminants have the capacity to override their importance at greater levels, which can lead to a variety of detrimental health impacts, including liver damage, renal failure, and occasionally fish death. These pollutants can be vital at very low concentrations (Sani et al., 2020).

In this research, the results of different substances present in the slaughterhouse were known. Water quality was assessed in both upstream and downstream areas. changes in parameters resulting from slaughterhouse effluents released into streams were discovered.

MATERIALS AND METHODS

Area of Study

In this research, the study area was Badri nullah stream in district Swabi. The stream originates in the Buner district of the north and travels to the Indus River by way of the Swabi region. A total of five locations were chosen to collect samples from across the stream channel for testing physiochemical parameters in relation to abattoir (slaughterhouse) effluents. In Fig. 1, the study area is depicted.

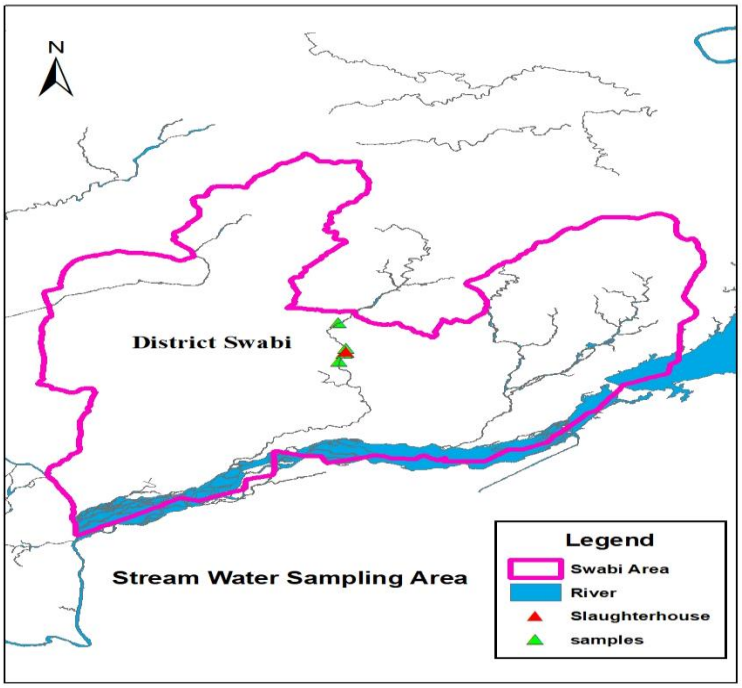

Fig. 1. Study area

Sample Collection

A sampling guide was created before heading out into the field to determine the proper water quality analysis. The instruction includes data, time, place, and the number of sample points. A systematic random sampling procedure was followed The samples were collected from water near bridges along the stream in a sequence manner from upstream to midstream and downstream. The Abattoir effluents were released to midstream going downstream.

Water sampling

Every five different locations along the stream had a sample of water taken. One liter of bottles was taken to gather the samples. The samples were taken from the middle of the stream, not too deep to

avoid any unnecessary impurities going into bottles. The bottles were first rinsed with stream water to make the bottles with the samples. Then these samples were taken into the laboratory for testing.

For sulfide test a separate sampling procedure was followed, because the stream water contained a number of surfactants due to which sulfide test was not possible. For this purpose, a solution of zinc acetate was made, which was used as a preservative for samples. The same sampling procedure was applied but this time with preservatives, and was brought to the laboratory for testing.

Data collection

Data was collected of the sampling sites with GPS points. Two out of five samples were taken upstream, one sample was taken midstream near Abattoir, and two samples were taken downstream.

Sample 1 was taken from Saleem Khan Bridge (Upstream), sample 2 was taken from Maneri Bridge (Upstream), sample 3 was taken from Swabi Bridge (Midstream), sample 4 was taken little below at some distance from Swabi Bridge (Downstream) and sample 5 was taken from Badri Bridge (Downstream). The Abattoir is located near Swabi Bridge from which waste effluents are released. besides Abattoir effluents, sewage from nearby homes and villages were also released into the streams. The data collection sites have been showed in fig 2.

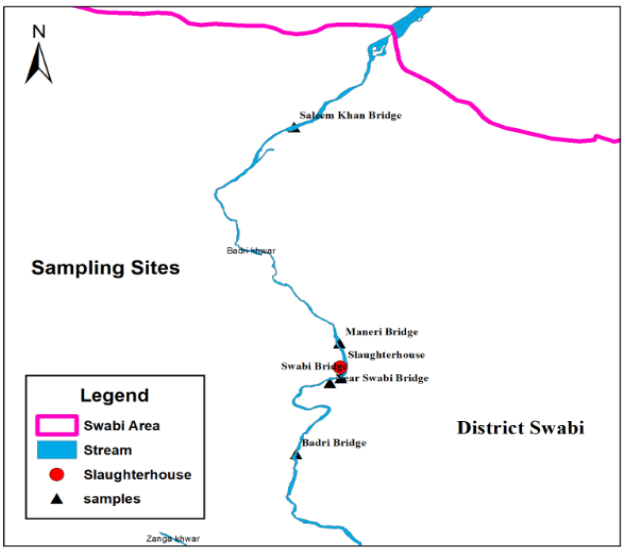

Fig. 2. A Digitized map of sampling sites

Analytical methods

Electrical conductivity (EC), pH, and total dissolved solids (TDS) were all measured utilizing meters (APHA 1985). Titration against H2SO4 with a methyl orange indicator was used to measure alkalinity. Chloride (CL), one of the anions, was examined using a potassium chromate indicator in a titration against $AgNO_3$. Titration against EDTA with the EBT indicator was used to determine the total hardness. Few drops of ammonium buffer PH 10 solutions were added to maintain solution. Calcium (Ca) was determined by titration against EDTA using murexide as an indicator. Total Suspended solids were determined by the method of filter paper and solution passed method, in this method filter paper is weighed and the water is passed through the filter paper and is oven dried for 24 hours at 120°C. Sulfide was analyzed using Iodometric method, it was titrated with starch indicator using Sodium Thiosulfate ($Na_2S_2O_3$) solution. Turbidity test was done through turbidity meter. First standards were set for turbidity test by applying standard test, which were 1, 10, 100, 1000 respectively.

RESULTS

Table 1 and 2 summarized the physical and chemical parameters of the upstream, midstream and downstream locations.

Physical parameters

The analytical data indicated that pH and TDS of all locations were ranging from 6.82 to 7.24 and 650 to 760 mg/l, respectively. The TDS results were compared with the standards of WHO and PSQCA (Pakistan standard quality control authority) and all were in the permissible limits of these standards. Total suspended solids values ranging from lowest (225 mg/l) to highest (280 mg/l). The lowest value occurred at sample 4 where the water is usually stagnant, so that would be the cause of lower value, while at sample 2 the value is highest, it is because of the high runoff speed of water which contain high number of TSS. They were in permissible limits of WHO standards.

Chemical parameters

The total hardness concentration ranged from 126.6 to 233.3 mg/l and all were in the permissible limits according to WHO standards. While Ca concentration in terms of hardness was

reported between 46.6 and 86 mg/l, in range. Sample 3 has more calcium than Sample 2. Because the sample location is next to an abattoir and the waste from the abattoir contains a variety of chemicals, including a high concentration of calcium, the hardness value is high here. We compared the results with NEQS and WHO standards and they were under the standards limit. The Total Alkalinity ranged from 336 to 440 mg/l. The high alkalinity at sample 4 site may be due to the carbonate ions present in water at high concentration, in the case of WHO, they were in the permissible limit. The Chloride concentration ranged from 32.03 to 48.05 and were in the permissible limits according to WHO. The values for Sulfide concentration ranged from lowest (0.4) of sample 1 (Upstream) to highest (2) of sample 3 (Midstream). The high concentration of sulfide at sample 3 was due to Abattoir (slaughterhouse) waste discharge into the stream, which increased the value of sulfide beyond the permissible limit. The values for Turbidity ranged from lowest (8.6 NTU) to highest (11.9 NTU). All the samples were exceeding the standards limit (WHO).

Table 1: Physical parameters of water samples

Electrical conductivity	**Lab Results**	**WWF Standards (2007)**	**WHO Standards**
Sample 1 (U-S)	1196 µS/cm	1500 µS/cm	1200 µS/cm
Sample 2 (U-S)	1180 µS/cm	1500 µS/cm	1200 µS/cm
Sample 3 (M-S)	1320 µS/cm	1500 µS/cm	1200 µS/cm
Sample 4 (D-S)	1255 µS/cm	1500 µS/cm	1200 µS/cm
Sample 5 (D-S)	1183 µS/cm	1500 µS/cm	1200 µS/cm
pH	**Lab Results**	**PSQCA Standards**	**WHO standards**
Sample 1 (U-S)	6.96	6.5 to 9.2	6.5 to 8.5
Sample 2 (U-S)	7.24	6.5 to 9.2	6.5 to 8.5
Sample 3 (M-S)	6.82	6.5 to 9.2	6.5 to 8.5
Sample 4 (D-S)	6.89	6.5 to 9.2	6.5 to 8.5
Sample 5 (D-S)	7.1	6.5 to 9.2	6.5 to 8.5
Total Dissolved Solids	**Lab Results**	**PSQCA Standards**	**WHO Standards**
Sample 1 (U-S)	690 mg/l	1000 mg/l	1000 mg/l

Sample 2 (U-S)	680 mg/l	1000 mg/l	1000 mg/l
Sample 3(M-S)	760 mg/l	1000 mg/l	1000 mg/l
Sample 4 (D-S)	685 mg/l	1000 mg/l	1000 mg/l
Sample 5 (D-S)	650 mg/l	1000 mg/l	1000 mg/l

Table 2: Chemical parameters of water samples

Total suspended solids	**Lab Results**	**PSQCA Standards**	**WHO standards**
Sample 1 (U-S)	260 mg/l	500 mg/l	500 mg/l
Sample 2 (U-S)	280 mg/l	500 mg/l	500 mg/l
Sample 3 (M-S)	230 mg/l	500 mg/l	500 mg/l
Sample 4 (D-S)	225 mg/l	500 mg/l	500 mg/l
Sample 4 (D-S)	245 mg/l	500 mg/l	500 mg/l
Total Alkalinity as Caco3	**Lab Results**	**NEQS**	**WHO Standards**
Sample 1 (U-S)	336 mg/l	200 mg/l	500 mg/l
Sample 2 (U-S)	346 mg/l	200 mg/l	500 mg/l
Sample 3 (M-S)	430 mg/l	200 mg/l	500 mg/l
Sample 4 (D-S)	440 mg/l	200 mg/l	500 mg/l
Sample 5 (D-S)	410 mg/l	200 mg/l	500 mg/l
Total Hardness ($CaCO_3$)	**Lab Results**	**PSQCA Standards**	**WHO Standards**
Sample 1 (U-S)	193.3 mg/l	500 mg/l	500 mg/l
Sample 2 (U-S)	226.6 mg/l	500 mg/l	500 mg/l
Sample 3 (M-S)	126.6 mg/l	500 mg/l	500 mg/l
Sample 4 (D-S)	153.3 mg/l	500 mg/l	500 mg/l
Sample 5 (D-S)	233.3 mg/l	500 mg/l	500 mg/l
Calcium Hardness	**Lab Results**	**NEQS**	**WHO Standard**

Sample 1 (U-S)	46.6 mg/l	200 mg/l	250 mg/l
Sample 2 (U-S)	66 mg/l	200 mg/l	250 mg/l
Sample 3 (M-S)	86 mg/l	200 mg/l	250 mg/l
Sample 4 (D-S)	65 mg/l	200 mg/l	250 mg/l
Sample 5 (D-S)	69 mg/l	200 mg/l	250 mg/l
Chloride	**Lab Results**	**NEQS**	**WHO Standard**
Sample 1 (U-S)	32.03 mg/l	<250 mg/l	250 mg/l
Sample 2 (U-S)	36.03 mg/l	<250 mg/l	250 mg/l
Sample 3 (M-S)	44.04 mg/l	<250 mg/l	250 mg/l
Sample 4 (D-S)	46.05 mg/l	<250 mg/l	250 mg/l
Sample 5 (D-S)	48.05 mg/l	<250 mg/l	250 mg/l
Sulfide	**Lab Results**	**Pak NEQS**	**WHO Standard**
Sample 1 (U-S)	0.4 mg/l	1 mg/l	1 mg/l
Sample 2 (U-S)	0.8 mg/l	1 mg/l	1 mg/l
Sample 3 (M-S)	2 mg/l	1 mg/l	1 mg/l
Sample 4 (D-S)	00	1 mg/l	1 mg/l
Sample 5 (D-S)	00	1 mg/l	1 mg/l
Turbidity	**Lab Results**	**PSQCA**	**WHO Standard**
Sample 1 (U-S)	8.6 NTU	5 NTU	5 NTU
Sample 2 (U-S)	11.9 NTU	5 NTU	5 NTU
Sample 3 (M-S)	10.7 NTU	5 NTU	5 NTU
Sample 4 (D-S)	10.8 NTU	5 NTU	5 NTU
Sample 5 (D-S)	10.3 NTU	5 NTU	5 NTU

DISCUSSION

Physical parameters

Water is a crucial component of the environment; but surface water and groundwater quality have long been deteriorating due to both natural and human-related activities. Natural factors that influence water quality are hydrological, atmospheric, climatic, topographical and lithological factors. (Uddin et al., 2018)

The values for total dissolved solids were ranging from lowest (650 mg/l) to highest (760 mg/l) for sample 5 (D-S) and sample 3 (M-S). The value of TDS at sample 3 is much greater than rest of the samples because the effluents from the Abattoir (slaughterhouse) are directly released here which contribute much to the greater amount of TDS. We compared the TDS results with the standards of WHO and PSQCA (Pakistan standard quality control authority) and all were in permissible limits of these standards. The pH of water determines the solubility (amount that can be dissolved in the water) and biological availability (amount that can be utilized by aquatic life) of chemical constituents such as nutrients (phosphorus, nitrogen, and carbon) and heavy metals (lead, copper, cadmium, etc.) (USGS 2016). The lowest to highest pH value was (6.82-7.24) in sample 2 and 3. By comparing the pH lab results with the Pakistan standard quality control authority (PSQCA) and with the standards of WHO, all the pH ranges of the samples were in permissible limits.

Water quality can be assessed by various parameters such as BOD, temperature, electrical conductivity, nitrate, phosphorus, potassium, dissolved oxygen, etc. (Bhateria and Jain, 2016). The electrical conductivity from lowest to highest was (1180- 1320) in sample 2 (Midstream) and 3 (Downstream). By comparing the lab results with the WWF and WHO standards, it shows that EC is in permissible limit according to WWF (world wide fund for nature) standards while in case of WHO, two samples were exceeding the WHO standards. The major reason for this would be slaughterhouse, because it is located near the area where these samples are taken from.

Total solids can be classified as suspended (non-filterable) or dissolved (filterable), by passing a known volume of sample through a glass-fiber filter (Whatman GF/C) (Sabari et al., 2018).

Total suspended solids values were ranging from lowest (225 mg/l) to highest (280 mg/l). The lowest value occurred at sample 4 where the water is usually stagnant, so that would be the cause of lower value, while at sample 2 the value is highest, it is because of the high runoff speed of water which contain high number of TSS.

Chemical parameters

Hardness of water is due to moisture and carbon dioxide reacts with calcium and magnesium ion present on the earth surface (Boyd et al., 2016). Total Hardness as $CaCO_3$ were ranging from lowest (126 mg/l) to highest (233 mg/l). All the sample were falling in the category of Hard and Very Hard water. We compared the analysis with the PSQCA and WHO standards, and all the samples were falling below the permissible limit. Similarly, Calcium Hardness as $CaCO_3$ were ranging lowest (46.6 US) to highest (86 M-S). Sample 3 has a higher value of calcium Hardness because the sample site is located near the Abattoir, the waste from Abattoir contains different substances including high calcium concentration, therefore the hardness value is high here. We compared the results with NEQS and WHO standards and they were under the standards limit

Total alkalinity is the concentration of titratable bases in water. A base will react to neutralize a hydrogen ion (H^+), for example, in the reaction $H^+ + OH^- = H_2O$, OH^- (hydroxyl ion) is the base. The word "total" is added to alkalinity because the contribution of different ions to total alkalinity may sometimes be reported separately, for example, hydroxide alkalinity (Boyd et al., 2016). Total alkalinity as Caco3 were ranging from lowest (336 mg/l) to highest (440 mg/l) for sample 1 (U-S) and sample 4 (D-S). The high alkalinity at sample 4 site may be due to the carbonate ions present in water at high concentration. We compared the results analysis with the National Environmental quality standards (NEQS) and with the WHO Standards. The sample were exceeding in the case of NEQS, while in the case of WHO, they were in the permissible limit.

The tests were performed for the P-Alkalinity to all the samples, with the addition of phenolphthalein and they remained colorless, therefore the P-Alkalinity was zero.

Chloride is mainly obtained from the dissolution of salts of hydrochloric acid as table salt (NaCl), $NaCO_3$ and added through industrial waste, sewage, sea water etc. Surface water bodies often have low concentration of chlorides as

compare to ground water. It has key importance for metabolism activity in human body and other main physiological processes. High chloride concentration damages metallic pipes and structure, as well as harms growing plants. (Meride and Ayenew, 2016). Chloride concentration in five samples were ranging lowest (32.03 mg/l) for sample 1 (U-S) and highest (48.05 mg/l) for sample 5 (D-S). We compared the results with national environmental quality standards and with WHO standards, all were falling below the permissible limit standards.

The oxidation of sulfide minerals within waste-rock stockpiles can lead to poor-quality drainage that presents an environmental risk and financial liability to mining companies (Amos et al., 2015). The values for Sulfide concentration were from lowest (0.4 mg/l) of sample 1 (Upstream) to highest (2 mg/l) of sample 3 (Midstream). The high concentration of sulfide at sample 3 was due to Abattoir (slaughterhouse) waste discharge into the stream, which increased the value of sulfide beyond the permissible limit. We compared the sulfide results with the Pak (NEQS) and other countries such as Australia and Malaysia. In case of Pakistan all sample were in guidelines limit except sample 3 which exceeded the permissible limit. In comparison with the other countries, samples which contained sulfide were exceeding its limit standards

Turbidity in drinking water is esthetically unacceptable, which makes the water look unappetizing (Omer, 2019).

The values for Turbidity were from lowest (8.6 NTU) to highest (11.9 NTU). All the samples were exceeding the standards limit. By comparing with the standards of PSQCA and WHO standards the Turbidity rate was higher and the water appeared to be turbid.

CONCLUSION

The research study was conducted on stream water for different physiochemical parameters, samples were collected from upstream and downstream with respect to slaughterhouse. Results showed that different parameters were exceeding the standard limits. E.C, Alkalinity, chloride and sulfide showed slight increase in downstream locations, as slaughterhouse waste effluents were entering at midstream location. Although few parameters showed increased in concentration downstream from the slaughterhouse waste effluent entry point. When measured against several standards, the majority of the evaluated metrics fell within the permitted range.

ACKNOWLEDGEMENT

The authors highly acknowledged the Department of Environmental Sciences, University of Peshawar for providing the analytical facilities.

CONFLICT OF INTEREST

There were no hidden conflicts of interest among the authors.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801324
Paper Submission: 17th Dec, 2021; Paper Acceptance: 10th March 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Evaluation of Probiotic Bacteria Isolated from Indigenous Honeybee Species of Pakistan

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ABSTRACT: *The honey bee gut is inhabited by many useful bacteria such as Lactic Acid Bacteria (LAB). These LAB possess active probiotic properties that are beneficial to other animals and even humans. The LAB is declared as generally recognized as safe (GRAS). The LAB is non-pathogenic and produces different metabolites having antimicrobial potential. In the current study LAB species isolated from four honey bee species (Apis melifera, Apis dorsata, Apis cerana, and Apis florae) prevalent in Pakistan were identified and investigated for their probiotic potential. The strains were identified using morphological, biochemical, and molecular techniques as genus Lactobacillus and Lactococcus. Current results confirmed that the honey bee gut has a rich source of lactic acid bacteria. Isolated strains were further evaluated for their ability to resist physiological stresses encountered in the human gastrointestinal tract. Safety testing of the isolates such as hemolytic assay revealed that these isolates have γ-hemolytic activity and were adjudged to be safe. Hence the present study concluded that honey bee gut is the source of potential probiotic bacteria and can be used in pharmaceuticals, fermented foods, and nutraceuticals and may be used as a natural food preservative.*

Keyword: Lactic Acid Bacteria, Gastro Intestinal Bacteria, pH and Bile Tolerance, *Apis spp, Lactobacillus*

INTRODUCTION

Honey bee belongs to the family *Apidae* of insects and contains approximately 20,000 species (Zareen et al., 2016). It is present all around the world excluding the extreme Polar Regions. During the last two centuries, honeybees were traditionally kept in Pakistan for honey production (Ansari et al. 2014). Out of four species of honeybee, that are present in Pakistan three of these species *Apis dorsata, Apis florae, Apis cerana* are native to Pakistan whereas *Apis mellifera* was imported from Australia or Russia in 1979 (Anjum et al., 2016). Declines of honeybee colonies are due to colony collapse disorder (CCD), which may occur because of infections, pesticides, contaminated water, antibiotic use, inadequate diet, and inappropriate breeding management have all been suggested as possible reasons for these large-scale losses (Borges and Goodwin, 2021).

Honey bees play a significant role in increasing crop yield production along with their fruit-bearing capacity in forest plants. They primarily consume pollen and nectar as food and produce a sweet liquid called honey (Nicolson and Thornburg, 2007). LAB enters and colonizes the honey stomach through pollen consumption and food exchange with older bees in the colony (Mardan et al., 2011). Probiotic bacteria are those microorganisms that improve the intestinal microbial flora of the host when it is taken in any supplemented food in an adequate amount and gives health benefits to the host. Bermudez-(Brito et al., 2013). Probiotics isolated from gut bacteria are frequently used in many dietary supplements and functional foods (Daisley et al., 2020) Many proteins and other metabolites are produced by these Lactic acid bacteria (LAB) which protect the honey bee from incoming microbial threats. Honeybee-associated Lactic acid bacteria like *Lactobacillus johnsonii, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus apis* are found to have inhibitory effects on honeybee pathogens such as *Paenibaccilus larvae* and *Melissococcus plutonius.*

Both lactic acid and acetic acid bacteria can tolerate very acidic conditions and they can also metabolize sugars to produce organic acid these unique characteristics of LAB aid their growth in the digestive tract of honey bees which is very rich in sugar content and it also inhibits the growth of acid-sensitive bee pathogenic bacteria (Balloi et al., 2011). Lactic acid production, low G + C %, without spore production, gram +ve, and catalase-ve are key characteristics of lactic acid bacteria and

are mostly used as a starting culture in food industries (Gomez, 2016). The presence of LABs in the gut of honey bees has gut-dwelling properties (Pattabhiramaiah et al., 2012). The objectives of the study were to identify the lactic acid bacteria from the honey bee gut of four different species and characterize their probiotic potential.

MATERIALS AND METHODS

Sample collection and culture condition:

Total eight honey bee gut isolates NPL 813 (*Apis florae*), NPL 814 and NPL 815 (*Apis cerana*), NPL 812, NPL 784, and NPL 785 (*Apis dorsata*), NPL 811 and NPL 786 (*Apis mellifera*) were obtained from the National Probiotic Laboratory (NPL) culture collection Faisalabad, Pakistan. These four different honey bee species were collected from hives of Honeybee Research Institute (HBRI), National Agricultural Research Center (NARC), Islamabad in sterilized bottles, and their gut samples were dissected out aseptically. These honey bee isolates were cultivated on MRS (De Man Rogosa Sharpe) agar plate (Neogen, USA) through the streak plate method. The MRS agar plates were incubated at 37 ºC for 24 to 48 hours under aerobic conditions in an incubator as mentioned by Singh et al. (2013).

Bacterial characterization

Morphological characterization

Bacterial plates were visually examined for colony morphologies i.e. size, shape, color, texture, and appearance. For microscopic observation, gram staining was performed according to the protocol of Erkus and Bannigidad (2011). The prepared slides were observed under an Intelligent Inverted Compound Microscope BX63 Olympus at 100X magnification using immersion oil. The microscopic characters (Gram stain reaction, shape, and arrangement) of the cells were 10 recorded.

Biochemical Characterization

The biochemical (catalase) test was performed by slide method. A small drop of 3% H_2O_2 was taken on a clean glass slide and one to two bacterial colonies were mixed in a drop with a sterilized inoculating loop. The appearance of gas bubbles indicated a positive catalase reaction (Rahman, 2015).

Molecular Characterization

The molecular identification of bacterial isolates was done by genomic DNA extraction followed by polymerase chain

reaction (PCR) using genus-specific primers.

In DNA Extraction, DNA of overnight growth culture was extracted using Thermo Scientific kit (USA) protocol with minor modification (Mattila et al., 2015).

Gel was prepared. Carefully loaded mixed 1 µl (6x loading dye) with 5 µl of each sample into separate wells and 1 µl DNA ladder into another well. The Gel was visualized under UV light and bands were compared with a DNA ladder this is done by usually documentation Costumbrado et al. (2012). The genomic DNA was amplified with different genus-specific primers by PCR (Bio-Rad, USA). Different ingredients used in a PCR reaction are mentioned below. *Lactobacilli* and *Lactococcus* primers were used in this study Lias et al. (2007) Pepe et al. (2002) Costumbrado et al. (2012). Bacterial species were determined by observing the band size.

Primer Sequence

The following (table 1 and 4) Reverse and Forward Primer sequence was used for NPL No. 811, 812, 813, 784, 785, 786, 788 at 16s rDNA identification of *Lactobacilli*.

Table 1: PCR reaction mixture

Sr. no.	Description	Volume	Final concentration
1	Green PCR reaction mix	12.5µl	1X
2	Forward primer	1.5 µl	0.6µM
3	Reverse primer	1.5 µl	0.6µM
4	Nuclease free water	4.5 µl	-
5	DNA template	5 µl	10pg-1µg

Table 2: Primers used in this study

Sr. #	Target genus	Primer sequence	Annealing temp.	Band size	Ref.
1	*Lactococcus lactis*	NPL-L-lactF GAAGTCGTAACAAGG NPL-L-lactR CAAGGCATCCACCGT	50°C	380	Blaiotta et al. (2002)
2	*Lactobacillus*	NPL-lactoF TGGAAACAGCTGCTAA TACCG NPL-lactoR GTCCATTGTGGAAGATT CCC	62°C	233	(Caufield et al., 2007)

Table 3: PCR Profile *Lactobacilli*

Profile	Temperature	Time	Cycle
Initial Denaturation	95ºC	3 Minute	1
Denaturation	95ºC	30 second	35
Annealing	62ºC	30 second	35
Extension	72ºC	1 Minute	35
Final Extension	72ºC	5Minute	1
Hold	4ºC	∞	-

Table 3: PCR Profile for *Lactococcus Lactis*

Profile	Temperature	Time	Cycle
Initial Denaturation	94ºC	1 Minute	1
Denaturation	94ºC	1 Minute	25
Annealing	55ºC	2 Minutes	25
Extension	72ºC	3 Minutes	25
Final Extension	72ºC	10 Minutes	1
Hold	4ºC	∞	-

Bacterial Growth Curve Experiment under Aerobic Condition

Overnight grown bacterial culture was centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded, and the pellet was washed twice with sterile PBS (pH 7.2). Then bacterial suspension was made by mixing the bacterial pellets in the sterile PBS (pH 7.2). The O. D 600 of bacterial cultures was adjusted 0.5 ± 0.05 through spectra max at 600nm wavelength.

The volume of PBS bacterial suspension was calculated through formula M1V1 = M2V2 for adjusting O.D 0.05±0.005 in MRS broth. Alone MRS broth was used as a negative control (without bacterial culture). The bacterial suspensions were inoculated into sterile fresh MRS broth and incubated at 37ºC. The O.D 600 bacterial cultures were taken in triplicate every 1 hour by transferring the 200µl bacterial culture in 96 well micro-titer plates through spectramax at 600 nm. The bacterial cultures O.D600 were measured and plotted in a line graph (Hu et al. 2017).

pH Assay

The NPL strains were revived from glycerol stocks in 5ml MRS broth at 37ºC for 16-18 hours. Bacterial isolates were sub-cultured twice till O. D600nm lies between 0.2 to 0.8. The bacterial culture was centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded, and the pellet was washed with PBS twice. The pellets were suspended in the PBS and vortex for

making the suspension. The Culture OD was adjusted between 0.2-0.8 through a spectrophotometer at 630 nm. Bacterial culture was calculated by using M1V1 = M2V2 for adjusting OD 0.05±0.005 and transferred into the PBS solution pH 1.5, 3.0, 6.5 and properly mixed. The culture OD was taken at 0 hour and incubated at 37ºC for three hours in PBS for stress treatment. After 3 hours, bacterial culture OD was observed. The bacterial culture was centrifuged at 5000 rpm for 5 minutes and cell pellets were suspended with fresh MRS broth. Initial O. D600nm was taken in triplicates and incubated at 37°C in the incubator. After 5h of incubation, O.D 600 nm of broth (positive control) revived from stress solutions was taken in Spectramax. Results were recorded in triplicates to analyze the pH tolerance of bacteria (Leong et al., 2011).

Bile Assay

The NPL strains were revived from glycerol stocks in 5ml MRS broth at 37ºC for 16-18 hours. Bacterial isolates were sub-cultured twice till O. D600nm lies between 0.2 to 0.8. The bacterial culture was centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded and the pellet was washed with PBS twice time. The OD was adjusted in PBS between 0.2-0.8 through spectramax at 600 nm. The bacterial culture was calculated by using the formula M1V1 = M2V2 to adjust the OD to 0.07. The culture was transferred into MRS broth containing 0%, 0.15 %, and 0.3% and mixed through overtaxing followed by incubation at 37ºC. The OD 600 nm was taken at 0 hour and log phase in triplicate in 96 well microplates. Results were recorded in triplicates to analyze the bile tolerance level of bacteria (Ehsani et al., 2012).

Hemolysis Assay

Overnight incubated strains were streaked on blood sheep Agar (Merck, Germany), and incubated at 37 º C for 48 hours. Zones were observed around the streaked culture Mandal et al., (2017). Hemolysis was categorized as no clear halos as non-hemolytic, clear hemolysis zone as β-hemolytic or completely hemolytic, and a greenish halo as α-hemolytic or partially hemolytic.

Glycerol Stock Preparation

The glycerol stocks of each purified isolate were made by subculturing on MRS broth. The broth cultures were preserved by adding glycerol (20% v/v). The mixture was transferred to labeled sterile eppendorf tubes. The label includes their respective NPL number, date of preservation, and initials of the related personnel. Each isolate was

preserved in triplicate cryovials one as mother stock and the other two as working. The stocks were kept in labeled cryo-boxes and stored at –40°C in a Biomedical freezer for further use (Sieo et al., 2014).

RESULTS

Morphological Characterization

In the present study, a total of eight bacterial isolates were taken from the National Probiotic Laboratory (NPL) culture collection Faisalabad, Pakistan. They were as follows: NPL 813 (*Apis florae*), NPL 814 and NPL 815 (*Apis cerana*), NPL 812, NPL 784, and NPL 785 (*Apis dorsata*), NPL 811 and NPL 786 (*Apis mellifera*) shown in figure 1. The identification was performed according to morphological and microscopic characteristics. The bacterial isolates were revived from glycerol stocks on MRS agar plates after 24 to 48 hrs, of incubation at 37ºC under aerobic conditions. All the isolates were gram-positive. Out of eight strains, morphologically six isolates NPL 813 (*Apis florae*), NPL 812, NPL 784, NPL 785 (*Apis dorsata*), NPL 811, NPL 786 (*Apis mellifera*) were bacilli and two isolates of NPL 814, NPL 815 (*Apis cerana*), were Coccus. All were found to be non-motile or non-spore former. The macroscopic characteristics of all the isolates were shown in Fig. 1.

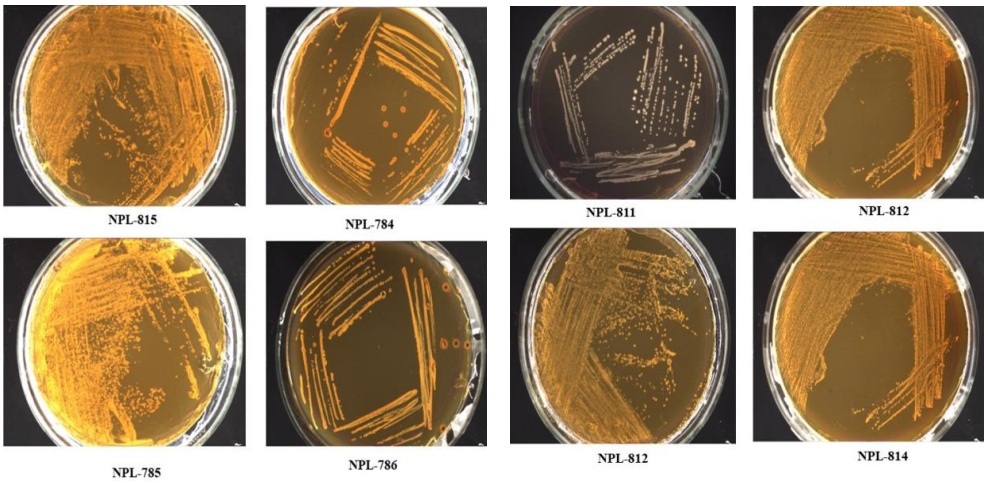

Fig. 1. Growth of honey bee isolates on MRS agar plates after 24-48 hours incubation at 37ºC

Biochemical characterization

All eight isolates of NPL was shown catalase negative. They are not formed bubble on glass slide when drop the H_2O_2 and converted it into H_2O.

Molecular Identification

The genus specific primers of *Lactobacilli* were used to amplify the genomic DNA of gram-positive rods whereas specie specific primers were used for gram positive cocci. The bands represent result after gel electrophoresis of PCR product. The DNA fragment of 380bp and 250 bp was separated on 2% agarose gel along DNA ladder. The observations on gel was taken by putting it in gel documentation system shown in Fig. 2.

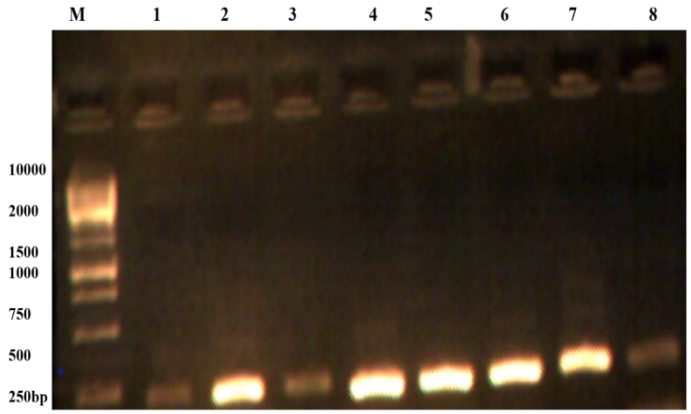

Fig. 2. PCR amplification of honey bee isolates of *Apis dorsata* (NPL-785, 784, 785), *Apis melifera* (NPL-811, 786), *Apis florae* (NPL-813) and *Apis cerana* (NPL-814, 815)

Bacterial Growth Curve Experiment

All the isolates were grown in MRS broth. The growth curve of *Apis dorsata*, isolates NPL784, 785, 812 showed that log phase from 2nd to 10th hours under aerobic condition Figure 3. The growth curve of *Apis melifera* isolates NPL 811 and 786 showed that the log Phase from 3rd to 7th hour hours, 2nd from 12th respectively under aerobic conditions are shown in Figure 4. The growth curve of *Apis florae* isolates NPL 813 showed that the log phase from the 2nd to 7th hour is presented in Figure 5. The growth curve of *Apis cerana* isolates NPL 813 showed

that the log phase from the 3rd to 7th hours is given in Fig. 6.

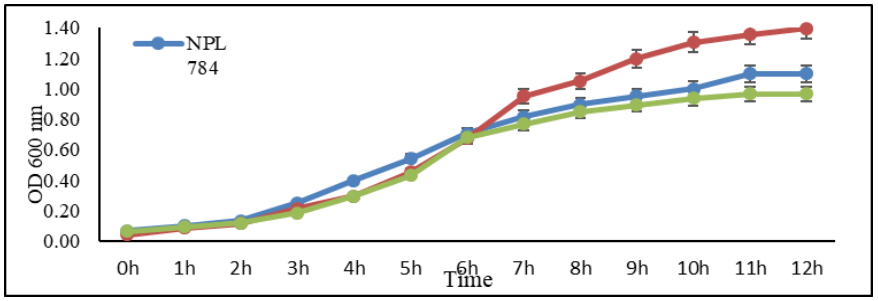

Fig. 3. Growth curve assays of honey be e isolate *(Apis dorsata)* under aerobic condition

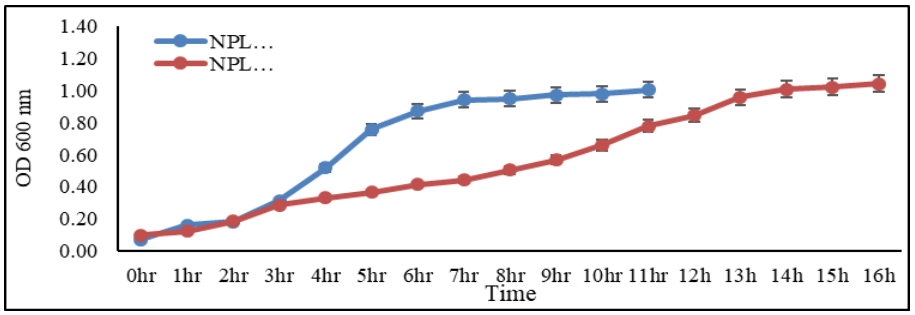

Fig. 4. Growth curve assays of honey bee isolate (*Apis melifera*) under aerobic condition

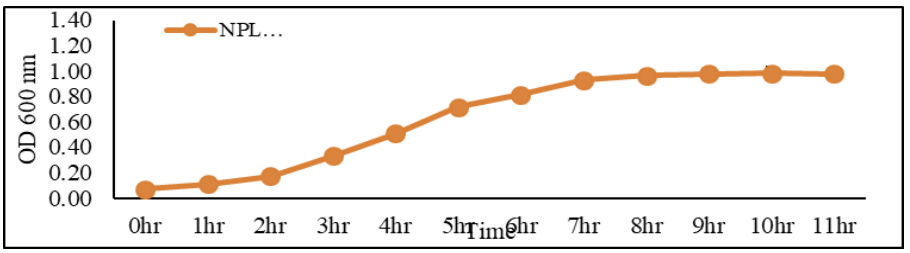

Fig. 5. Growth curve assays of honey bee isolate (*Apis florae*) under aerobic condition

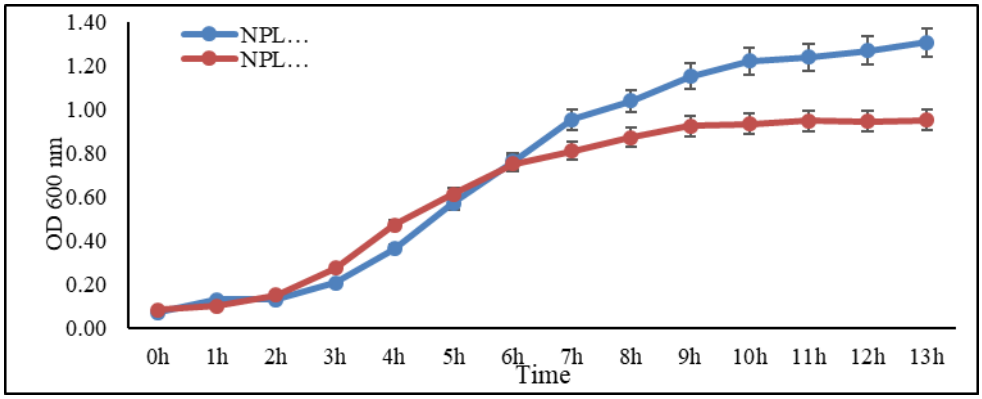

Fig. 6. Growth curve assays of honey bee isolate (*Apis cerana*) under aerobic condition

pH Assay

The results of the present study are shown in figure 7. The sustainability of *lactobacilli* decreased after incubation at pH 1.5 and pH 3.0. The comparison of all strains showed similar pH tolerance by showing significant differences in the cell viability after 3 hours of treatment. The strains were not stressed at pH 6.5 which was thought to reduce the viability of the test bacteria. As a result, all strains continued to exhibit normal growth.

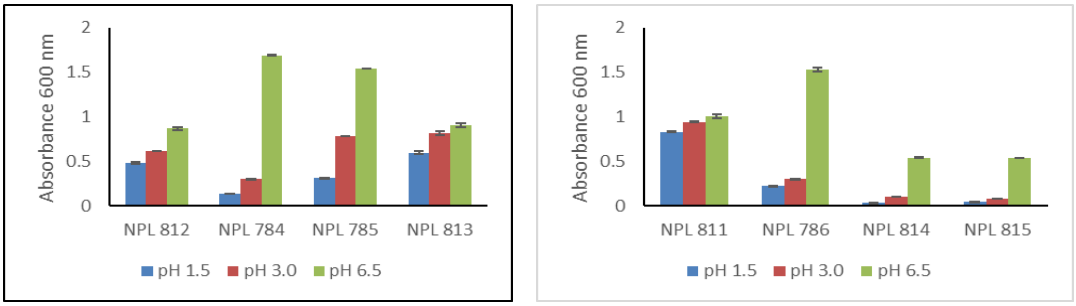

Fig. 7. pH assay for (A) *NPL 812, 784, 785 (Apis dorsata) and NPL 813 (Apis florae)* (B) NPL 811, 786 (*Apis melifera*) and NPL 814, 815 (*Apis cerana*) under aerobic condition

Bile Assay

All NPL strains were resistant to bile concentration with a minor reduction of growth. As all the strains tolerated bile salts so were surviving well in the host intestine which showed their capacity to be resistant to bile salts that may be considered as an antimicrobial molecule. This consequently showed the potential use of LAB as a probiotic, because if LAB can resist the concentration of bile salt then they could

manage to colonize in host intestine as its normal flora. The bile tolerance results of all the NPL strains. Nine NPL were able to grow when cultured at bile salt concentrations of 0.3% and 0.15% (average concentration of bile salt depending upon individual's gastric condition and food ingested) at 1, 2, and 7 hours of incubation Fig. 8 and 9.

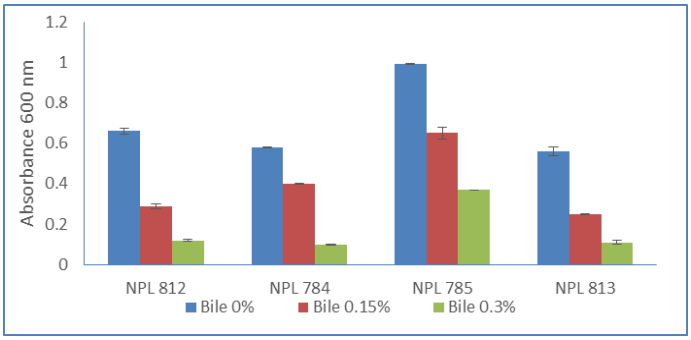

Fig. 8. Bile assay for NPL 812, 784, 785 (*Apis dorsata*) and NPL 813 (*Apis florae*) under aerobic condition

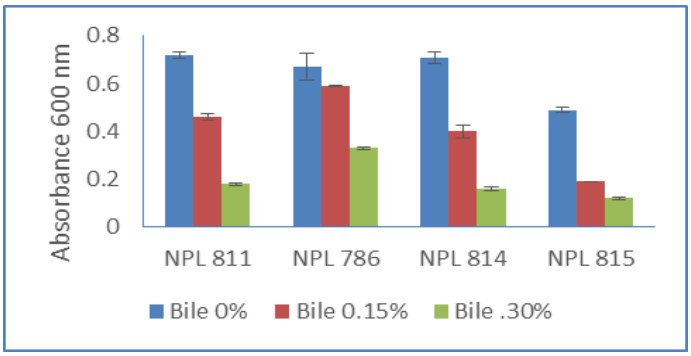

Fig. 9. Bile assay for NPL 811, 786 (*Apis melifera*) and NPL 814, 815 (*Apis cerana*) under aerobic condition

Hemolysis Assay

None of the honey bee isolates formed any zones around the colonies streaked on the blood agar.

Glycerol Stock

The easy way to store bacterial strains for a longer period is to preserve them in glycerol stock. All eight NPL isolates

glycerol stock was placed at -40˚C for further use.

DISCUSSION

The current study was designed to evaluate the probiotic properties of some lactic acid bacteria (LAB). These LAB were isolated from honey bees collected from NARC Islamabad, Pakistan. MRS agar medium was used for the isolation of lactic acid bacteria (LAB). As the cultures were grown on MRS medium, all the isolates were subjected to morphological and biochemical characterization. Selected isolates were found to be gram-positive and catalase negative. *Lactobacilli* were gram-positive rods that occur in chains, pairs, or singly. They were facultative anaerobes, non-endospore forming, and non-motile bacteria. *Lactococcus lactis* isolated from honeybees found was coccoid, gram-positive, facultative anaerobes, non-endospore forming, non-motile, catalase-negative that occurs in chains, pairs, or singly.

The NPL strain was isolated from the gut of *Apis Mellifera* honey bee species of Pakistan obtained from an apiary maintained by NARC Islamabad. The physiology and biochemical characteristics of selected LAB isolated are similar to those described in Bergey's Manual Determinative of Bacteriology for genera *lactobacillus* and *Lactococcus* Grimont and Grimont (2005).

Probiotic properties of the isolated bacteria were evaluated and their pH and bile assay. Probiotics help to improve digestion and boost non-specific immunity Elzeini et al. (2021; Safonov (2020). Out of these isolated bacteria, all were able to grow at as low as pH 1.5 and at pH 3.0. All isolates retained their viability at this low pH, but their growth was insignificant. Our study was corroborated by a study, where isolated strain showed insignificant growth withstanding low pH of 1.5 and 3.0 El Sohaimy et al. (2016). When the pH of the medium was increased from pH 3 to pH 6.5, there was a gradual increase in the growth of bacteria. This shows that our isolated strains can survive in a human gastric environment which has a pH of 1.5 to 3.0. All eight NPL strains were grown at low pHs of 1.5 and 3.0 in PBS. After exposure to pH, all eight strain retain their viability. These results agree with other studies, where *lactobacillus* strains can maintain their viability when exposed to low pH.

Bile concentration in the small intestine varies and ranges up to 0.3% w/v in humans which is necessary for the metabolic activity of bacteria and in

contributing balance of intestinal microflora Shehata et al. (2016). LAB enduring the bile concentration is considered as bacteria of choice for probiotic activity. All eight strains were able to tolerate bile concentrations of 0.15% and 0.3 %. The NPL strain 785 was more tolerant to this bile concentration concerning other strains. The strain was more tolerant of 0.15% bile than 0.3% concentration. Similar results were found in two different studies observing reduced viability and diminished growth at similar concentrations Arcand et al. (2005) Bianchi et al. (2014). Thus strain 785 proved to be a more beneficial probiotic bacteria based on its characteristics. All other strains maintained their viability and downscale culture at the given conditions with lesser probiotic activity but fulfilled the basic criteria.

Hemolysis on blood sheep agar is a safety consideration that should not be observed in the potential probiotic strains for their selection. Along with hemolysis, antibiotic resistance is another phenomenon that if present excludes the potentially probiotic strain from being applied for human or animal consumption (Food, 2002). In the current study, all strains were screened for hemolysis, and all the strains with γ-hemolysis (i.e. no hemolysis). The findings were supported by previous studies on hemolysis with similar results Zoumpopoulou et al. (2013) Viale et al. (2014).

Molecular analysis is used for the definitive identification of the strains. Out of eight isolated strains, six isolates belonged to the *lactobacillus* genus (75%) and two belonged to the *Lactococcus* genus (25%). The ratio of *Lactobacilli* species was higher as compared to *Lactococcus* showing that they are more prevalent probiotic bacteria. The *Lactococcus* strains were identified as *Lactococcus lactis* species. The *lactobacilli* were predominant in a similar conducted study with (10%) and *Lactococcus lactis* were found to be (10%) in the study Edward et al. (2018).

CONCLUSION

It was concluded in the study that the bacteria isolated from the honey bee gut have probiotic potential. The bacteria were gram-positive and molecular technique identified them as *Lactobacillus* and *Lactococcus*. *Lactobacillus* were found in abundant and comprised 78% of the isolates. The probiotic potential activity can be used in food, pharmaceutical, and nutraceutical industries for the well-being of living organisms.

ACKNOWLEDGEMENT

The author is highly thankful to the National Probiotic Lab (NPL) at the National Institute of Biotechnology and Genetic Engineering in Faisalabad for this research.

CONFLICT OF INTEREST

Author's declare there is no conflict of interest.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801325
Paper Submission: 20th Dec, 2023; Paper Acceptance: 10th March 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Antibacterial and Antibiofilm Activity of *Eucalyptus camaldulensis* Derived Fe_3O_4 Nano-particles against Foodborne Pathogens

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ABSTRACT: *Foodborne pathogens are zoonotic and multidrug resistant, which are not only affecting economy but also accountable for public health burdens. The present study was aimed to evaluate the efficacy of medicinal plant i.e., Eucalyptus camaldulensis extract mediated Fe_3O_4 and ZnO nanoparticles against the 27 food-borne pathogenic strains, isolated from milk, meat, dry fruits and vegetable samples collected from Multan, Pakistan. In phytochemical screening, the plant extract was found to contain numerous bioactive compounds including flavonoids, phenolic compounds, tannins, quinones, and anthocyanins. Fe_3O_4 NPs synthesized from Eucalyptus camaldulensis displayed the highest antibacterial activity with zones of inhibition of 12-13 mm against pathogens. Fe_3O_4 NPs were found to have highest anti-inflammatory potential with recorded percentage of 67 % at 40 µg/ml. Fe_3O_4 NPs also demonstrated the highest antibiofilm activity after 120 hours of incubation. For DDPH antioxidant assay, the highest antioxidant activity was displayed by Fe_3O_4 NPs and their absorbance recorded was 1.43. Therefore, Eucalyptus camaldulensis mediated Fe_3O_4 NPs proved as an effective and eco-friendly approach to combat multidrug resistance in bacterial infections through characteristic antibacterial, antibiofilm and antioxidant properties.*

Keyword: Foodborne pathogen, *Eucalyptus camaldulensis* extract, Nano-particles

INTRODUCTION

Food-borne illness is caused by a variety of pathogenic species like *Salmonella*, *Shigella*, *Vibrio Campylobacter*, *C. perfringens*, *Clostridium botulinum*, some *Escherichia coli* sero groups, *Bacillus cereus* and *Listeria monocytogenes* which are either taken intentionally or un-intentionally in body through different sources like raw or improperly cooked food, unprocessed dairy products, unhygienic food packaging and contaminated water etc. (Aladhadh, 2023). Immuno competent persons are not affected by them, but immuno suppressed patients, old people, young children, and pregnant women are susceptible to these pathogens. Later suffers from spontaneous abortion of the baby, septicemia, stillbirth and even death of the child. Pathogens are a small group of microbes, consisting of ≤10 species, responsible for severe illnesses due to annual global food poisoning (Elbehiry et al., 2023).

Variety of bacterial species present in raw milk cause zoonotic diseases like brucellosis, salmonellosis, tuberculosis and Q- fever (Alves de Aguiar Bernardo et al., 2021). Poultry is known as a prime source of pathogens which cause economic losses for the poultry industry and potential health risks like irritable bowel syndrome and long-term arthritis (Tarabees et al., 2017). Certain strains of *Salmonella* spp., and *Listeria monocytogenes* remain alive on dry fruits and vegetables for an extended period and are far more challenging to kill (Sheng and Wang, 2023). According to a report of WHO, diarrheal diseases, lower respiratory infection, malaria, HIV/AIDS and tuberculosis are top diseases associated with morbidity and mortality (Reygaert, 2018).

These pathogenic microbes are MDR because they have drug resistant genes which cause enzymatic inactivation, drug permeability reduction, antimicrobial target modification, and drug efflux. Moreover, pathogens form biofilms to protect themselves from the action of drugs, thus antibiotics are not effective to treat such infections (Castro-Vargas et al., 2020). Biofilm necessitates a physical surface to grow; formed on sutures, medical devices, catheters, dental implants, human and animal tissues, damp building materials, and aquatic habitats; and produce toxic substances (Dutt et al., 2022). Researchers are taking a look at different methods that could get rid of biofilm and so far, plant derived nano-particles appears to be the most successful way of preventing the formation of biofilm (Liu et al., 2023). Gene expression is increased in biofilm forming microbes; and extracellular

matrix secretion is a major factor which causes successful adherence, colony formation and maturation (Mohamad et al., 2023).

Medicinal plants possess therapeutic potential and thus are traditionally being used for phyto-therapy, aromatherapy, and home remedies (Villa et al., 2022). One of these plants is *Eucalyptus* having antibacterial properties. It contains compounds like eucalyptol and essential oils which not only kill bacteria like *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Trichophytonmentagrophytes* etc; but also inhibit biofilm formation. *Eucalyptus globulus* from *Myrtaceae* family, is a valuable source of many phytochemicals and has numerous applications ranging from medicinal to cosmetic. It is known to have antibacterial, anti-inflammatory and antioxidant properties (Surbhi et al., 2021).

In nanotechnology, various metals like Gold (Au), Silver (Ag), Copper (Cu), Zinc (Zn), Platinum (Pt), Iron (Fe), Nickel (Ni) and Cobalt (Co), are used to synthesize metallic and green nanoparticles, which have proven to be effective for antimicrobial therapy, and are involved in the treatment of cancer, bacterial infections and inflammation (Chandrakala et al., 2022). Iron oxide NPs are proven to be effective in eliminating various types of heavy metal ions and organic compounds (Tan et al., 2023). ZnO NPs are antimicrobial agents and one of the theories explains "Trojan Horse effect" for them which means nanoparticles are degraded inside the cellular lysosomes which results in the release of metal ions and other harmful elements that ultimately disrupt the cellular reproduction (Pushpalatha et al., 2022). This research was focused assess the antibacterial, anti-biofilm, antioxidant and anti-inflammatory potential of *Eucalyptus camaldulensis* extract mediated Fe_3O_4 and ZnONPs against the food-borne pathogens.

MATERIALS AND METHODS

Isolation and characterization of food-borne pathogens

Food samples of various types *i.e.*, milk, meat, raw vegetables, and dry fruits were collected from different areas of Multan Pakistan. A 25 g of each sample was added separately in 250 ml of water, blended for 5minutes and then filtered via sieve of 4 mm pore size. 10ml of each filtrate was then serially diluted upto 10^{-7}; spread on SS Agar and Nutrient Agar; and incubated for 24 hours at 37°C. Selected colonies were characterized morphologically and streaked on MacConkey and EMB agar to examine the colony appearance.

Isolated strains were then subjected to biochemical characterization following protocol given by Cappuccino and Welsh (2019).

Antimicrobial susceptibility and slime production test of food-borne pathogens

These tests were performed according to the protocols of Dela Cruz and Torres (2012), Ronavari et al. (2021) and Wilson et al. (2017) respectively. Antimicrobial susceptibility was checked against following antibiotics; carbapenem/CRO (30 µg), erthyromycin/E (10 µg), amikacin/Ak (30 µg), Penicillin G/P (10 units), tetracycline/TE (30 µg), levofloxacin/LEV (5 µg), amoxacillin/AML (10 µg), ampicillin/AMP (10 µg) and ciprofloxacin/CIP (5 µg).

Preparation of plant extract and nanoparticles

Fresh leaves of *Eucalyptuscamaldulensis* collected from the vicinity of Multan city (species identified and confirmed by The Department of Botany, The Women University Multan) were gently washed with tap water, dried at 60°C and powdered using mortar and pestle. 20 grams of Eucalyptus powder was then transferred to 100 ml distilled water and boiled at 80°C for 60 minutes in water bath. After that it was filtered through Whatman qualitative filter paper no. 1. Different concentrations of *Eucalyptus* aqueous extract *i.e.*, 2%, 4% and 8% were prepared and stored at 4°C till further use. Phytochemical screening of Eucalyptus aqueous extract was done according to the method described by Shaikh and Patil (2020) with slight modifications.

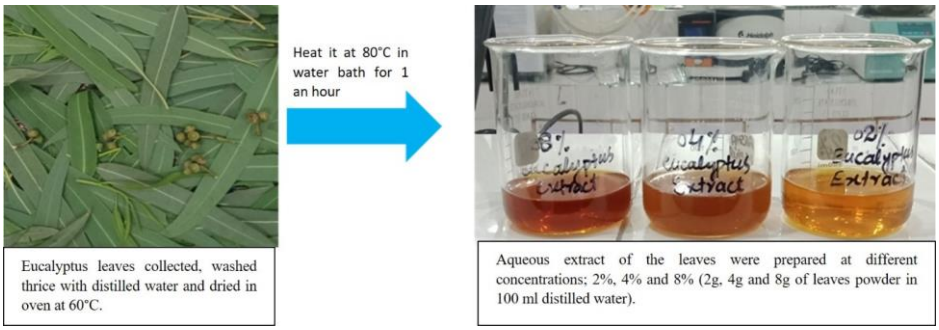


Fig. 1. Preparation of *Eucalyptus camaldulensis* aqueous extract from the collected plant leaves

Iron oxide nanoparticles were synthesized by using the method of Andrade-Zavaleta et al. (2022) with slight modifications. 0.1 M ferric chloride solution was prepared in distilled water and added to 2% aqueous extract of *Eucalyptus* in a 1:2 ratio. It was homogenized in rotary shaker for 30 minutes and then lyophilized for 3 hours, following Christ LCG lyophilization manual.

Zinc oxide nanoparticles were synthesized by using the protocol of Barzinjy and Azeez (2020) with slight modifications. 30 ml of the extract was added in beaker and heated at 60°C. 3g of zinc sulfate was then added and stirred continuously for about an hour until it turned into yellow paste. After that it was placed in oven at 400°C for two hours, and then rinsed with ethanol and distilled water repeatedly and the resulting powder was then dried in oven at 100°C.

Minimum inhibitory concentration of ZnO and Fe_3O_4 nanoparticles

MIC of the NPs was checked by using the protocol of Mann and Markham (1998) with slight modification. In this method, NPs of varying concentration were prepared as: 2.4, 1.2, 0.6, 0.3, 0.15, 0.075, 0.0375, 0.018 and 0.009 µg/ml and inoculated in the media. 20 µL of this was then added in 96 well flat bottom polystyrene plate and incubated for one day and read using microtiter plate reader. The absorbance was measured at 600 nm.

Antibacterial, anti-inflammatory, antibiofilm and antioxidant activity of plant extract and NPs Antibacterial effect of plant extract and nanoparticles

Agar well diffusion method was used to evaluate antibacterial activity of plant extract and NPs. For this purpose, isolate was spread on MHA using cotton swab, wells were made on agar using Pasteur pipette and 100 µL of the extract, iron oxide nanoparticles and zinc oxide nanoparticles were added in the wells. The plates were then incubated at 37°C for 24 hours. Then antibacterial activity was assessed by measuring the diameter of zones of inhibition in millimeter.

Anti-inflammatory assay

0.2% w/v BSA working solution was prepared and 5ml of this reagent was added in 50 µL of the different concentrations of the nanoparticles (200, 400, 800) µg/ml. Tubes were placed in a water bath at 75°C for 5 minutes and cooled at room temperature. Ascorbic acid was used as a standard control and absorbance was recorded at 600nm-660nm against the standard control.

Anti-inflammatory activity assay was performed in triplicates and results are described in terms of % activity, by using the following formula:

Anti-inflammatory assay (%) = (OD $_{control}$ −OD $_{sample}$) / OD $_{control}$ × 100]

Qualitative biofilm and anti-biofilm assay

Protocol of Mathur et al. (2006) was used with slight modifications to determine biofilm formation potential. BHI was prepared with 2% sucrose to check biofilm formation. In 96-well micro-titer plate, 180 µL of fresh broth was dispensed and 20 µL of bacterial culture (McFarland standard 0.5) was inoculated. Plate was then incubated at 37°C for (72, 120,168) hours. The test was performed in triplicate and repeated 3 times. Broth from each well was decanted and deionized water was used to wash planktonic cells from wells. The biofilms were fixed with 2% sodium acetate, stained with 0.1% w/v crystal violet and excess stain was rinsed off by thorough washing with deionized water. Plates were kept for drying and then read using microtiter plate reader at the OD of 570 nm.

Protocol of Kalishwaralal et al. (2010) was followed slight modifications for anti-biofilm assay. 160 µL of BHI broth was added in individual wells of 96-well-flat bottom TCP and inoculated with 20 µL of overnight culture (McFarland standard 0.5). A 20 µL of extract was then added and plate was incubated at 37°C for (72, 120 and 168) hours. The test was performed in triplicate and repeated 3 times. Further steps were performed as mentioned for qualitative biofilm assay.

Antioxidant assays

FRAP Assay: Protocol given by (Schlesier et al., 2002) was followed where five different concentrations of extract and NPs (20, 40, 60, 80 and 100)µg/ml were used in which potassium phosphate buffer (pH set at 6.6) was added following the addition of 2000 µL of the potassium ferric cyanide was added. The tubes were incubated in water bath at 50°C for 20 minutes. After that 2500µL of 10% tri-chloroacetic acid was added and subjected to centrifugation at 5000 rpm for 5 minutes. Afterwards 2 ml of supernatant was transferred to new tube and mixed with 200 µL of 0.1% ferric chloride and 2 ml of distilled water was added in the tubes. Left the tube left for 10 minutes and absorbance was recorded at 765 nm. Same procedure was performed with ascorbic acid which was used as a standard. The test was performed in triplicate and %age of the antioxidant activity was measured using following

formula: [FRAP (%) = FRAP $_{blank}$– FRAP $_{sample}$ / FRAP $_{blank}$ x 100].

RESULTS

Isolation and Characterization of food-borne pathogens

Food samples of various types (milk, meat, raw vegetables, and dry fruits) were collected from different areas of Multan Pakistan. A total of 27 strains were isolated and subjected to morphological and biochemical characterization with their results listed in Table 1.

Table 1: Morphological and biochemical characterization of food-borne pathogens

Strain isolated from food samples	Colony color on MacConkey Agar	Colony color on EMB Agar	Gram Staining	Catalase test	Oxidase test	Citrate test	Triple Sugar Iron (TSI) Test. *Y= yellow R=Red* ✓*=Yes,* ×*=No*				MR-VP test	
			Positive (+) Negative (-)				*Slant*	*Butt*	*CO_2*	*H_2S*	*Methyl-Red*	*Vogues-Proskauer*
S1D1	Light Pink	Pink	-	+	-	+	Y	Y	✓	✓	Red	Rose Red
S2D1	Light Pink	White	-	+	-	-	Y	Y	✓	✓	Pink	Yellow
S3D1	Yellow	-	+	+	+	-	R	Y	✓	×	Red	Rose Red
S4D1	Light Pink	Pink	-	+	+	+	R	O	✓	✓	Yellow-Orange	Rose Red
S5D1	Light Pink	Purple	-	+	+	-	R	Y	×	✓	Red	Rose Red
S6D1	Light Pink	Purple	-	+	-	-	Y	Y	✓	×	Pink-Red	Yellow
S7D1	-	White	-	+	+	+	R	R	×	✓	Red	Yellow

S1D2	-	Green	-	+	+	-	R	Y	✓	×	Yellow-Orange	Yellow
S2D2	-	Green	-	+	-	-	Y	Y	✓	×	Pink-Red	Yellow
S3D2	-	Pink	-	+	+	+	R	R	✓	×	Pink-Red	Rose Red
S4D2	White	Wheat	-	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S5D2	-	-	+	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S1D3	-	-	+	+	-	+	R	R	×	✓	Yellow-Orange	Yellow
S2D3	-	-	+	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S3D3	-	-	+	+	+	+	R	R	×	✓	Pink-Red	Rose Pink
S4D3	White	Pink	-	+	+	+	R	R	×	✓	Red	Pink
S5D3	Colorless	Pink	-	+	-	+	R	R	×	✓	Orange	Yellow
S6D3	Light Pink	Pink	-	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S7D3	Baby Pink	Pink	-	+	+	+	R	R	✓	✓	Pink-Red	Rose Pink
S8D3	-	-	+	+	+	+	R	R	×	✓	Pink-Red	Pink
S1D4	White	White	-	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S2D4	Colorless	Pink	-	+	+	+	R	R	×	✓	Orange	Yellow
S3D4	-	-	+	+	+	+	R	R	×	✓	Pink-Red	Rose Pink
S4D4	Wheat	Purple	-	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S5D4	yellow	Pink	-	+	-	+	Y	Y	✓	×	Pink	Rose Pink
S6D4	-	-	+	+	+	+	B	R	×	✓	Pink-Red	Yellow

S7D4	-	Green	+	+	-	-	R	Y	✓	×	Yellow-Orange	Rose Pink

Antimicrobial susceptibility and slime production test of food-borne pathogens

Certain microorganisms produce (proteolytic extracellular enzyme) gelatinase, which is involved in liquefaction process and hydrolyzes protein to amino acids. Even extremely low temperatures of 4°C won't be able to recover the gel characteristic once this degradation has taken place. In this study, only strains S3D1, S5D1 and S7D1 gave the positive results which means that they have the enzyme gelatinase. For slime production test, the appearance of black coloredcolonies in the presence of Congo red dye and sucrose indicated the strong slime production and results are given in Table 2. Strain S4D1 showed the maximum sensitivity against ceftriaxone (CRO). Most strains were resistant to penicillin, tetracycline and erythromycin. Most bacterial strains were sensitive to ciprofloxacin, amikacin and levofloxacin as shown in Table 3.

Table 2: Slime production test of food-borne pathogens

Strain	*Congo Red Agar without Sucrose*	*Congo Red Agar with Sucrose*	Strain	*Congo Red Agar without Sucrose*	*Congo Red Agar with Sucrose*	Strain	*Congo Red Agar without Sucrose*	*Congo Red Agar with Sucrose*
S1D1	Light Pink	Black	S3D2	Baby Pink	Red	S7D3	Pink	Light Red
S2D1	Red	Black	S4D2	Pink	Black	S8D3	Pink	Light Red
S3D1	Red	Red	S5D2	Baby Pink	Red	S1D4	Red	Orange
S4D1	Light Red	Black	S1D3	Pink	Pink	S2D4	Red	Orange
S5D1	Bright Red	Red	S2D3	Pink	Orange	S3D4	Light Pink	Red
S6D1	Baby Pink	Light Black	S3D3	Pink	Light Red	S4D4	Red	Red
S7D1	Baby Pink	Black Color	S4D3	Pink Color	Light Red	S5D4	Light Pink	Black
S1D2	Red	Red Color	S5D3	Pink	Pink	S6D4	Peach	Black

S2D2	Baby Pink	Black Color	S6D3	Color Pink Color	Light Red	S7D4	Peach	Black

Table 3: Antibiotic sensitivity profiling of food-borne pathogens

Strain	**Antibiotic Sensitivity profiling (millimeter)**								
	P	**E**	**CRO**	**AMP**	**CIP**	**AK**	**LEV**	**AML**	**TE**
S1D1	R	R	R	R	R	S	S	S	R
S2D1	R	R	S	R	S	S	S	R	R
S3D1	R	S	S	R	S	S	S	R	R
S4D1	S	S	S	S	S	S	S	S	S
S5D1	R	S	S	R	S	S	S	R	R
S6D1	S	S	S	R	S	S	S	R	S
S7D1	R	S	S	R	S	S	S	S	S
S1D2	R	S	S	S	S	S	S	R	S
S2D2	S	S	S	S	S	S	S	S	S
S3D2	R	S	S	R	S	S	S	R	S
S4D2	R	S	S	R	S	S	S	R	S
S5D2	R	S	R	R	S	S	S	R	R
S1D3	R	R	R	R	S	S	S	R	R
S2D3	R	R	R	R	S	S	S	R	R
S3D3	R	R	R	R	S	S	S	R	R
S4D3	R	R	R	R	S	S	S	R	R
S5D3	R	R	R	R	R	R	R	R	R
S6D3	R	R	R	R	S	S	S	R	R
S7D3	R	R	R	R	S	S	S	R	R
S8D3	R	R	R	R	S	S	S	R	R
S1D4	R	R	S	R	S	S	S	R	R
S2D4	R	R	R	R	S	S	S	R	R
S3D4	R	R	R	R	S	S	S	R	R
S4D4	R	R	S	R	S	S	S	R	R
S5D4	R	R	S	R	S	S	S	R	R
S6D4	R	R	S	R	S	S	S	R	R
S7D4	R	R	S	R	S	S	S	R	R

*R=Resistant, S=Sensitive

Preparation of plant extract and synthesis of metallic NPs

Plant extract was prepared from the leaves of *Eucalyptus camaldulensis* and subjected to phytochemical screening. Carbohydrates, reducing sugars, flavonoids, phenolic compounds, tannins and quinones were found in the extracts (Table 4).

Table 4: Phytochemical profiling of *Eucalyptus camaldulensis* leaves extract

Compound	Observation	Results
Alkaloids	Yellow color appeared	-
Carbohydrates	A violet color ring formation	+
Reducing Sugars	Green color appeared	+
Glycosides	Yellow color appeared	-
Proteins and Amino Acids	Yellow color appeared	-
Flavonoids	An intense yellow color	+
Phenolic Compounds	Black color appeared	+
Tannins	Formation of emulsion	+
Quinones	Red color appeared	+
Anthocyanin	Yellow color	-

***+=Positive, -=Negative**

MIC determination of Iron Oxide Nanoparticles

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent (such as nanoparticles) that inhibits the visible growth of a microorganism. The lowest MIC value recorded for the Iron Oxide nanoparticle was 0.04 microgram / milliliter against S5D1.

Antibacterial, anti-inflammatory, antibiofilm and antioxidant activity of extract and NPs

Order of antibacterial activity from highest to lowest, with recorded zones of inhibition was found as Fe_3O_4 NPs (12-13mm), ZnO NPs (7-8mm) and *Eucalyptus* extract (2-3mm) respectively. So Fe_3O_4 nanoparticles performed the maximum antibacterial activity (Fig 2 (a)). While Fe_3O_4 NPs were also found to have highest anti-inflammatory potential with recorded percentage of 67% at 40 µg/ml (Fig 2 (b)). Fe_3O_4 NPs also performed the highest antibiofilm activity after 120 hours of incubation (Fig 2 (c)). For DDPH assay, the highest antioxidant activity was performed by Fe_3O_4 NPs and their absorbance recorded was 1.43. In FRAP antioxidant assay, the highest

activity was performed by *Eucalyptus* (Fig. 3). extract with recorded absorbance of 1.07

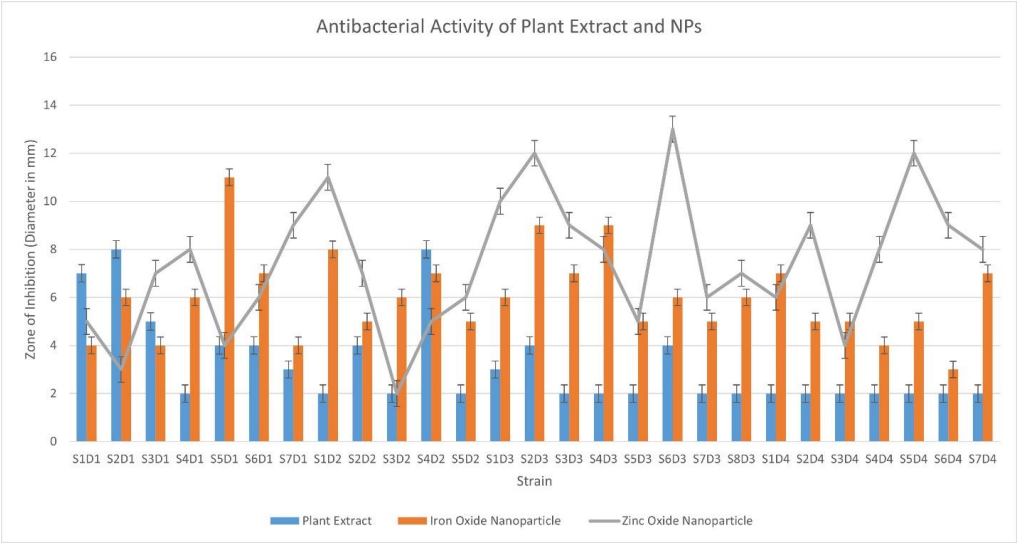


Fig. 2a. Antibacterial activity of plant extract and NPs

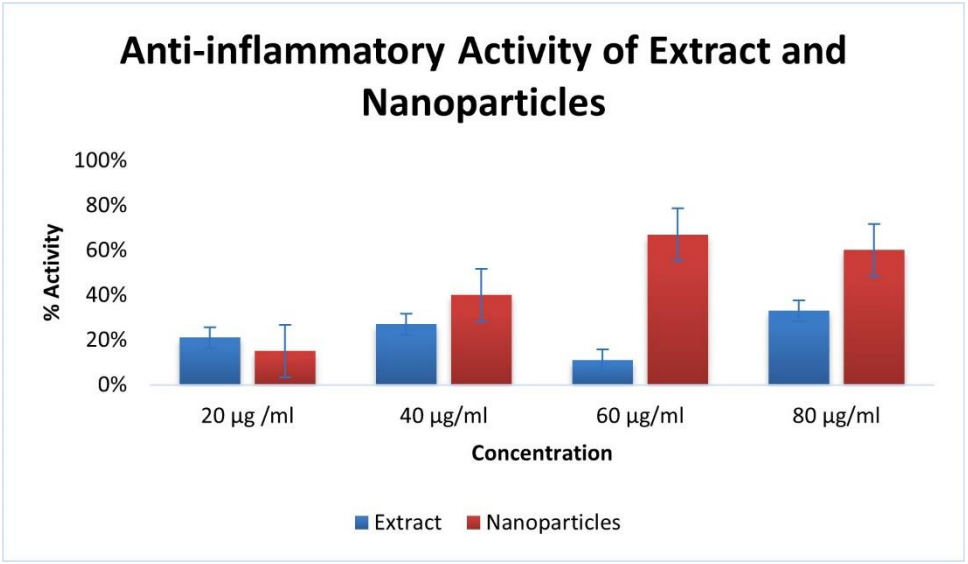
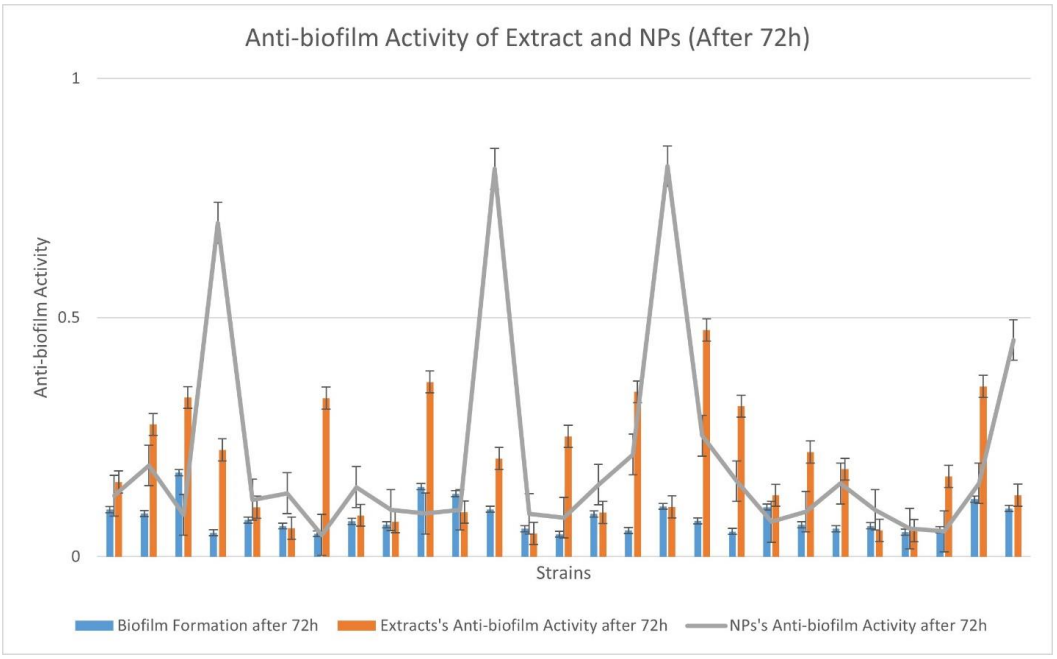


Fig. 2b. Anti-inflammatory activity of plant extract and NPs



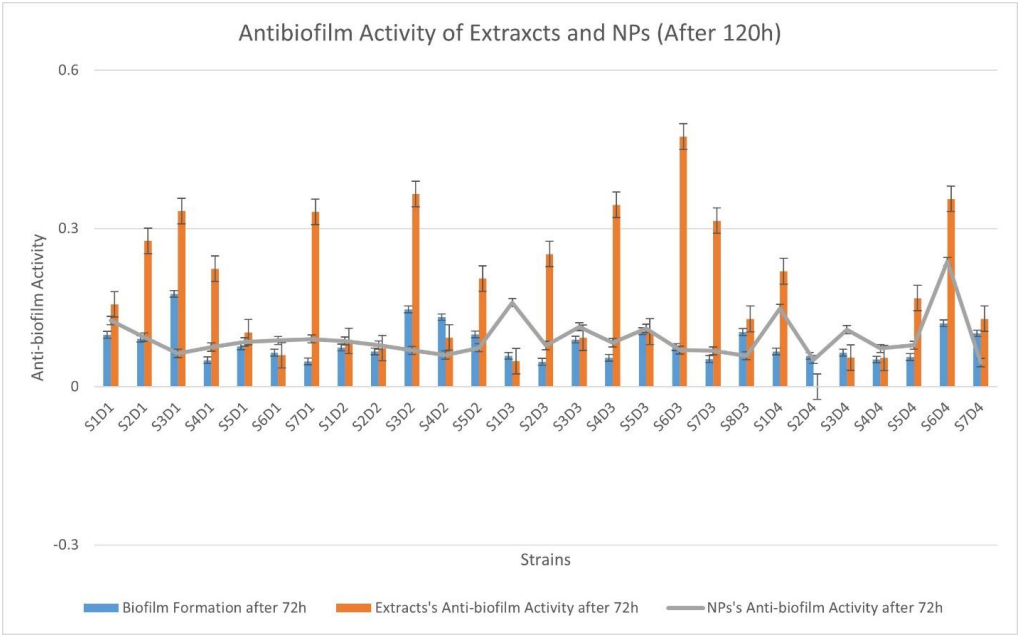


Fig. 2c and 2d: Anti-biofilm activity of plant extract and NPs (After 72 and 120 hours)

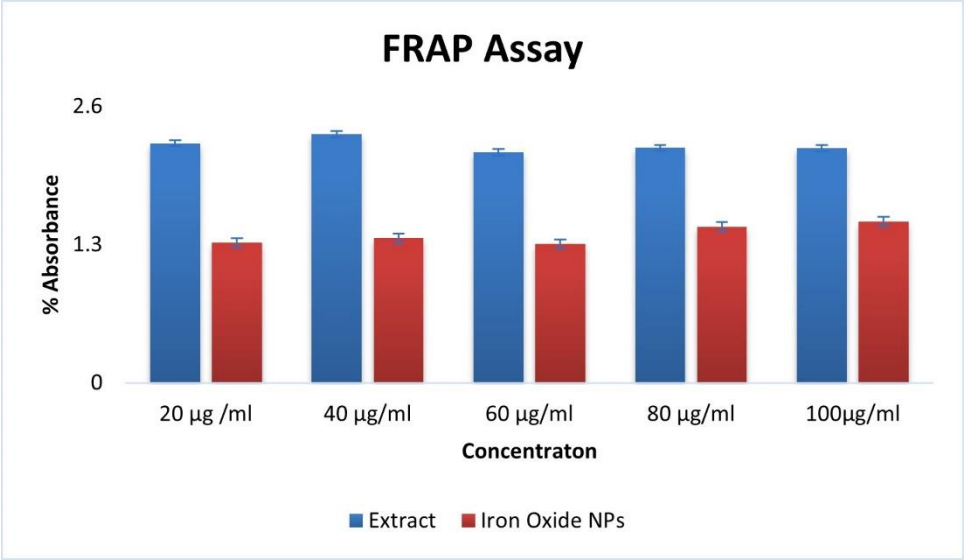


Fig. 3. Antioxidant potential of plant extract and Fe_3O_4 NPs

DISCUSSION

Food-borne pathogens are one of the main cause of infections, encountered in the population of any age group. Drug resistance is continuously booming in microbes and there are multiple factors involved in this phenomenon like the biofilm formation, excess dosage, and misuse of antibiotics. More than 80% of the bacterial species live by the formation of biofilm, which is extracellular polymeric substance, forms a protective layer around microbial communities and helps the bacteria to escape from action of drug or antibiotics. Thus, the infections are becoming difficult to treat. As biofilms are formed in five different steps, so if at any step its formation is interrupted by a strong agent, it will ultimately lead to stop their growth. Under the consequence of these prevailing circumstances, sources other than antibiotics are thought to be potential to ameliorate the infections. More than 5,700 species of the medicinal plants are there in Pakistan. Nanotechnology and medicinal plants are found to be very effective in this regard and are thus being employed for treatment purposes. Nanotechnology is a promising tool for treating the ailments and infections, using nanosized materials applicable in medicine, agriculture, dairy industry and fisheries, drug delivery system and fabric industries etc. Nanoparticles can either be metallic in nature or can be synthesized from plants or they can be bio-metallic NPs i.e., they are synthesized from microbes like bacteria. Aim of this study was the isolation of foodborne pathogens and evaluation of

the efficacy of plant extract and metallic nanoparticles against them effect.

Samples were collected from different food items like meat, milk, vegetables, dry fruits etc. They were serially diluted and spread on two different media (Nutrient Agar and SS Agar). A total of twenty-seven strains were isolated which were streaked and re-streaked to obtain pure colonies. Their colony morphology was observed on different media like EMB and MacConkey Agar. They were then subjected to biochemical characterization. Isolated strains belonged to *Salmonella, Shigella, Enterobacter aerogenes, Pseudomonas aeruginosa, Staphylococci, Escherichia. coli, Klebsiella pneumoniae,* Coagulase positive *Staphylococci* and non-pathogenic *Shigella spp.* Similar results were reported in literature by (Abdaslam et al., 2014; Al-Hilua & Al-Shujairib, 2020; Bantawa et al., 2019; Dib et al., 2018; Tassew et al., 2010).

Gelatin hydrolysis test was performed. Certain microorganisms produce (proteolytic extracellular enzyme) gelatinase, which is involved in liquefaction process and hydrolyzes protein to amino acids. In this study only strains S3D1, S5D1 and S7D1 gave the positive results which means that they have the enzyme gelatinase. Biofilm formation ability of the strains was checked in 96 well micro titer plate. The strains growing could form biofilm in the wells of polystyrene plate. Biofilm or slime producing ability of the strains were checked by Congo red agar method, the slime production was best as the media is supplemented with sucrose. The slime producing ability of the strain depends on the availability of the sucrose. Without sucrose there were a less amount of slime is produced. For slime production test, Black coloration of strain in the presence of Congo red dye and sucrose indicates the strong slime production of the strain. Strain S4D1 showed the maximum sensitivity against ceftriaxone (CRO). Antimicrobial susceptibility test was done by Kirby-Bauer Disc Diffusion method to check if isolated strains were resistant to antibiotics. Most strains were resistant (R) to the thus these were found to be the most exhausted antibiotic which did not give any zone of inhibition. However, CRO, ciprofloxacin, erythromycin and Levofloxacin were the antibiotics for which most strains were found susceptible. Strains were also found sensitive to antibiotics like amoxicillin, ampicillin, and amikacin. Similar results were reported by (Ardila & Vivares-Builes, 2022; Ben Mhenni et al., 2023; Pino-Otín et al., 2022; Singhal et al., 2023).

Eucalyptus plant was selected in this study as it was reported in literature ((Efdi et al., 2023; (Mousa et al., 2023; Serag et al., 2023; Song et al., 2022) that this medicinal plant has important application in the treatment of foodborne pathogens. Extract was prepared from the leaves of selected plant and then phytochemical screening of this extract was done. It was found to have Carbohydrates, Reducing sugars, Flavonoids, Phenolic Compounds, Tannins and Quinones. Similar results were also cited in previous studies (Kwansa-Bentum et al., 2023; Lenny et al., 2023; Obembe, 2023). Two different types of metallic nanoparticles were prepared i.e., ZnO NPs and Fe_3O_4 NPs. It was reported in literature that these had antibacterial, anti-inflammatory, antibiofilm and antioxidant properties (Gul et al., 2023; Hamk et al., 2023; Lee et al., 2023; Murali et al., 2023; Mushtaq et al., 2023; Patil et al., 2023; Smaoui et al., 2023). MIC of Fe_3O_4 NPs was also evaluated, and it was found to be 0.004 µg/ml which was the lowest concentration of an iron oxide nanoparticle that is required to inhibit the growth of a specific bacterial culture.

If we talk about antibacterial activity, the order observed for it, with (mm) diameter of zones of inhibition was as: Fe_3O_4 NPs(11-13mm) >ZnO NPs (7-8mm) >*Eucalyptus* extract (2-3mm). So, metallic nanoparticles showed high antibacterial activity in comparison to plant extract and Fe_3O_4 NPs were found as best candidate for this activity. That's why, Fe_3O_4 nanoparticles were selected for further studies. The anti-inflammatory activity of the extract and nanoparticles was also analyzed. The results revealed that Fe_3O_4 NPsare the best anti-inflammatory compounds. The anti-inflammatory activity of the nanoparticles was seen to be directly proportional to the concentration and highest activity observed was 67% at 40 µg/ml. Anti-biofilm assay was performed using 96-well micro titer plate. 10 µL of the stress was given to the biofilm growing strains and anti-biofilm activity was checked. Fe_3O_4 NPs also performed the highest antibiofilm activity after 120 hours of incubation. Antioxidant assays (FRAP and DPPH) were performed. In FRAP assay, absorbance is directly proportional to antioxidant activity while in DDPH assay it is inversely proportional. In FRAP antioxidant assay, highest activity was performed by *Eucalyptus* extract with recorded absorbance of 1.07. For DDPH assay, the highest antioxidant activity was performed by Fe_3O_4 NPs and their absorbance recorded was 1.43.

CONCLUSION

Fe_3O_4 NPs synthesized from *Eucalyptus camaldulensis* extract have displayed the largest zone of inhibition, the highest percentage of anti-inflammatory activity, the highest antioxidant activity, and the maximum biofilm inhibition after 120 hours of incubation against foodborne pathogens. It is therefore concluded that Eucalyptus mediated iron oxide nanoparticles are potent effective alternative cutting edge therapeutic agents against foodborne pathogens.

ACKNOWLEDGEMENTS

Department of Microbiology and Molecular Genetics, The Women University Multan, Pakistan is acknowledged for providing laboratory facilities and chemicals required for this research work.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801326
Paper Submission: 22nd Nov, 2023; Paper Acceptance: 10th March 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Dietary Habits and their Impact on Physical Symptoms Severity in Young Females Diagnosed with PCOS

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ABSTRACT: *Polycystic Ovary Syndrome (PCOS) is characterized as chronic anovulation or oligo-menorrhoea and clinical hyper-androgenism. It involves excessive Gonadotrophin releasing hormone (GnRH) leading to over-production of luteinizing hormone (LH). Advanced glycation end products (AGEs) are formed as a result of consuming thermally processed and highly modified foods in body. AGEs are expressed as pro- inflammatory receptors and their production is exaggerated in women with PCOS. The purpose of this to check whether unhealthy dietary habits result in increased severity ofphysical symptoms of PCOS in diagnosed females. And to determine the importance of diet in dealing with PCOS. Cross-sectional study was conducted on a sample size of 45 participants (n=45) diagnosed with PCOS. For statistical analysis, SPSS was used and the values of Pearson's Correlation Coefficient were used to identify the correlation between severity of symptoms and poor dietary habits. The results of our study ranged from weak to moderate which demonstrates that there is a correlation between severity of physical symptoms of PCOS and poor dietary habits. The results of the findings of this study stand true to the hypothesis that there is a correlation between severity of physical symptoms and poor dietary habits.*

Keyword: Polycystic ovary syndrome, poor dietary habits, life style factors, autoimmune disease

INTRODUCTION

NIH conference in 1990 was the first to define the term Polycystic Ovary Syndrome (PCOS) as the amalgamation of chronic anovulation or oligo-menorrhoea and clinical hyperandrogenism (Haase et al., 2023). PCOS is a disease that involves multiple genes preceded by multiple factors leading to systemic and inflammatory dysregulation, also coined as an autoimmune disease establishing chiefly as a result of lifestyle errors (Patel, 2018).

The pathophysiology of PCOS involves excessive Gonadotrophin releasing hormone (GnRH) leading to over-production of luteinizing hormone (LH), that affects androgen production from ovaries and progression of oocyte. Altered feedback from the hypothalamus heightens the gonadotropin anomalies (Lewis et al., 2023).

Worldwide PCOS has affected the woman of reproductive age without any racial and ethnical discrimination (Bozdag et al., 2016). The rate of prevalence can be variable while following specific diagnostic criteria or due to varying environmental and genetic factors (Radwan et al., 2023). In South Asian, particularly Pakistani women the occurrence of PCOS is about 52% that is more advancing as compared to the women of United Kingdom that is around 20%-25 % (Wolf et al., 2018).

With the range of varying phenotypical symptoms such as abnormal menstrual cycle, excess hair growth and acne PCOS is considered to be a complex heterogeneous syndrome (Archer and Chang, 2004; Zafar et al., 2019). PCOS results in a number of clinical manifestations most of them being menstrual irregularities, weight abnormalities, hyperandrogenism and infertility. Prolonged, untreated PCOS can possibly lead to clinical manifestations like Type II diabetes mellitus (Balen, 2017) (Ashraf et al., 2022). Symptoms that will be assessed in the subjects participating in this study include menstrual disturbances, weight abnormalities, androgenic alopecia (hair loss), hirsutism (excessive facial hair production), and acne and dark patches on folds of the body called Acanthosis Nigricans (Asadi et al., 2023) (Dewailly, 2016).

Some loci have been researched as PCOS genes but no specified gene has been attributed to develop PCOS phenotype (Meixiong et al., 2022). Furthermore, there is an amplified risk of obesity and metabolic syndrome in young females who experienced

oligomenorrhea and hyperandrogenism during adolescence (Sharma, 2023).

This shows a contributory relationship between obesity and PCOS (Alenezi et al., 2023).

Abdominal obesity is very prevalent in PCOS (Islam et al., 2019). Ongoing researches show that glucose intolerance and type II diabetes is a common factor among women with PCOS (Azhar et al., 2020). Insulin resistance in PCOS women is shown to be the chief contributing factor of blood pressure, abdominal lipids and androgens, when compared with normal women (Polinski et al., 2023).

Advanced glycation end products (AGEs) are formed in the body by consuming thermally processed and highly modified foods such as butter, margarine, cream cheese, mayonnaise, oils, fried eggs, certain cheeses, nuts and meat (particularly red meat) (Memon et al., 2020). AGEs are expressed as pro-inflammatory receptors and their production is exaggerated in women with PCOS. They cause alterations in metabolism and reproductive processes (Garg and Merhi, 2015).

Researches have shown that women with increased consumption of fast food, carbohydrates and soft drinks are twice likely to develop in obesity and consequently hyper insulinemia and PCOS as compared to women with healthy eating pattern and lifestyle (Sedighi et al., 2015).

The medical and the scientific research field came up with the three distinct ways to diagnose PCOS over the period (Barrea et al., 2021). The process of each diagnostic criterion to evolve either PCOS is present or not has somewhat different clinical and imaging reports. In order to assess an appropriate finding on the prevalence of PCOS we follow different criteria of diagnosis (Albogami et al., 2023).

According to National Institute of Health consensus (1990 NIH Criteria) a woman is said to have PCOS if she has irregular ovulation and increase androgen levels in her clinical lab findings (Mohammad and Seghinsara, 2017).

To widen the range of PCOS diagnostic criteria The Rotterdam (ROT-2003) was evolved and in order to get the PCOS diagnosed via Rotterdam 2003 a woman must have at least two symptoms out of three symptoms. (Ehrmann and Crowley, 2021). ROT-2003 shows the prevalence of PCOS in about 83% identified population (Sahmay et al., 2014). The Androgen Excess-PCOS (AE-PCOS 2006) criteria was evolved to progressthe PCOS diagnostic process which stresses the requirement of lab

proofs for excess androgen levels/ hyperandrogenism to assess PCOS presence (Juliawan et al., 2022). Under AE-PCOS 2006 the prevalence is about 70.6% (Lizneva et al., 2016). The aim of this study was to assess unhealthy dietary habits and its relation in an increased severity of physical symptoms of PCOS in diagnosed females. Moreover, the study also focused to determine the importance of diet in dealing with PCOS

MATERIALS AND METHODS

Research design

A cross-sectional study was conducted in 2022 to assess the correlation between poor dietary habits and physical symptoms among patients who have been diagnosed with PCOS. The aim of the questionnaire was to check the severity of symptoms among the participants and their dietary habits.

Sample/Participants

For this survey, females between the ages 19-34, who have been diagnosed with PCOS were selected. Data was collected from two hospitals of Sialkot namely Amina Hospital and Dr. Abdul Kareem Surgi-Med Center as well as from the students of different universities namely UMT, USKT and GCWU Sialkot. A total of 50 questionnaires were filled out of which 5 were excluded because they were above 35 years of age. So. 45 participants were considered eligible for this study. A 35 participants weren't taking any medication, 7 were taking Metformin and 3 were taking supplements of iron and folic acid.

Inclusion criteria

Females diagnosed with PCOS.

Exclusion criteria

Females above the age of 35 years.

Data collection

Data was collected in the premises of the hospital after the patients were diagnosed bythe gynecologist. From the university students, a standard questionnaire was filled on campus as well as online through Google document. Survey conductor explained the content of the questionnaire wherever needed. Females actively participated and were well aware about their symptoms.

Statistical analysis

For statistical analysis, SPSS version 26 was used. Pearson's correlation was used to measure the strength of relationship between severity of symptoms (dependent variable) and dietary factors (independent variable). Descriptive statistics was used to show

the mean, standard deviation and frequency of BMI in the sample.

RESULTS

This chapter explained the intricate correlation that exists between unhealthy eating practices and the degree of physical symptoms experienced by young girls who have been diagnosed with PCOS, or polycystic ovarian syndrome. By examining empirical data, our work clarified the intricate relationship between food and PCOS manifestation and advanced our knowledge of how dietary decisions affect the severity of symptoms in this particular group of people.

Table 1: A Pearson's Correlation Coefficient (r) between severity of symptoms and dietary habits

	Chicken more than thrice a week	Meat more than thrice a week	Sugar and sugary products almost daily	Processed foods	Fried foods more than twice a week	Spicy food	High fat dairy	A bowl of salad daily	A bowl of fresh fruits daily
Acne	.131	.376	.292	.154	.278	.117	.040	-.015	.103
Facial hair	.116	.403	.153	.157	.298	.127	.149	-.232	-.011
Irregular menstruation	.095	.447	.308	.193	.175	.182	.153	.086	.073
Weight gain	.097	.525	.288	.053	.168	.125	.043	.001	-.015
Difficulty losing weight	-.051	.514	.171	.014	.183	.093	.035	.105	.071
Oily skin	.030	.151	.049	.178	.302	.111	-.049	.030	.146
Hyperpigmentation	.010	.364	.199	.268	.182	-.020	.245	.103	.152
Severity of hair loss	.099	.278	-.007	.184	.234	-.065	.040	-.074	.012

The result from Table 1 showed various symptoms such as:

1. **Acne:** There's a weak to moderate, positive relationship between severity of acne and consumption of high fat dairy (r = 0.040), a bowl of fresh fruits (r = 0.103), spicy foods (r = 0.117), chicken (r = 0.131), processed foods (r = 0.154), fried foods (r = 0.175), sugary products (0.292) and red meat (r = 0.376).
   This indicates that as the consumption of these products increase, the severity of acne increases as well but the relationship is not strong.
   However, there's a weak, negative relationship between severity of acne and salad (r = - 0.015) consumption. This indicates that as the consumption of salad increases, there's a decrease in severity of acne.
2. **Facial hair:** There's a weak to moderate, positive relationship between severity of facial hair and consumption of chicken (r = 0.116), spicy foods (r = 0.127), high fat dairy (r = 0.149), sugary products (r = 0.153), processed foods (r = 0.157), fried foods (r = 0.298), and red meat (r = 0.403). This indicates that as the consumption of these products increase, there's an increase in facial hair growth as well but the relationship is not strong.
   However, there's a weak to moderate, negative relationship between severity of facial hair and salad (r = -0.232) and fruits consumption (r = -0.011). This indicates that as the consumption of salad and fruits increases, there's a decrease in the growth of facial hair.
3. **Irregular menstruation:** There's a weak to moderate, positive relationship between severity of irregular menstruation and consumption of fruits (r = 0.073), salad (r = 0.086), chicken (r = 0.095), high fat dairy (r = 0.153), fried foods (r = 0.175), spicy foods (r = 0.182), processed foods (r = 0.193), sugary products (r = 0.308) and meat (r = 0.447). This indicates that as the consumption of these products increase, there's an increase in irregular menstruation as well but the relationship is not strong.
4. **Weight gain:** There's a weak to moderate, positive relationship between severity of weight gain and consumption of salad (r = 0.001), high fat dairy (r = 0.043),

processed foods (r = 0.053), chicken (r = 0.097), spicy foods (r = 0.125), fried foods (r= 0.168), sugary products (r = 0.288) and meat (r = 0.525). This indicates that as the consumption of these products increase, there's an increase in weight gain as well but the relationship is not strong.

However, there's a weak, negative relationship between severity of weight gain and fresh fruits (r = -0.015) consumption. This indicates that as the consumption of fresh fruits increases, there's a decrease in weight gain.

5. **Difficulty losing weight:** There's a weak to moderate, positive relationship between difficulty losing weight and consumption of processed foods (r = 0.014), high fat dairy (r = 0.035), fruits (r = 0.071), spicy foods (r = 0.093), salad (r = 0.105), sugary products (r = 0.171), fried foods (r = 0.183) and meat (r = 0.514). This indicates that as the consumption of these products increase, there's an increase in difficulty of losing weight as well but the relationship is not strong.

   However, there's a very weak, negative relationship between difficulty losing weight and chicken (r = -0.051) consumption. This indicates that as the consumption of fresh fruits increases, there's a decrease in difficulty losing weight.

6. **Oily skin:** There's a weak to moderate, positive relationship between severity of oily skin and consumption of chicken (r = 0.030), salad (r = 0.030), sugary products (r = 0.049), spicy foods (r = 0.111), fruits (r = 0.146), meat (r = 0.151), processed foods (r = 0.178), and fried foods (r = 0.302). This indicates that as the consumption of these products increase, there's an increase in difficulty of losing weight as well but the relationship is not strong.

   However, there's a very weak, negative relationship between severity of oily skin and high fat dairy (r = -0.049) consumption. This indicates that as the consumption of high fat dairy increases, there's a decrease in severity of oily skin.

7. **Hyperpigmentation:** There's a weak to moderate, positive relationship between severity of hyperpigmentation and consumption of chicken (r = 0.010), salad (r = 0.103), fruits (r = 0.152), fried foods (r = 0.182), sugary products (r = 0.199), high fat dairy (r = 0.245), processed foods (r = 0.268) and meat (r = 0.364). This indicates that

as the consumption of these products increases, there's an increase in severity of hyperpigmentation as well but the relationship is not strong.

However, there's a very weak, negative relationship between severity of oily skin and spicy foods (r = -0.020) consumption. This indicates that as the consumption of spicy foods increases, there's a decrease in severity of hyperpigmentation.

8. **Hair-loss:** There's a weak to moderate, positive relationship between severity of hair- loss and consumption of fruits (r = 0.012), chicken (r = 0.099), processed foods (r = 0.184), fried foods (r = 0.234) and meat (r = 0.278). This indicates that as the consumption of these products increases, there's an increase in severity of hair-loss as well but the relationship is not strong.

However, there's a very weak, negative relationship between severity of hair-loss and spicy foods (r = -0.065), sugary products (r = -0.007), salad (r = -0.074) consumption. This indicates that as the consumption of spicy foods and salad increases, there's a decrease in severity of hair-loss.

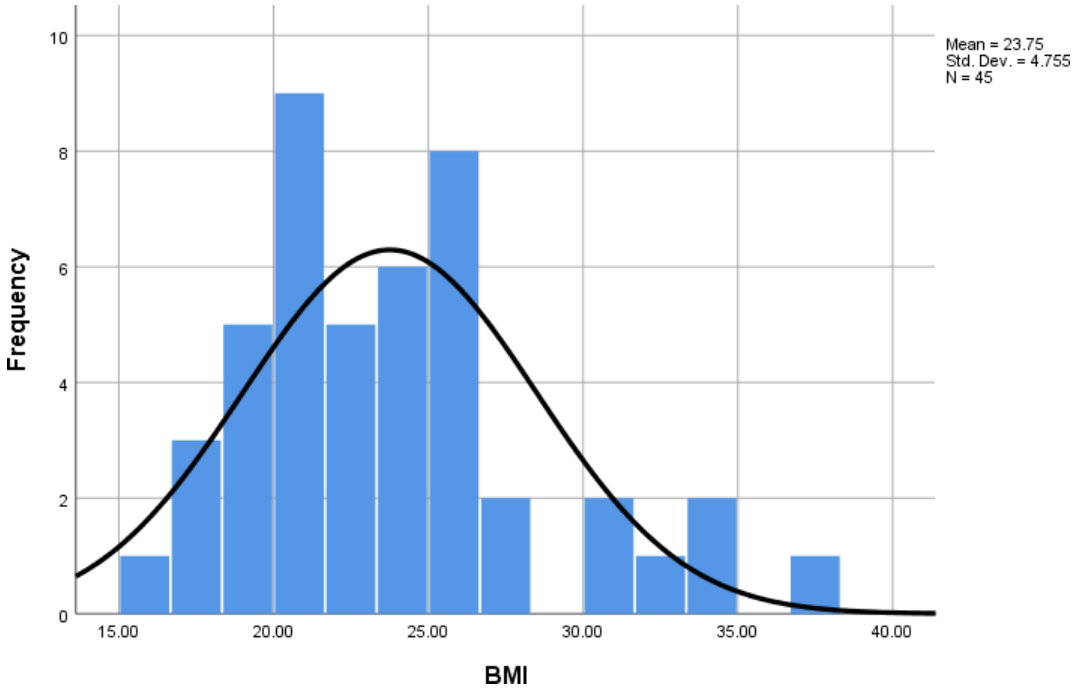

Fig. 1. The values and frequency of BMI from sample

The mean BMI is 23.7 and standard deviation is 4.7. This can be used to make interferences about weight status and overall health of the individuals in the current sample. The mean BMI is within a normal range but the standard deviation showed that the individuals of this sample have a significant variability in their BMIs. This suggest that some individuals have BMIs within normal range, some are underweight and some individuals are in the overweight or obese range (Fig. 1).

DISCUSSION

Our study showed that there's a correlation between severity of physical symptoms of PCOS and dietary habits although the correlation in our findings were statistically weak to moderate. This showed that an increase in unhealthy dietary habits can aggravate the physical symptoms of PCOS. Furthermore, it was found through other researches that only dietary habits can't induce such drastic changes. Other factors such as hormones, genetics and lifestyle factors can also affect the severity of symptoms of PCOS even if the person is taking generally a healthy diet.

The mean BMI of the women in our sample fell within the normal range. Studies suggest a strong correlation between severities of physical symptoms of PCOS with fluctuations in BMI. Additionally, dietary habits can also cause changes in the BMI and ultimately in the overall severity of PCOS symptoms.

Unexpectedly, there were some negative associations between certain dietary factors and symptoms but those associations are statistically very weak and go against the majority of researches available so they can be ignored.

The positive relationship, although weak in strength, found in this study between unhealthy diet and severity of PCOS symptom goes in accordance with numerous researches done in the past. In a randomized controlled trial, the weight loss group was told to consume a diet high in vegetables, fruit and fiber content and low in saturated fats (such as meat and fried foods). By the end of the trial, it was noted that dietary weight loss in women with PCOS lead to significant reduction in BMI, irregular menstruation, waist circumference and facial hair (Marzouk and Sayed Ahmed, 2015).

A study was conducted that a diet consisting of lean animal protein, low sugar fruits and low dairy products resulted in increased insulin sensitivity, weight loss and testosterone reduction in PCOS patients which as a result lead to

decreased symptom severity (Haas et al., 2023). These goes in accordance with the result of the current study that consumption of lean animal protein (chicken) aids in weight loss and high dairy consumption may result in increased weight and difficulty losing weight although the statistical correlation between these factors is weak in this study.

In this current research, there is a moderate, positive relationship between meat consumption and weight gain. This suggested that as meat consumption increases, there are increased chances of weight gain. However, there are various other factors such as frequency, portion size and leanness of meat that influence weight changes. A prospective cohort investigation was conducted among women to find the relationship between weight gain and meat consumption over 20 months. It was concluded in it that consumption of meat (other than lean meat) resulted in significant weight gain in women (Alomran & Estrella, 2023).

Statistics used in this current research suggested that an increase in sugar intake leads to an increase in weight gain but there was a positive moderate relationship. A cross- sectional study was performed among Korean adults that showed that consuming sugary beverages frequently was linked positively with metabolic syndrome and obesity (Shin et al., 2018).

Various studies suggest that acne, oily skin, obesity and hirsutism in PCOS patients is linked with hype-rinsulinemia (Fareg and Dadoush, 2023). Hyper-insulinemia can be prevented with the help of lifestyle and dietary interventions (Witchel et al., 2020). This results in improvement of insulin sensitivity and weight loss which subsequently leads to decrease in severity of PCOS symptoms (Muscogiuri et al., 2022).

According to our research, eating processed meals has a moderately positive link with the severity of hyperpigmentation. This suggests that the severity of hyperpigmentation increases along with the use of highly processed foods. Using Rotterdam's criteria, a prospective case control study was done on patients who had just received a PCOS diagnosis. The purpose of this study was to look at the connection between eating processed foods (items with a high GI) and the degree of hyperpigmentation and other skin issues. Principal component analysis and logistic regression were also employed to examine the association between food patterns and the odds of developing PCOS related problems (hyperpigmentation, oily

skin). According to the findings, the highly processed food pattern gradually increased the severity of skin issues in PCOS patients (Patel and Shah, 2018; Panjeshahin et al., 2020)

According to our analysis, consuming dairy products high in fat has a moderately positive link with the severity of hyperpigmentation. This shows that the severity of hyperpigmentation worsens with increased consumption of high-fat dairy foods. Dairy products, a staple of the typical diet, can potentially have an impact on PCOS-related issues. There isn't much research done in this area, though. In order to assess the connection between dairy product consumption and PCOS-related skin issues, a descriptive cross-sectional study including 400 women was carried out in 2023. High fat milk consumption was directly correlated with a considerable rise in the risk of PCOS; with every additional unit of milk consumption resulting in a 1% increase in the risk factors for skin issues such acne and other skin conditions among PCOS women (da Luz et al., 2023).

Our results suggest a weak to moderately positive correlation ($r = 0.184$) between both the severity of hair loss and dietary intake of processed foods. This shows that although there is a weak correlation between the severity of hair loss and processed food consumption, it does occur. In 2013 a study discovered that eating a diet high in processed foods can cause hair loss since these foods frequently lack vital nutrients crucial for the health of the hair. The study also found that women with PCOS are more likely to have hair loss than those without the illness. The study also claimed that eating more processed foods while having PCOS can result in nutrient inadequacies such those in iron, zinc, and vitamin and a balanced diet is essential for maintaining hair health (Jain et al., 2013).

Our study found a weak to moderate correlation between poor dietary habits and symptoms severity of PCOS which has also been found in a case-control study conducted in 2022-2023 High glycemic load diets, dairy consumption and sugary foods consumption led to the development of acne (Bykowska-Derda et al., 2023)

A research also confirms the development of acne in individuals who have been taking high glycemic index foods like chicken, sugary foods, milk and meat etc. however, the pathogenesis of acne is highly dependent on gender and ethnicity apart from dietary factors (Süli et al., 2023).

A study was conducted in Kerala, India that investigated the effect of diet on symptoms of PCOS that concluded that those girls who consume non-veg diet are more susceptible to the development of symptoms of PCOS as compared to those girls who were taking a vegetarian diet (Roberts et al., 2017). The poor dietary habits disturbed the menstrual cycle. This also goes well with our findings which show a moderate correlation between meat and sugary foods consumption and menstrual abnormalities. The study also showed that the girls who were taking non-veg diet had increased facial hair growth which has been found in our study as well George and Alex (2021).

CONCLUSION

In conclusion, the results of the findings of this study stand true to the hypothesis that there is a correlation between severity of physical symptoms and poor dietary habits. Stronger statistical correlation can be achieved if the limitations are overcome.

The most significant limitation of the current study is the small sample size. The time span given to conduct the research was short. The dietary analysis was limited to the information provided by the participants in the present setting. Some participants were receiving medications that may have altered the severity of their symptoms. The current study has some limitations, including a small sample size and a short study time span. The study was restricted to Sialkot and diagnosed patients of PCOS, thus thesmall sample size.

AUTHOR'S CONTRIBUTIONS

Anam Tariq, Fatima Shahid done the survey and analyzed the experimental data. Shyeda Alizeh wrote this article. Sumaira Saeed and Fatima Shahid contributed to revise the manuscript. All authors approved the final version.

FUNDING

N/A

CONFLICT OF INTEREST

All authors declares that there is no conflict of interest.

CONSENT OF PUBLICATION

All authors gave their consent for publication.

ACKNOWLEDGEMENT

I would like to acknowledge the sub campus of University of Management and Technology, Sialkot that help me in this research. Special thanks to all students, faculty and staff members that support me in this article.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801328
Paper Submission: 11th Dec, 2023; Paper Acceptance: 10th March 2024; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Assessment of Cyclical Spreading and Ecological Extortions to the Avian Species in District Jhang, Punjab, Pakistan

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ABSTRACT: *The study was conducted to estimate the bird diversity and the risks they confront at Head Trimmu, District Jhang, Punjab, Pakistan. A number of field surveys were managed over 12-months by employing both direct and indirect means during certain hours of a day. The study found a total of 54 species from 15 orders and 31 families. The bird species were classified as residents (64.4%), breeders (8%), migrants (26%), and passage migrants (1.4%). The most frequent species observed during the study were house sparrow, house crow, domestic rock pigeon, ring-necked dove and common myna. Overall, the study revealed a diversified avian community with Shannon-Wiener diversity index of 3.3367. Only two species found near threatened like the Houbara bustard (Chlamydotis undulata)and Alexandrine parakeet (Psittacula eupatria) but the majority of the species are of least concern. The ecological richness of the area is under threat by urban development due to growing human population. So, the major threats to avian species in the study area are habitat degradation, Illegal hunting and lack of awareness. It is suggested to preserve avian diversity by the involvement of government agencies, private groups (WWF, IUCN, BirdLife International) and the general public.*

Keyword: Avian diversity, Cycling spreading, Ecological extortion, Head Trimmu

INTRODUCTION

Birds are endothermic, bipedal, oviparous, vertebrate animals with feathers on their bodies that live in hilly regions, agricultural fields, urbanised areas, rural areas, and water bodies at different elevations (Govender, 2021; Umar et al., 2018). The Aves are the main class of birds, which includes the flightless birds (Palaeognathae), the weak-flying species (Tiramisu), and the flying birds (Neognathae) (Platt et al., 2021). Birds are great producers of ecosystem services; they may disseminate seeds, eat pests, scavenge carrion, pollinate flowers, cycle nutrients, and improve the environment for the benefit of humans and other animals (Sekercioglu, 2006; Whelan et al., 2015).

Numerous zoogeographical zones, i.e., the Ethiopian, Palaearctic, and Oriental are home to a variety of bird species. Among them, Pakistan is recognised for its miscellaneous climate and floral zones, which are considered one of the most significant homelands for avian species (Khan and Pervaiz, 2001). There are a total of 10960 bird species worldwide (BirdLife, 2016), while 2700 avian species have been reported from Asia (Altaf et al., 2012). Pakistan harbours a very diverse avian fauna due to its geographical structure and is home to more than 750 species of birds (Tanveer et al., 2002; Khalid et al., 2017; Mehmood et al., 2018). The winter season in Pakistan is particularly significant for certain migratory bird species (Grimmett et al., 2001; Mirza and Wasiq, 2007). To avoid extremely cold weather, a large number of birds migrate from Middle Eastern countries to Europe and to the wetlands of Pakistan (Ali, 2005; Iqbal et al., 2007). Now there is a decline in the population of migratory birds in Pakistan too. One particular instance is the decreasing number of Pakistani wetland bird species, *Sterna acuticauda*, due to habitat degradation, i.e., overgrazing, poaching, and summer floods (Altaf et al., 2018). With the rapid urban expansion of the late 20th century in response to the growing human population, maintaining and restoring ecosystems in urban settings became a difficulty (Riaz et al., 2019). This is in accordance with the conservation strategies suggested at several environmental conferences (Khera et al., 2009).

The earth biodiversity is negatively impacted by unfavourable effects of environmental changes, including climate change, pollution, habitat loss, deforestation, and the introduction of

invasive species (Thomas et al., 2004; Khan et al., 2017). In the observed area, the primary risks to species variety are habitat degradation, human population pressure, and illegal hunting. Parrots are rare and endangered bird group (Pruett-Jones, 2021), with 95 (28.6%) of the 354 recognised species facing challenges. One of them is the Alexandrine Parakeet (*P. eupatria),* which is distributed in South and Southeast Asia, extending to Pakistan and India (Abed et al., 2020). The houbara bustard is on the IUCN red list, and Pakistan uses it as a fungible reserve (Adler et al., 2020). The Asian houbara (*C. macqueenii*) is a migratory species, and its number has been affected by unsustainable hunting and trapping (Dolman et al., 2018). Both the Asian (*C. macqueenii*) and African (*C. undulata*) houbara are listed as vulnerable due to uncertainties that the release of captive-bred birds could have an adverse effect on wild populations (IUCN, 2019).

Several species diversity indices are used to evaluate variety; these include the Shannon-Weiner, Simpson, Evenness, and Richness indices. The indices rise as the number of species, populations, and similar numbers within species groups rise (Altaf et al., 2013). The diversity of various birds at Taunsa Barrage, Head Trimmu Barrage, Changa Manga, Head Qadirabad, and Ravi Siphon as well as their ecological impacts, have already been examined (Ali, 2005; Mahboob, 2009; Altaf, 2010; Munir, 2010; Irfan, 2010). So, we are the first to investigate the avian diversity of the Head Trimmu junction area, which is regarded as a wetland in the Jhelum and Chenab rivers. According to the irrigation and power department, the diversity of flora and fauna in that region is also poorly impacted by the pH of the water, which varies from 7.1 to 8.1 (Irrigation and Power Department, Punjab, 2007). pH levels can stress animal systems, reduce hatching and survival rates, and increase mortality rates. Species with higher sensitivity are more affected by pH changes.

So, the current study has been designed to access cyclical spreading and ecological extortions to the avian species in Distt. Jhang, Punjab, Pakistan. This work would provide valuable resources on the conservation of cyclical spreading and ecological extortions to the avian species in Distt. Jhang, Punjab, Pakistan.

MATERIALS AND METHODS

Study area

Head Trimmu is considered an agro-forest land with a highly variable avian

diversity. Its ecosystem includes a multifacet of aquatic, woodland, and agricultural territory. It is located in Tehsil 18 Hazari, district Jhang, at the intersection of the rivers Chenab and Jhelum in the village “Basti Sanga." The climate of this region is temperate, with four seasons. Summer is comparatively longer, and temperatures range from 3°C to 45°C, with very rare excursions below 2°C or above 44°C. Relative humidity is typically between 20 and 25% in the summer and 40 to 65% in July and August (Fig. 1) shows a study area map by Arc GIS 10.8.2 https://desktop.arcgis.com/en/arcmap.

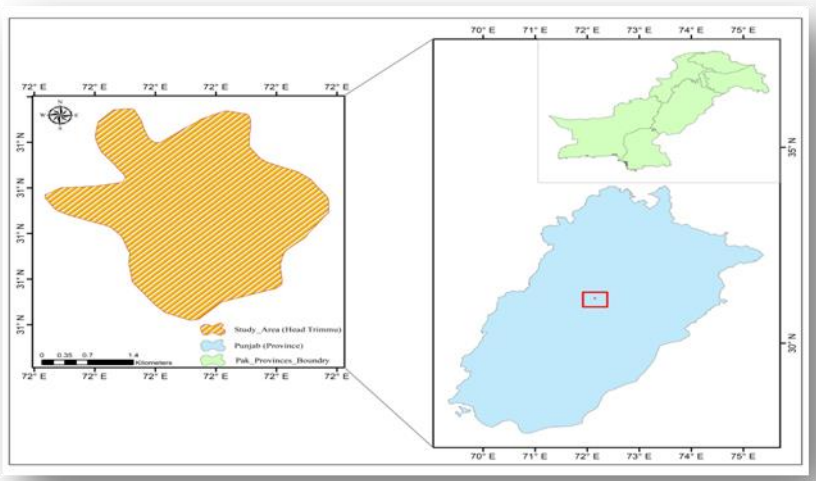

Fig. 1. A map of the study area Head Trimmu, showing sampling site

Study design

All the field information being used in the assessment was gathered based on direct surveillance of the birds, i.e., numbering birds through vision and echo (Aves surveys and information collection). The present study was conducted on the birds' fauna at Head Trimmu from January 2019 to December 2019, specifically focusing on the distribution, diversity, and ecology of the birds. Data were collected from dawn (5:00 a.m. to 8:00 a.m.) to the early sunset (5:00 p.m. to 7:00 p.m.) throughout the assessment period. Throughout the assessment process, a range of field techniques, including direct land observations and indirect interpretation i.e., filling questionnaire and interviews from the restricted community, were employed.

The parallel observational area was divided into three main groups: stream areas, woodlands (with trees and

orchards surrounding the living enclosures), and vegetation/greenery planted (around the grassy territory and housing colony) to be observed and drawn by using Arc GIS 10.8.2 https://desktop.arcgis.com/en/arcmap in order to estimate the number of birds (Fig. 2). Small cameras were installed to document the habitats and landscapes. Additionally, focused group debates and interviews were conducted. "Fauna of Pakistan" as a field reference was used to identify the bird's life in the observation area (Grimmett et al., 2008).

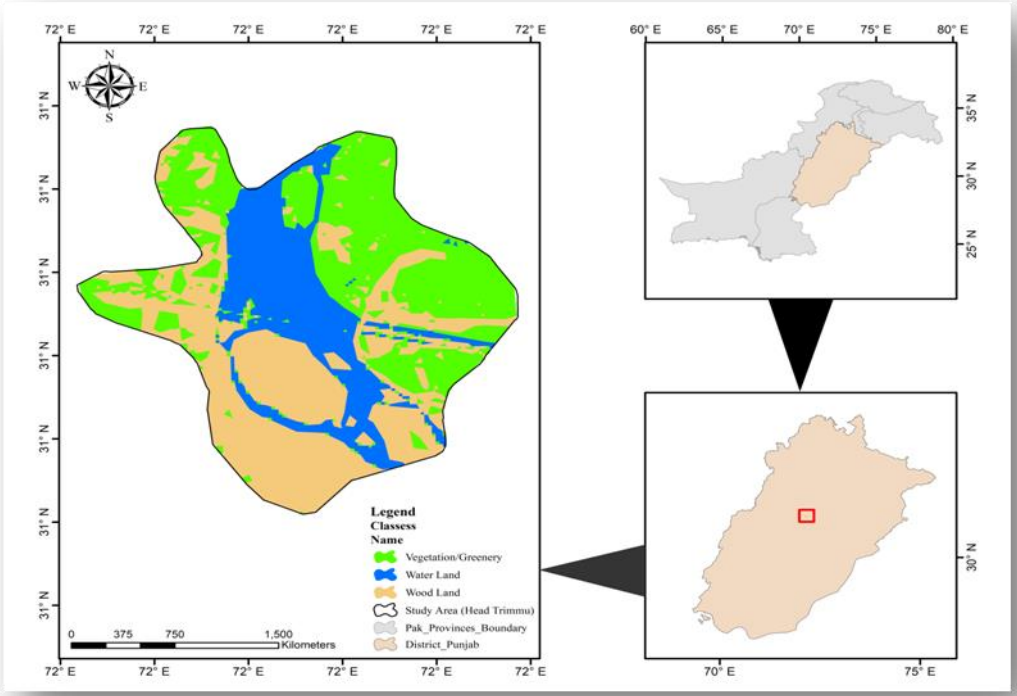

Fig. 2. Map showing the area in study site covered by water land, woodland, and vegetation/greenery

Five diversity indices (Dominance, Shannon-Wiener diversity Index, Simpson Index, Margalef and Evenness) were used to calculate the avian diversity by means of documented data (Hammert et al., 2001; Khan and Ali, 2014).

Shannon-wiener diversity Index

Shannon-wiener diversity Indices was designed with the following formula (Shannon and Weaver, 1963):

$H' = -[\Sigma PI \ln PI]$

H' is Shannon-wiener diversity Indices

PI is relative abundance of bird species to entire population (Ashraf et al., 2019).

Species richness (SR)

Species richness calculated with the formula given below (Margalef, 1951).

"$SR = (S - 1)/\log N$"

S is the whole numeral of bird's species

N is the numeral of birds there in the sample

Species evenness (E)

Species evenness was considered with the formula presented forward (Pielou, 1966).

"$E = H'/ \log S$"

Where

S is Whole numeral of avian species

H' is Shannon-wiener diversity indices

RESULTS

The Trimmu barrage is a dominant site for avian fauna as it shows the features of both terrestrial and aquatic habitats. The study area offers abundant food resources, including insects, crustaceans, invertebrates, arachnids, grains, seeds, fruits, and human waste, supporting Passeriformes species like the common myna, barn swallow, and house sparrow. The area features a variety of tree species, including Siris, Egyptian mimosa, Bo tree, River redgum, Date palm, Rosewood, Indian jujube, and Wild jujube. Ground flora includes lamb's quarters, Cheese weed mallow, Camel thorn, Toothed medick, Sweet Clover, Bermuda grass, Syrian rue, and Puncture vine. Aquatic plant species include Water-thyme, Striate vallis, Sacred lotus, Bengal cane, Wild cane, Tamarisk, and Southern cattail. Out of the five dominant species, four are Passeriformes species, influenced by food abundance. The number of birds is linked to breeding season, habitat quality, and food enrichment (Bibi and Ali, 2013).

During the 12-month field survey, 54 species of birds were identified from 15 orders and 31 families (Table. 1). In the present study, the most dominant order was Passeriformes, representing 19 species of birds belonging to 14 families, followed by Columbiformes with 9 species, Accipitriformes with 5 species, Pelecaniformes with 4 species, Falconiformes, Galliformes with 3 species, Cuculiformes, and Psittaciformes with 2 species, while Apodiformes, Otidiformes, Strigiformes, Bucerotiformes, Charadriiformes, Gruiformes, and

Anseriformes are represented by single specie each. We took references of classification from (BirdLife International, 2019).

Out of 54 bird species observed during our survey, we reported 37(69%) species as Residents including the house crow, tufted titmouse, house sparrow, common starling, rosy starling, bank myna, oriental magpie robin, black drongo, indian silverbill, red vented bulbul, common babbler, steep grey shrike, common tailorbird, rock doves, ring necked doves, Laughing doves, red collared doves, Asian green bee eaters, Indian roller, little swifts, Eurasian sparrow hawks, black kites, shikras, golden eagles, night herons, cattle egrets, little egrets, Pied Crusted Cuckoo, peregrine falcons, ring necked pheasants, Harlequin quails, Common quail, rose ringed parakeets, alaxandrine parakeets, spotted owlets, African hoopoes and red wattled lapwings. 17 (31%) species as migrants, including common myna, river tern, yellow wagtail, pied winged swallow, sand martin, purple sunbird, blue cheeked bee eaters, desert wheat eaters, white throated kingfishers, common buzzards, Indian pond herons, great coucals, laggar falcons, common kestrels, houbara bustards, common coots, and northern shovlers (Table. 1) (Fig. 3).

Table 1: Summary of diversity Index and distribution at Head Trimmu Jhang

Order	Family	Scientific name	Common name	Diversity Index	Distribution
Passeriformes	Corvidae	*Corvus splendens*	House crow	-0.255	RESIDENT
	Paridae	*Baeolophus bicolor*	Tufted titmouse	-0.1657	RESIDENT
	Passeridae	*Passer domesticus*	House sparrow	-0.23165	RESIDENT
	Sturnidae	*Acridotheres tristis*	Common myna	0.22104	MIGRANT
		Sturnus vulgaris	Common starling	-0.07659	RESIDENT
		Pastor roseus	Rosy starling	0.06119	RESIDENT

		Sterna aurantia	River Tern	-0 .1245	MIGRANT
		Acridotheres ginginianus	Bank Myna	0.05255	RESIDENT
	Muscicapide	*Copsychus saularis*	Oriental Magpie Robin	-0.0727	RESIDENT
	Motacillidae	*Motacilla flava*	Yellow wagtail	-0.0272	MIGRANT
	Dicruridae	*Dicrurus macrocercus*	Black drongo	-0.1249	RESIDENT
	Estrildidae	*Euodice malabarica*	Indian silverbill	-0.0264	RESIDENT
	Hirundinidae	*Hirundo leucosoma*	Pied winged swallow	-0.0288	MIGRANT
		Riparia riparia	Sand martin	-0.002	MIGRANT
	Nectariniidae	*Cinnyris asiaticus*	Purple sunbird	-0.0611	MIGRANT
	Pycnonotidae	*Pycnonotus cafer*	Red vented bulbul	-0.0721	RESIDENT
	Leiothrichidae	*Argya caudata*	Common babbler	-0.0699	RESIDENT
	Laniidae	*Laniu sexcubitor*	Steppe grey shrike	-0.0611	RESIDENT
	Cisticolidae	*Orthotomus*	Common	-0.028	RESIDENT

		sutorius	tailorbird		
Columbiformes	Columbidae	*Columba livia*	Rock dove	0.175	RESIDENT
		Streptopelia capicola	Ring necked dove	-0.1523	RESIDENT
		Spilopelia senegalensis	Laughing dove	-0.0676	RESIDENT
		Streptopelia tranquebarica	Red collared dove	0.0311	RESIDENT
	Meropidae	*Merops orientalis*	Asian green bee eater	-0.1023	RESIDENT
		Merops persicus	Blue cheeked bee eater	-0.0248	MIGRANT
		Oenanthe deserti	Desert wheat eater	-0.0272	MIGRANT
	Coraciidae	*Coracias benghalensis*	Indian roller	-0.01634	RESIDENT
	Alcedinidae	*Halcyon smyrnensis*	White throated kingfisher	-0.0198	MIGRANT
Apodiformes	Apodidae	*Apus affinis*	Little swift	-0.02727	RESIDENT
Accipitriformes	Accipitridae	*Accipiter nisus*	Eurasian sparrow hawk	-0.019	RESIDENT
		Milvus migrans	Black kite	-0.0916	RESIDENT

		Buteo buteo	Common buzzard	-0.0699	MIGRANT
		Accipiter badius	Shikra	-0.0333	RESIDENT
		Aquila chrysaetos	Golden eagle	-0.0525	RESIDENT
Pelecaniformes	Ardeidae	*Ardeola grayii*	Indian pond heron	-0.0961	MIGRANT
		Nycticorax nycticorax	Night heron	-0.0377	RESIDENT
		Bubulcus ibis	Cattle egret	-0.1186	RESIDENT
		Egretta garzetta	Little Egret	-0.0398	RESIDENT
Cuculiformes	Cuculidae	*Centropus sinensis*	Great coucal	-0.067	MIGRANT
		Clamator jacobinus	Pied Crusted Cuckoo	-0.0296	RESIDENT
Falconiformes	Falconidae	*Falco jugger*	Laggar falcon	-0.0163	MIGRANT
		Falco tinnunculus	Common kestrel	-0.0419	MIGRANT
		Falco peregrinus	Peregrine falcon	-0.0377	RESIDENT
Galliformes	Phasianidae	*Phasianus colchicus*	Ring necked pheasant	-0.0405	RESIDENT

		Coturnix delegorguei	Harlequin quail	0.01724	RESIDENT
		Coturnix coturnix	Common quail	-0.0377	RESIDENT
Psittaciformes	Psittaculidae	*Psittacula krameria*	Rose ringed parakeet	0.0041	RESIDENT
		Psittacula eupatria	Alaxandrine parakeet	-0.0075	RESIDENT
Otidiformes	Otididae	*Chlamydotis undulata*	Houbara bustard	-0.0053	MIGRANT
Strigiformes	Strigidae	*Athene brama*	Spotted owlet	-0.0126	RESIDENT
Bucerotiformes	Upupidae	*Upupa africana*	African hoopoe	-0.0154	RESIDENT
Charadriiformes	Charadriidae	*Vanellus indicus*	Red wattled lapwing	-0.0215	RESIDENT
Gruiformes	Rallidae	*Fulica atra*	Common Coot	-0.0135	MIGRANT
Anseriformes	Anatidae	*Anas clypeata*	Northern shovler	-0.0135	MIGRANT

The study reveals 54 species among 5405 individuals, with relative abundance (IP) of 1.004. Shannon Wiener's index (H') of 3.3367 and Simpson's index (S) of 0.997 that provide insights into species diversity and dominance. Evenness index (E) of 0.5218 and Richness (R) of 0.733 indicate species abundance distribution. Margalef index (I) of 6.1645 shows species richness relative to the number of individuals. These indicators provide valuable understandings into the avian diversity and ecological dynamics of the population (Table. 2). Only a two species found near threatened like the Houbara bustard (*Chlamydotis undulata*)and Alexandrine parakeet (*Psittacula eupatria*) but the majority of the species are of least concern.

Table 2: Diversity Indices of Avian Fauna at Head Trimmu

DIVERSITY INDICIES	VALUES
Number of species(S)	54
Population (P)	5405
Relative Abundance (IP)	1.004
Shannon wiener index (H')	3.3367
Simpson index (s)	0.997
Evenness index (E)	0.5218
Richness (R)	0.733
Margalef index (I)	6.1645

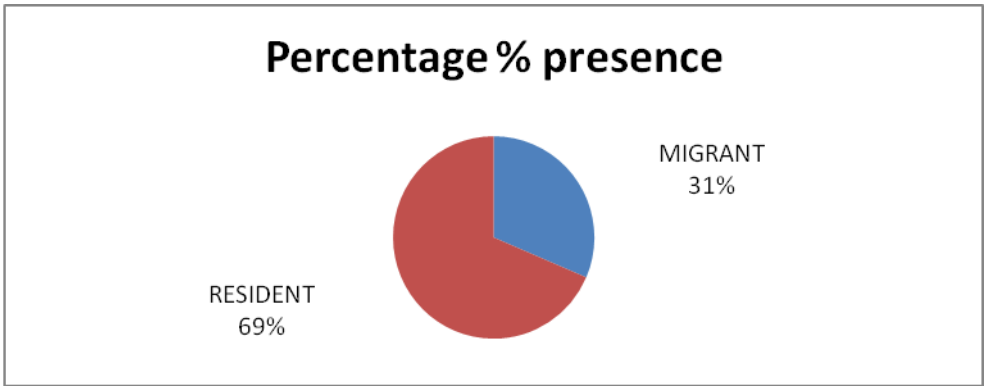

Fig.3: Percentage of resident, migratory bird species

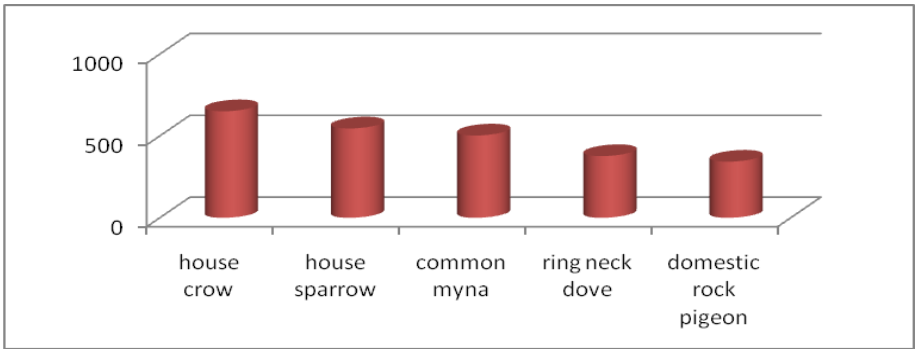

Fig.4: Relative abundance and percentage of 5 most dominant species

Threats: A few common threats recognized were deforestation, rapid and haphazard urbanization, and a lack of public awareness to care for avian diversity.

Deforestation: As part of the barrage renovations, some bird houses have

been cleared, which has led to a decrease in the number of birds.

Rapid and haphazard urbanization, particularly in biodiversity-rich areas, is a lethal cause of diversity decline due to increased illegal hunting, netting, and shelter decline, despite deforestation alone having fewer adverse effects.

Lack of awareness: The primary factor contributing to the decrease in avian diversity in developing nations like Pakistan is a lack of knowledge about the value of wildlife.

DISCUSSION

A 12-month field survey recognised 54 bird species from 15 orders and 31 families, with Passeriformes being the most dominant order. This aligns with previous conclusions by Shahid et al. (2018), who reported Passeriformes as the main order, representing 37.52% of avian species. Raza et al. (2015) noted Passeriformes as the most common order, with 55.7% of bird species belonging to 17 families. Researchers at Head Khanki observed 51 waterfowl species, including 33 genera and 16 families. Other studies have found 59 bird species, with 24 being residents, 14 winter visitors, and 11 summer visitors. Irfan (2010) recorded 57 summer avifauna species, with 51 being SP. residents, 5 summer breeders, and 1 annual visitor. However, the exact percentages and number of families may vary due to differences in locations, methodologies, and survey durations.

This study highlights the need for continued conservation efforts to protect avian diversity. Despite the ban on extinctions since the CBD's inception, several species remain threatened and require constant conservation efforts (Ghalib et al., 2018; Díaz et al., 2019; Khan and Baig, 2020). The detrimental consequences of pollution, deforestation, climate change, loss of territory, and the influx of non-native species must be addressed. The study area is rich in diverse flora and fauna species, which provide food, shelter, and habitat for various bird species. However, the financial vulnerability of developing nations, coupled with dysfunctional political systems, an impotent judiciary, and ineffective regulatory mechanisms, can erode laws protecting the environment and the survival of threatened bird species. Greater efforts are needed to prevent extinction and improve the condition of 6,811 species listed as seriously endangered on the Red List (IUCN, 2020).

CONCLUSION

Data from the agriculturally wetland revealed 54 bird species, which was a

sign of a healthy ecosystem. Based on the results, it was recorded that the Public and private organizations have made progress in saving endangered species. Future research on avian diversity resources is recommended for improved conservation and management measures.

ACKNOWLEDGEMENTS

The authors gratefully appreciate the cooperation from the department of wildlife, the department of irrigation, and the field staff of Head Trimmu for supporting and directing us to perform this study.

CONFLICT OF INTEREST

All the authors declared no conflict of interest in this publication.

AUTHORS CONTRIBUTION

Iffat Abid, Ayesha Zulfiqar, and Sidra Hussain designed the study. Iffat Abid, Ayesha Zulfiqar, and Alina Sikandar collected the field data, interpretation results, and put up to writing the Research article. Alina Sikandar, Tariq Ahmad, Tahir Raza, and Nusrat Liaqat participated in statistical analysis and also revised the draft. The authors examined and officially permitted the manuscript.

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LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2024.0801329
Paper Submission: 9th Oct, 2022; Paper Acceptance: 21st Aug 2023; Paper Publication: 15th March 2024

Review Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

A Review on Advancement of Cancer Therapy with Metal Based Nanoparticle

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ABSTRACT: *The primary cause of numerous diseases that frequently affect the human body is a compromised immune system. For instance, it can increase the body's susceptibility to infection, which also increases the body's resistance to medicines, by triggering pro-inflammatory reactions and even the loss of good cells and tissues. Metal nanoparticles have the potential to overcome the challenges caused by conventional chemotherapies. Metal nanoparticles play an essential role in the treatment of cancer by targeting, gene suppression, and drug delivery to the targeted area. The best suitable metal nanoparticles better work with targeted ligands to control the energy deposition within the tumors. Other than therapeutic advantages, metal nanoparticles are also helpful as a diagnostic tool for the imaging of cancerous cells. Moreover, metal nanoparticles provide beneficial options in controlled targeted drug delivery, instant diagnosis, and therapeutic methods. Nanoparticles are useful as therapies and diagnostics because cancer cells are heterogeneous. Breast cancer anti-cancer therapy is difficult due to the disease's aggressive spread to the body. Noble metal nanoparticles are used in the treatment of cancer because of their distinctive features, great biocompatibility, surface effects, and tiny size effects. Numerous cancer treatments have been developed thanks to advances in science and technology, but the global mortality rate for cancer patients is still astronomically high, necessitating conventional treatment approaches in order to preserve lives. Scientists are trying to find a cancer cure, working day and night. Due to their effectiveness and promising outcomes, nanoparticles for the treatment of cancer have acquired relevance.*

Keyword: Nanoparticles, Nanomedicine, Cancerous Cell

INTRODUCTION

The immune system of humans is a complicated system that is comprised of cells, physical barriers and proteins. All these components work together to prevent diseases in the body and to keep the body fully functional for the healthy survival of the person. If the immune system is working accurately, it has the ability to protect the body from foreign particles with the help of some specific cells and their relevant functions (Tasic et al., 2018).

The underperformance or malfunctioning of immune system is the major reason of different disease that often occurs in human body. For example, if the immune system is working higher than normal, the autoimmunity responses will generate in the body leading to pro-inflammatory responses and even destruction of healthy cells and tissues (Becher et al., 2017). Contrary to this, if the immune system is under active, it can make the body susceptible to infection which also makes the body resistant to antibiotics. Tumorigenesis showed that the immune system is mainly responsible to control the abnormalities related to proliferation of cells by which they become malignant (Huang et al., 2020). This review focuses on the fundamental principles of nanotherapeutics application, current challenges, and future research directions.

Cancerous Cells and their formation

Cancer is the second leading cause of mortality worldwide. The number of annually diagnosed cases of cancer is 18 million. It is stated in the literature that successful growth of tumors included a prolonged journey with various factors which are not clearly known yet (Razak et al., 2021). The growth of the tumor requires a proper mechanism through which immune evasion will be possible (Dhatchinamoorthy et al., 2021). Studies have revealed that the formation of tumors in the human body is only possible when the surrounding environment support its growth by suppressing immune responses. Mainly, cellular signaling, extracellular matrix and secretion of cytokine and growth factor are altered for the formation of cancerous cells (Dhatchinamoorthy et al., 2021)

Immunotherapies

Immunotherapies include all those treatments that make the immune system capable of eliminating malignant cells growth not only protect the other cells but also destroy the further proliferation of cancerous mass of the cells. Immunotherapy has different classes that can target different stages of

immune system from initial antigen occurrence to final effector stage. Considering specificity of the cancer and its stages, it has concluded that early immunotherapies give promising results and highly potent in the situations where many different clinical standards failed to provide successful outcomes. One of the effective directions for the treatment of cancer include Nanomedicines that provide prolonged changes in the body to fight against cancer. Drug delivery system by the use of nanoparticles has unique importance in nano-medicines (Huang et al., 2020).

Nanoparticles (NPs) and their synthesis

Nanoparticles are defined as the particles or small bodies whose sizes range within nanometers. Due to their smaller size, these particles cannot view through naked eyes. Nanoparticles have significant properties as they are composed of some specific material that can target the cancerous cells (Tasic et al., 2018).

The formation of organic nanoparticles is based on lipids and polymers along with selective solvents. Nano-participation, emulsion solvent evaporation pathways including nano-gel, liposome and poly-caprolactone are the pathways included in the formation of nanoparticles. Inorganic materials can also be made within nanoparticles that include gold, iron oxides, copper sulphates, silica and silicon. The core of the resulting inorganic nanoparticles has high capacity to move with different optical, magnetic and electrical features. The material selected for the core of nanoparticles depends upon the working, cargo and application of nanoparticles within the human body. It is also known that nanoparticles play a significant role in facilitating the size and shape of cell membrane camouflaged nanoparticles, drug releasing mechanism and pharma-kinetic behaviour (D'Acunto et al., 2021).

Anticancer mechanism of Nano particles

It is stated in different literature that anticancer mechanism of nanoparticles follows different biosynthetic routes and also cause cytotoxicity. The size and the shape of nanoparticle is majorly responsible cytotoxicity, for example, the nanoparticles made by using the material of plants and are spherical in shape plays a significant role against cancerous lines. The process angiogenesis involves the formation of new blood vessels by existing vessels which are helpful in wound healing and in the formation of granulation tissues. Relevant to it, the growth of solid tumor

is also occurred due to the formation of new blood vessel as it supplies oxygen and nutrients equally to the cancerous mass. Resultantly, the cancerous cells proliferate and spread in the body. Recent studies have revealed that nanoparticles exhibit a prominent role in the treatment of retinal neovascularization diseases by inhibiting the vascular endothelial growth factor and also block the activation of extracellular signal related kinase by the help of phosphorylation of vascular endothelial growth factor receptor 2. In other words, anti-angiogenesis properties have been used in the treatment of cancer using different approaches related to nano-particles (Ratan et al., 2020).

Nanoparticles and nanomedicine

The field of oncology is strongly focused on nanomedicine due to its remarkable features in the treatment of cancer. The basic outcomes obtained by using nanoparticles for cancer treatment includes their therapeutic nature which results in the structural and chemical modifications according to the requirement of cellular environment. The diagnosis and imaging with the idea of alternation in the vaccine development is also obtained by the nano-medicine treatment (Siegel et al., 2011).

Nano-therapies are competitive in the tumor forming environment that did not improve the circulation of drug but also reduce the toxicity. The results appeared after using the techniques of nanoparticles are outstanding and promising for the clinical development of therapeutic drugs (Hanahan and Weinberg, 2011).

Many therapeutic nanoparticle technologies including polymeric micelles, albumin nanoparticles, liposomes etc. are well known categories for cancer treatment while many other similar technologies including radiation therapy, chemotherapy, immunotherapy, RNA interference therapy, hyperthermia etc. are under clinical trials and investigation (Wicki et al., 2015).

With the advancement in the field of nanomedicine, there are various outstanding outcomes attained but studies have revealed that there are many challenges present yet that need to be resolved in the field of nanotechnology. For example, the chemical modification of tumor with heterogeneity and complexity makes the selection of nanoparticles difficult. Thus, careful selection of the patient is needed to benefit from nano-medicine (Sinha et al., 2006).

Nanoparticles with physico-chemical properties

Nanomedicines have revolutionized the treatments of cancer by offering different health opportunities to the patients. The nanoparticles can easily be modified in shape, size and surface characteristics to treat specific type of tumor. The customization of size is very essential because nanoparticles must travel in the blood stream and deliver the nano-carrier to the targeted tissue. In other words, the optimization of nanoparticle size may help improve the uptake of nanocarrier into the tumor site (Mavaddat et al., 2015).

Solubility and degradation

There are many anti-cancer drugs used for targeting cancer cells but these drugs failed to perform their task because of their poor solubility in water and ultimately eliminated from blood (Wicki et al., 2015). On the other hand, hydrophilic nanoparticles have excellent solubility and allow effective delivery in the tumor environment. The improvement in the development of nanoparticle can make the drug available for longer period of time in the circulation of blood without degradation which can help in suppressing the tumor (Wicki et al., 2015).

Targeting

Nanocarriers can be designed to work as active or passive target tool to reach tumor tissue (Heidel and Davis, 2011). Active targeting involves nano-carrier attachment with ligand that has high specificity for the receptors and target cells. While, passive nano-carrier are designed within the tissues where blood circulation is higher for the supply of drug (Lovrić et al., 2005).

Nanoparticles in combination therapies

Nanomedicine could conduct different therapeutic agents for the purpose of treatment. Loading different siRNA could increase the rate of sensitivity in the treatment. These features of nanoparticles make them extraordinary unique for the treatment of cancer (Wang et al., 2013).

Cancer cell treatment Methodology

Nanoparticles are useful as therapies and diagnostics because cancer cells are heterogeneous. Breast anti-cancer therapy is difficult due to the disease's aggressive spread to the brain, bone, lung, and liver. For the diagnosis and defense against metastatic breast cancer, magnetic nanoparticles are essential (Subhan et al., 2022). Metal based nanoparticles are undoubtedly versatile molecules that can be used in different

biomedical treatments as they possess high diagnostic characteristics like sensitive assays, radiotherapy based enhanced treatments, thermal ablation, targeted gene as well as drug delivery etc. It has also been studied that nonmetal-based nanoparticles offered as nontoxic carriers for the purpose of gene and drug delivery (Johnson et al., 2010). Nanoparticles with noble metal-based nanoparticles can help providing simultaneous diagnostic options and therapeutic benefits. These nanoparticles can penetrate in better way and track the targeted area for drug delivery that ultimately decreased the risk as possible in conventional therapies (Thornburg et al., 2008).

Noble metal nanoparticles, such as gold, silver, platinum, and palladium nanoparticles, are frequently utilized in the treatment of cancer because of their distinctive optical features, great biocompatibility, surface effects, and tiny size effects (Zhao et al., 2022).

Metal nanoparticles-based therapies

Tumor Targeting

Metal based nanoparticles provided two types of tumors targeting strategies that are known as active and passive targeting. In passive targeting, metal nanoparticles faced the tumor based defective vasculature and failed lymphatic drainage due to instant proliferation of solid tumors. Thus, metal nanoparticles entered the tumor environment through angiogenic vasculature that enhanced the targeted permeation and retention of the drug as shown in fig 1. In case of active targeting, the metal-based nanoparticles function to perform various task in the form of different biological moieties including peptides, antibodies, RNA or DNA cellular receptors. The monoclonal antibodies based on metal nanoparticles successfully target cancerous cells or surface proteins and initiate the anticancer process and decrease the risk of damaging healthy cells (Vogelstein and Kinzler, 2004).

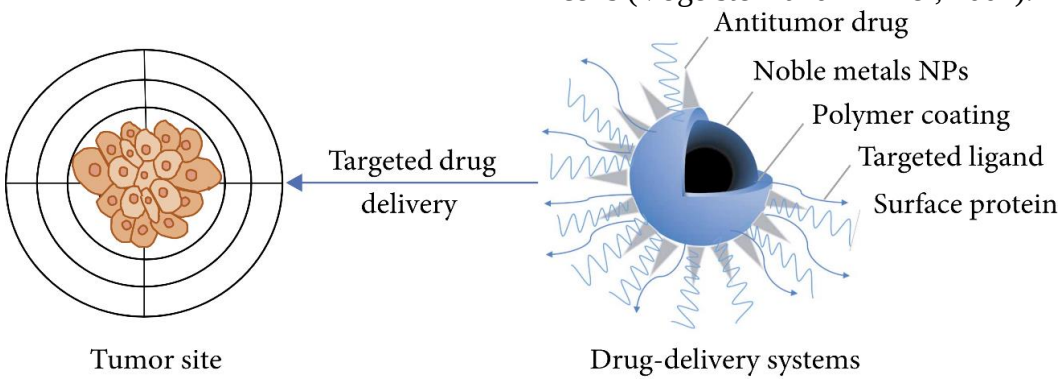

Fig. 1. Brief schematic of drug delivery systems (Ding et al., 2013)

Gene Silencing

The studies have revealed that gold nanoparticles have effective intracellular drug delivery option that can work as antisense vehicle for oligonucleotides and for siRNA and protect it against RNA's and promote selective targeting. The gold nanoparticles attached with single stranded oligodeoxy nucleotides have powerful ability to work in gene therapy as efficient gene regulator. These molecules provide high loading capacity of antisense DNA without causing toxicity which is ultimately a beneficial property in anticancer treatment (Ferrari, 2005).

Hyperthermia

Hyperthermia therapy in cancer has been widely accepted both by direct irradiation and by customizing temperature vector which are metal nanoparticles. These nanoparticles increase the temperature of the cancerous cells beyond their tolerance ability but lower than the temperature of normal tissues by failed blood circulation and selectively kill them. This technique is used by exposing the patient or the targeted area to the magnetic field or intense light range that cause the nanoparticles to increase the temperature and cause thermal ablation of the cancerous cells (Meyer et al., 2015).

Drug Delivery

The studies have revealed that metal-based nanoparticles have potential to work as vector for targeting cancer cells and help optimizing the distribution of drugs. Gold nanoparticles have been used as tool for the anticancer drug delivery including platinum-based drugs i.e., oxaliplatin, cisplatinetc (Sperling et al., 2008).

Radiotherapy

The technique is widely used for the treatment of cancer by the use of radiation on the proliferating cells. This treatment has a major challenge of damaging healthy tissues located near the cancerous environment that uptake intense radiations. Radiotherapy has been modified by the introduction of noble metal nanoparticles that act as antennas to target the radiations on the specific cancerous environment and protect the healthy tissues. The radiations are also targeted in a control manner to avoid the risk of healthy cells damage (Hainfeld et al., 2004).

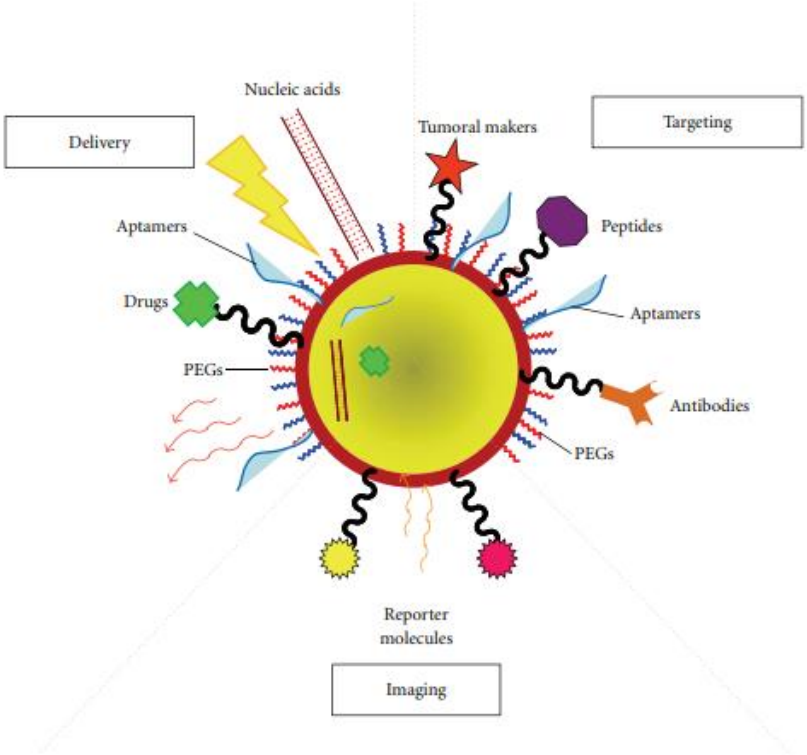

Fig. 2. Multifunctional Metal based nanoparticle system for tumor targeting, delivery and imaging (Van Vlerken et al., 2006)

Table 1: Nanoparticles applications in different types of cancer recognition

	Platform	Application	Indication	Reference
1	Silica Nanoparticles	Lymph node imaging	Head and neck melanoma	(Maggiorella et al., 2012)
2	Hafnium oxide nanoparticles	Radiotherapy	Solid tumor, head and neck cancer, lymph node cancer	(Diaz-Gil et al., 2016)
3	Iron oxide nanoparticles	Magnetic resonance imaging	Prostate cancer	(Fortuin et al., 2013)
4	Gold coated silica nanoparticles	Photo-thermal ablation	Head and neck cancer	(Gupta et al., 2013)
5	Colloidal gold nanoparticle	Tumor necrosis factor delivery	Solid tumors	(Libutti et al., 2010)

Table 2: Use of nanoparticles in different cancer therapies

Nanoparticle	Drug	Cancer type	Approval
Albumin bound nanoparticle	Pacilitaxel	Breast cancer, pancreatic cancer, lung cancer	2005
Liposome based nanoparticle	Doxorubicin	Ovarian cancer, Breast cancer, Lung cancer, ovarian cancer	1996
Liposome based nanoparticle	Paclitaxel	Breast cancer, ovarian cancer	1999
PEG polymeric nanoparticle	L-asparaginase Styrene maleic anhydride neocarzinostatin (SMANCS)	Liver cancer, Renal cancer	2007
Iron oxide nanoparticle	Doxorubicin	Thermal ablation glioblastoma	2010
Polymeric protein nanoparticle	Vincristine	Leukemia	2006

Tissue compatibility and inflammation of nanoparticles

Nanoparticles are made using different natural and artificial polymers, dendrimers, ceramics, lipids and metals. Nanoparticles has the ability to deliver therapeutics in a reliable manner and can access intracellular components by different biological processes i.e., phagocytosis and endocytosis. The decrease in the size of particles assists material to penetrate biological barriers of the cells and ultimately cause inflammation. It has been known that nanoparticles can trigger the inflammatory responses as they enhance the production of reactive oxygen species in lung epithelial cells and macrophages that resultantly cause lung infections and even injury (Table 1). Thus, it is important to consider nanoparticles inflammatory response when designing a drug to cure a specific organ of the body (Powell et al., 2010).

(i) Generally, biomaterials are considered as the foreign molecules and bodies that are

responsible to start inflammatory responses in the body. Inflammatory reactions that occur during the implantation of nanoparticles are mainly caused by the interaction of proteins with the surface of reactive site (Xie, 2013).

(ii) The level of inflammation varied from the type of proteins which is to be implanted along with nano-particles. Commonly, albumin, fibrinogen, vitronectin and fibronectin are responsible for the inflammatory reactions of implanted nanoparticles (Table 2). Inflammatory reactions also depend upon other factors including surface charge and chemical composition of the material. It has been studied that the topography is also responsible for the adsorption of plasma proteins within extracellular matrix(Eniu et al., 2008).

(iii) The acute inflammatory responses are generated in different conditions. For example, any injury that includes the implantation of biomaterials resultantly introduced neutrophils that ultimately activate the mast cells. The absorption of proteins on the surface of cells also leads to acute inflammatory response. The cells resultantly release reactive oxygen species (ROS) and cytokines for example, interleukin 3 and interleukin 4 that cause oxidative stress and joining of monocytes in the blood. Moreover, addition of biomaterial enhances the production of inflammasomes. Inflammasomes are the intracellular multi-protein bodies that involved in the activation of pro-inflammatory cytokines for example, Interleukin-18 and interleukin 1-β. The phagosome rupture after the uptake of material through phagocytosis and their interaction with plasma membrane also resulted in the production of inflammasomes (Ferraz da Costa et al., 2012).

(iv) The use of nanoparticles in tissue engineering creates the same inflammatory scenario as created by other biomaterials, i.e., cytokines, inflammatory cells and enzymes etc. but their response is quite different from these biomaterials. Nanoparticles possess different features like surface nano-topography and inflammatory cell response. For example, surface architecture of nano-porous scaffolds influenced the production

of cytokines in macrophages (Gupta et al., 2014).

It is worthy to mention that the nanoparticles and carbon nanotubes are utilized as individual entities instead as a component of bulk biomaterials. It means that two classes of nanomaterials are different from each other even at nano-scale features. That is why they are taken up by cells through phagocytosis or by other means. The specificity in the responses of the cells with the interaction of nano-particles explains the activation of inflammatory cells (Haynes et al., 2006). On the other hand, the cells in contact with nano-scale surface features are activated via adsorbed protein-receptor interaction. Nanoparticles geometry including size, shape, structure and reactive capacity is responsible for inflammatory responses (Wang et al., 2011). The inflammatory responses of nanoparticles are regulated by shape and size and their characterized features are associated with the level of inflammation. The inflammatory responses of nanoparticles can be optimized by addition of material that shows necessary biocompatibility, anti-inflammatory molecules association with nanoparticles and chemical modifications when the drug is introduced in the body (Chapekar, 2000). The chemical composition of nanoparticles also involved in eliciting inflammatory responses. Nanoparticles with non-biodegradable composition and cationic polymers have greater chance to induce more inflammatory responses compared with biodegradable composition with anionic behaviour (Harrison and Atala, 2007).

CONCLUSION

With the advancement in medicines and technology, there are many cancer treatments have been introduced but still the death rate of cancer patients in the world is exponentially high which requires conventional treatment strategies to save lives. The technique of radiotherapy is widely used for the treatment of cancer, but this is challenging due to highest rate of damaging healthy tissues. The studies have revealed that metal-based nanoparticles have potential to work as vector for targeting cancer cells and help optimizing the distribution of drugs. Recent studies based on nanoparticles for the cancer treatment gained importance because of efficiency and promising results. The formation of nanoparticles using green chemistry is environment friendly, cheap to afford and nontoxic in nature. These nanoparticles increase the temperature of the cancerous cells beyond their

tolerance ability but lower than the temperature of normal tissues by failed blood circulation and selectively kill them. The process of delivering nanoparticles in the cancerous patients is quite simple and cause less side effects compared with radiotherapy and chemotherapy.

CONFLICT OF INTEREST

Authors declare there is no conflict of interest.

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DOI: https://doi.org/10.54692/lgujls.2024.0801330
Paper Submission: 5th April, 2023; Paper Acceptance: 21st Aug 2023; Paper Publication: 15th March 2024

Research Article | LGU J. Life. Sci
Vol 8 Issue 1 January - March 2024 | ISSN 2519-9404
eISSN 2521-0130

Serum Levels of Biomarkers in COVID-19 Patients in a Hospital-based Population in Lahore

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ABSTRACT: *COVID-19 disease has affected more than 1.58 million people in Pakistan. The disease has been associated with a number of inflammatory markers such as serum CRP, d-dimer and ferritin. The present study was undertaken to investigate the role of these 3 biomarkers with the severity of disease in a hospital-based population in Lahore. Ninety-three COVID-19 patients (52 males, 41 females, age 18-70 years) were recruited with informed consent. The blood was analyzed for the serum levels of CRP, d-dimer and ferritin using commercially available kits. Severity of disease was determined on the basis of clinical symptoms. Analysis of the data revealed that although there were elevated serum levels of CRP and d-dimer in most patients (54.8% and 60.2%, respectively), yet no significant differences were observed in mean levels of these biomarkers in males and females. Similarly, mean serum ferritin levels were not significantly different between male and female patients. Since the normal ranges of serum ferritin in females and males were different, the comparison among patients showed more males with normal ferritin levels than females. Thus, there were significantly more females with elevated serum ferritin levels compared to males (56.1% vs. 32.7%; p=0.015) indicating an important role of this biomarker in Pakistani female patients. No relationship was found between levels of serum ferritin and severity of disease.*

Keyword: COVID-19 disease, serum ferritin, serum d-dimer, serum CRP, biomarkers

INTRODUCTION

COVID-19 disease has severely affected people of Pakistan. According to the WHO dashboard by the first week of February, 2023 there have been 1.58 million confirmed cases of this disease in Pakistan, while the mortality has been reported to be 30,639 [http://covid-19.who.int> region>emro>country].

The involvement of serum biomarkers in COVID-19 disease has been examined in a number of studies. Variability in levels has been reported and it is based upon the differences in patients' exposure to the virus and status of the immune system of the infected patients. For example, elevated serum levels of ferritin (a non-specific marker of inflammation) and d-dimer (a marker for thrombotic status) have been reported in those individuals who had a poor survival rate (Tang et al., 2020; Kermali et al., 2020). Another study conducted by Xiang et al. (2020), reported significantly elevated serum markers ferritin and C-reactive protein (CRP; a marker for inflammation) in critically ill COVID-19 patients compared to those who had mild symptoms of this disease. Similarly, a study involving 1500 COVID-19 disease patients showed significant differences in these biomarkers' levels on admission in patients who survived compared to those who did not survive (Loomba et al., 2020). These studies showed a relationship of these biomarkers with severity of COVID-19 disease.

There are only a few studies that have been carried out in Pakistan to investigate the relationship of these biomarkers with COVID-19 disease. (Saeed et al., 2022; Hassan Shah et al, 2021). The present study was undertaken to investigate the relationship of serum levels of CRP, d-dimer and ferritin with severity of COVID-19 disease in a hospital-based population in Lahore.

MATERIALS AND METHODS

In a cross-sectional study, 93 adult consecutive COVID-19 disease suspected patients (52 males and 41 females with ages in the range from 18-70 years) were recruited with informed consent from the Umer Shoaib Surgical Hospital, Lahore from January 21, 2021 to December 11, 2021. They had the confirmed diagnosis of COVID-19 disease based on clinical examination and lab results. Blood samples (10 ml) were collected for the determination of serum levels of biomarkers CRP, d-dimer and ferritin using commercially available kits and following manufacturers' instructions. CRP kit

was obtained from Pol. Ind. Can Castells. C, Barcelona. The method adopted in this kit had a sensitivity of 74% and a specificity of 75%. The d-dimer kit was purchased from EDAN, Shanghai International Corporation, GMbH, Humburg having a sensitivity of 98.9% and a specificity of 92.5%. Ferritin kit was obtained from Cal biotech Inc. California. It was based on sandwich ELISA with sensitivity and specificity exceeding 98%. In order to ensure quality and accuracy, a negative control and standards with known concentrations of biomarker were run along with patients' samples in each assay of these 3 biomarkers. Severity of the disease was assessed by the clinician based on clinical symptoms and the National Guidelines pertaining to symptoms of COVID-19 for diagnosis of mild, moderate and severe disease as mentioned by Hassan Shah et al (2021). These have been briefly outlined below:

Mild: Respiratory rate < 24/min; SpO2 > 94 % at room temp

Moderate: Respiratory rate 24-30/min; SpO2 90% -94% at room temp

Severe: Respiratory rate > 30/min; SpO2 < 90% at room temp

The study had been approved by the Bioethical and Safety Committee of the Department of Life sciences, University of Management and Technology.

STATISTICAL ANALYSIS

The data were analyzed in the Statistical Package for Social Sciences (SPSS) version 21.0 using various statistical tests such as Independent Sample t-test and Chi-Square test. P-value < 0.05 was considered significant.

RESULTS

Among the 93 recruited COVID-19 patients, 52 were males and 41 were females. Table 1 shows the biochemical characteristics of female and male COVID-19 patients. Mean CRP levels among female and male patients were not found to be statistically significant (54±58.2 mg/l vs. 76.6±104 mg/l; p = 0.088). However, 30 male patients (57.7%) and 21 female patients (51.2%) had CRP levels above the normal levels (< 6 mg/l). The mean serum levels of d-dimer in both female and male patients were not statistically different (217±234 µg/ml vs. 131±216 µg/ml; p = 0.603), but 31 males (59.6%) and 25 females (60.9%) had d-dimer levels above the normal levels (< 200 µg/ml). Mean serum ferritin levels in female patients below the age of 40 years were higher compared to the highest normal value of this biomarker in this age group (137 ng/ml). On the other hand, mean serum ferritin levels in male patients above the age of 40 years were well above the

maximum normal serum ferritin levels of 464 ng/ml in this age group. However, the mean serum ferritin levels in both these age groups were not found to be statistically significant among males and females. The data also showed that among the 93 patients, 17 males and 23 females had more than the maximum normal value of serum ferritin level for that gender and age group (Table 2). When the proportions of male and female patients having serum ferritin levels above the maximum normal value for their categories were compared using Chi Square test, there was significantly higher percentage of females having elevated levels of serum ferritin compared to male patients ($p = 0.015$), indicating serum ferritin as an important biomarker of inflammation in COVID-19 disease, especially among the female patients. Fifty-two percent of our patients had severe to moderate disease. However, no significant difference was observed between male and female patients in terms of severity of disease (Table 3).

Table 1: Biochemical characteristics of female and male COVID-19 patients in a population in Lahore, Pakistan

Variables	**Total N**	**Female**		**Male**		**P-value***
		N	**Mean ± SD**	**N**	**Mean ± SD**	
C-Reactive Protein (CRP) mg/l	93	41	54 ± 58.2	52	76.6 ± 104.1	0.088
D-dimer µg/ml	93	41	217 ± 234	52	131 ± 216	0.603
Ferritin with respect to 2 age groups (ng/ml)						
≤40*	43	16	278 ± 410	27	341 ± 374	0.236
> 40**	50	25	188 ± 408	25	486 ± 458	0.598

* P-value was obtained by comparing means of males and females using an Independent sample t-test.

** Normal range of serum ferritin in age groups less than or equal to 40 years for females is 6.24 to 137ng/ml and for males is 17.9 to 464ng/ml

*** Normal range of serum ferritin in age group above 40 years for female is 11.1 to 264ng/ml and for male is 17.9 to 464ng/ml.

Table 2: Frequency distribution of male and female COVID-19 patients having serum ferritin levels above the maximum normal value for their categories.

Gender	N	n (%)	Chi-Sq. value	P- value*
Male	52	17 (32.7)	6.652	0.015
Female	41	23 (56.1)		

* P-value was obtained by comparing the percentages of male and female COVID-19 patients having serum ferritin levels above the maximum normal value for their categories by using the Chi-square test.

Note: Maximum normal ferritin value in males of any age is 464 ng/ml, while the maximum normal value in females below 40 years is 137 ng/ml and above 40 years is 264 ng/ml.

Table 3: Comparison between Males and Females with respect to the severity of the disease

Disease	Male n(%)	Female n(%)	p-value*
Severe	11 (21.1)	6 (14.6)	0.680
Moderate	18 (34.2)	14 (34.1)	
Mild	23 (44.2)	21 (51.2)	

*P-value was obtained by comparing the proportions between each of the three rows pertaining to severe, moderate, mild disease using Chi Square test

DISCUSSION

The serum levels of biomarkers CRP, ferritin and d-dimer were found to be elevated in most of the study participants, nonetheless, the most significant increase was observed in serum ferritin levels in our female patients. These results are different from the results of another study from Pakistan showing higher serum levels of ferritin in COVID-19 male patients with moderate to severe disease (Hassan Shah et al., 2021). This could be due to the reason that they did not take into account the fact that the normal serum levels of ferritin are lower in females compared to males (6.24 ng/ml to 264 ng/ml for females and 17.9 ng/ml to 464 ng/ml for males). However, their results pertaining to serum levels of d-dimer indicating no statistically significant difference between male and female patients with moderate and severe disease are in line with findings of this study. Another study carried out in Pakistan showed significantly higher levels of serum ferritin and serum d-dimer in 48 critically ill patients with COVID disease (Alam et al., 2021). However, their sample size was too

small to be grouped into mild disease patients and those suffering from moderate to severe disease.

Ferritin is one of the major mediators of immune system dysregulation and contributes to development of "cytokine storm" in COVID-19 disease patients (Vargas-Bargas and Cortes-Rojo, 2020). In a meta-analysis, significantly elevated serum levels of ferritin were reported in severe to critically ill COVID-19 disease patients indicating its prognostic value (Kaushal et al., 2022). According to a study carried out in China, nearly 63% of the COVID-19 patients had serum levels of ferritin well above the normal range (Chen et al., 2019). Results of our study are in line with this study and 43% of our patients had levels of ferritin well above the normal range.

The above mentioned studies showed a relationship between levels of this biomarker and the severity of COVID-19 disease. However, in our recruited patients, we could not find any relationship between levels of serum ferritin and severity of the disease. This could be due to the small sample size (n = 93) of patients analyzed for this biomarker or the immune system of these patients was robust enough to minimize the inflammatory response to viral attack. This is further supported by the fact that there was only one mortality among the 93 recruited patients.

Another study carried out at the Allama Iqbal Medical College/Jinnah Hospital reported that higher levels of serum ferritin (> 200 ng/ml) and d-dimer (> 400 ng/ml) could be a predictor of mortality among COVID-19 disease patients. However, the recruited patients in that study were critically ill with mortality up to 35%. (Yousaf et al., 2022). We had several patients with levels greater than the cut-off values reported in that study, nonetheless there was only one mortality among our recruited patients. This shows that clinical condition of patients apart from these biomarkers is also important in predicting the outcome of the disease.

The current study though with a relatively small sample size is a useful addition to the existing body of knowledge in the country related to coronavirus infection and the role of serum biomarkers, especially serum ferritin in this disease. However, more prospective studies with an increased number of samples are required to clearly determine the role of biomarkers' levels in predicting the disease, its prognosis, and its final outcome.

CONCLUSION

Serum ferritin levels were elevated above the normal levels in significantly more female patients suffering from COVID-19 disease than male patients. This showed that ferritin was an important biomarker for predicting COVID-19 disease in Pakistani female patients presenting with common symptoms of this disease.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help provided by staff of the Umer Shoaib Hospital Lahore. We are also thankful to Mr. Koukab Imran for proper formatting of the paper.

ETHICAL APPROVAL

The study have been approved by the Biochemical and Bioethical Safety Committee of the Department of Life Sciences, UMT, Lahore.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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