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Siamotragulus (Tragulidae: Artiodactyla: Mammalia) from the Siwalik Group of Indian Subcontinent (Pakistan)

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***Siamotragulus*, Middle miocene, Dhok Bun Ameer Khatoon, Siwaliks, *Dorcatherium*, Chinji Formation**

ABSTRACT

The present study deals with the description of a right mandibular fragment having p2-m3 referred to the genus *Siamotragulus.* This specimen was recovered from the Chinji Formation outcrops (Middle Miocene) of Dhok Bun Ameer Khatoon, District Chakwal, Punjab, Pakistan. The dentition of this mandibular ramus under study is characterized by selenodonty, premolars with very sharp edges, molars with preproto- and premetacristids are longer than the postproto- and postmetacristids, lingual cusps are flat without any prominent bend or rib and lack *Dorcatherium* platform. These characters differentiate it from the genus *Dorcatherium* and help to associate the mandibular fragment with the genus *Siamotragulus*. Based on its size, being larger than *S. nagrii* and *S. minimus*, it is referred to as *Siamotragulus* sp. nov.

INTRODUCTION

The Siwalik Group of Indian subcontinent represents the fluvial sedimentation phase in the Himalayan Foreland Basin, which developed south of the rising Himalayan in response to the collision between the Indian and Eurasian tectonic plates which started 50 million years ago and even continues today (Nanda *et al*., 2022). Siwaliks are very famous for vertebrate fossils pertaining to almost all the major mammalian groups such as Rodentia, Artiodactyla, Perissodactyla, Proboscidea and Carnivora etc. (Colbert, 1935). Of these, the fossil artiodactyls are very well known in different formations of the Siwaliks. Artiodactyls are represented by a variety of suids, hippos, camels, cervids, giraffids, bovids and tragulids. There are four extinct genera of the family Tragulidae in the Siwaliks; *Dorcatherium*, *Afrotragulus*, *Dorcabune*, and *Siamotragulus* Among these, *Dorcatherium*, *Dorcabune*, *Afrotragulus* are known mostly by maxillary and mandibular fragments and isolated upper and lower teeth while the genus *Siamotragulus* is known only by postcranials except that have previously been described as *Dorcatherium*.

We describe here the Middle Miocene *Siamotragulus* from the Chinji Formation outcrops exposed west of village Dhok Bun Ameer Khatoon (DBAK), Chakwal district, Punjab, Pakistan. The specimen under study is a right mandible which provides the basis to discuss the taxonomic problems of the Siwalik tragulid allocated to the genus *Dorcatherium*. The geographic and geological settings of Dhok Bun Ameer Khatoon are given below.

The fossil site, Dhok Bun Ameer Khatoon (Lat. 32° 47' 34.6" N: Long. 72° 55' 36.1" E and altitude 1942.43 ft) is in the northeastern region of the Chua Saydan Shah town, district Chakwal, Punjab, Pakistan (Fig. 1) and about 50 km northeast of the Chinji stratotype, Chinji Formation. The studied deposits are located northwest of the Dhok Bun Ameer Khatoon village. Locally, the outcrops area in the west from where the specimen is

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collected is known as Jallowala. The exposed sediments cover a few hundred square meters, approximately 40 m thickness, bearing mammalian remains. The outcrops consist of shales, siltstones and sandstones deposited in a fluvial environment, mainly filled by unweathered igneous minerals (Cheema, 2003). Based on the faunal elements, the age is dated between 14 and 11.6 Ma.

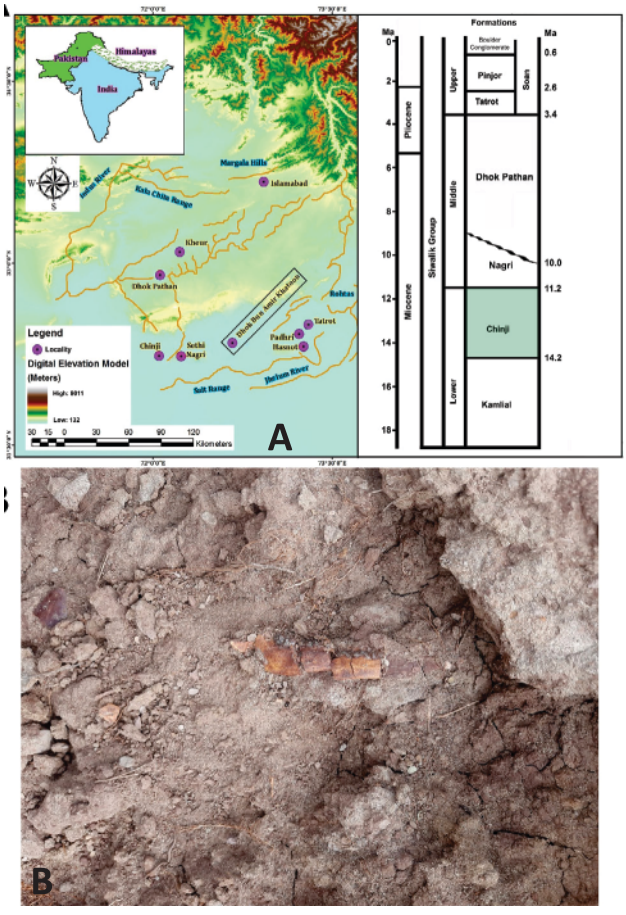

Fig. 1. A: The geographic position of Dhok Bun Ameer Khatoon from where the described mandible was collected. B: PUPC 25/34 in the field.

MATERIALS AND METHODS

The material includes a mandibular fragment with p2-m3 during the 2024 field visits. The specimen was found partially buried in the sediments (Fig. 1B) and recovered with the help of geological hammer and chisel in five pieces that were later glued with Elfy after preparation of the specimen. Fine needles and brushes were used to remove the sediments and clay. A hand lens was used for keen observations of very small and ambiguous morphological features of the teeth of the mandible under study. Photographs were taken with the help of DSLR camera (Canon 6D). The measurements of the specimen were taken millimeters (mm) with the help of digital Vernier caliper. The specimens collected show the collection year and the serial number of that year e.g., PUPC 25/34. The upper figure denotes the collection year and the lower one the serial number of the respective year.

Abbreviations: PUPC, Punjab University Palaeontological Collection; PG, Pong.

SYSTEMATIC PALAEONTOLOGY

Mammalia Linnaeus, 1758
Artiodactyla Owen, 1848
Ruminantia Scopoli, 1777
Tragulina Flower, 1883
Traguloidea Gill, 1872
Tragulidae Milne Edwards, 1864
Genus *Siamotragulus* (Thomas *et al*., 1990)
Type species: *Siamotragulus sanyathanai* (Thomas *et al*., 1990)

Holotype

PG1, left mandible with p2 (partly broken) to m3 (Thomas *et al*., 1990).

Type locality and age

Pong, Phayao Province, Thailand and Middle Miocene in age (Thomas *et al*., 1990).

Diagnosis

Small Tragulidae with a size intermediate between that of *Dorcatherium minus* Lydekker and *Dorcatherium minimus* west. The lower premolars, in particularly the p3, are very pointed and sharp, differing thereby from those of *Dorcatherium* and *Dorcabune.* The p4 possess an elongate posteriorly directed metaconid but no other lingual feature which may correspond to the entoconid or entostylid. The p3, long, treanchant and strongly compressed, has a tall protoconid, a minute paraconid and a distinct hypoconid. No posterolingual ridge (metaconid) is present on p3. Upper canines of males saberlike. The limb bones are relatively long and slender (Thomas *et al*., 1990).

***Siamotragulus* sp. nov.**

(Fig. 2)

Referred specimen

PUPC 25/34, a right mandible bearing p3-m3, partially broken p2 and root of p1 (Fig. 2).

Locality and age

Dhok Bun Ameer Khatoon, Chakwal district, the

Punjab province, Pakistan.

Description

PUPC 25/34 is a mandible having p3-m3, partially broken p2, and root of p1 (Fig. 2).

Fig. 2. *Siamotragulus* sp. nov. PUPC 25/34 is a mandible having p3-m3, partially broken p2, and root of p1. (a) Occlusal view, (b) lingual view (c) labial view.

Corpus

The corpus though cracked at various places and thin, but it is extremely stout. The corpus is concave lingually and convex labially (Fig. 2A). It also preserves a small part of symphysis, diastema, angular process, and ascending ramus. A large mylohoid line is present at the base of the corpus stating under the p2 on the lingual side (Fig. 2B). There are two mental foramina: the anterior and larger under the diastema and a posterior and smaller under the p3 (Fig. 2C). The diastema is very small and has a length between mandibular symphysis and p2 is 6.5 mm. The depth of the corpus increases under the teeth from p1 to m3 and the vertical depth of the mandible below the m1 is about 13 mm and transverse width is about 6 mm. The antero-posterior length of mandible is 80 mm and of p2-m3 series is 62.36 mm. Length of premolar series 29.01 mm length of molar series 33.35 mm.

Premolars

The dentition is also well preserved except for the p1 and p2. Only the root of p1 is preserved. The p2 is in early stages of wear and partially broken anteriorly resulting in loss of paraconid and some part of the protoconid which is large and connected to the hypoconid and metaconid through its postcristid. A prominent valley is present between the metaconid and hypoconid. The p3 is also in early stage of wear and well preserved. It has a large paraconid which is connected to the large and bulbous protoconid through its postcristid. A shallow valley is present between the paraconid and protoconid lingually and small groove labially. The metaconid is large, more lingually and posteriorly oriented and is in contact with large and round hypoconid. A large groove is present between the protoconid and hypoconid and a large, lingually open valley is present between the metaconid and hypoconid. The p4 is most worn among premolars and is in the early middle stage of wear. It is slightly smaller than p3 (Table I) and is most developed. Most of its morphology is similar to p3 except the posterior half in which the posthypocristid is large the posterior valley is extremely narrow.

Molars

Except the extreme wear, the molars are also well preserved. The m1 is the most worn and smallest among the molars. The metaconid is partially broken resulting in loss of the enamel in the center. It is in the late stage of wear resulting in loss of both anterior and posterior fossette. However, the distinction between the cusps is still possible. A large ectostylid is present in the median valley. The tragulus fold has been lost due to wear, but the *Dorcatherium* fold is still recognizable. The m^2 is less worn and it is also in late stage of wear. The anterior posterior side of the anterior fossette is preserved rest is lost due to wear. It is a selenodont and brachydont tooth. The preprotocristid is longer than the postprotocristid and premetacristid which is longer than the postmetacristid. Similarly, the prehypocristid is longer than the posthypocristid and preentocristid while postentocristid is extremely reduced. The *Dorcatherium* fold present on the postmetacristid is large and prominent while a small part of the *tragulus* fold is preserved. The median valley is blocked by a moderate sized ectostylid. The posterior cingulid is present at the posthypocristid that runs posterotransversly. The m^3 is least worn, most preserved in terms of its morphology and trilobed. It is extremely selenodont. The cusp morphology is similar to the m^2. Both fossettes are well preserved and these are narrow and crescentic especially the posterior fossette. The M/Σ structure is complete and pronounced as both the *Dorcatherium* fold on the postmetacristid and *tragulus* fold on the postprotocristid are intact and complete. The median valley has a weak ectostylid and a small cingulid is present at the base of postprotocristid. The postentoconid groove is open. The third lobe is composed of hypoconulid and very small entoconulid. The hypoconulid has prominent pre- and postcristids while a small entoconulid completely closes the prominent back fossette of m3 lingually. The posthypocristid is bifurcated and makes a small fold which is in contact with the hypoconulid. *Dorcatherium* platform is completely absent. The lingual walls of the metaconid and entoconid are flat. The enamel is extremely rugose.

Table I. Comparative measurements of the lower cheek teeth of middle miocene tragulids.

Taxa/Catalog No.	Position	Length	Width	References
Siamotragulus sp. nov.				
PUPC 25/34	p1	-	-	This study
	p2	8.71 est.	2.95	
	p3	9.46	3.51	
	p4	8.84	3.80	
	m1	8.87	6.57	
	m2	9.81	7.11	
	m3	14.67	7.14	
Siamotragulus nagrii				
VPL/AS/H/101	p2	5.2	2.3	Gaur, 1992
	p3	7.0	2.6	
VPL/AS/H/102	p4	8.2	3.3	
	m1	5.8	3.4	
	m2	7.1	4.2	
	m3	11.0	4.3	
Siamotragulus sanyathanai				
PG1	p3	9	3	Thomas *et al.* 1990
	p4	8	3	
	m1	6.7	4	
	m2	8	5	
	m3	11.7	5.1	
Dorcatherium minus				
SNSB-BSPG 1956 II 2489	p3	12.0	3.5	Guzmán-Sandoval and Rössner, 2021
	p4	10.0	4.4	
	m1	10.5	6.2	
	m2	11.4	6.6	
PUPC 68/313	m1	8.95	5.60	Khan and Akhtar, 2013
	m2	10.25	6.70	
	m3	15.65	7.40	
GSI-B 594	p4	10.0	4.8	Pilgrim, 1915
	m1	10.8	6.8	
	m2	12.5	7.5	
	m3	16.7	8.3	
Dorcatherium guntianum				
SNSB-BSPG1956 II 2554	m1	7.7	4.3	Guzmán-Sandoval and Rössner, 2021
	m2	9.1	5.0	
	m3	14.5	6.6	
SNSB-BSPG 1881 IX 737	m1	8.6	5.5	
	m2	10.0	6.1	
	m3	14.7	6.4	

Comparison

The studied specimen proves strongly its inclusion in the family Tragulidae, based on the selenodont pattern, rugosity of enamel and the presence of M/Σ structure. The family Tragulidae in the Siwalik hills are represented by two extinct genera *Dorcatherium* and *Dorcabune*, with many species. The *Dorcabune* is a large extinct tragulid of the Siwaliks and very close to anthracotheriids having bunodont molars. The paraconus rib is more conspicuous in *Dorcabune* than in *Dorcatherium* (Colbert, 1935; Farooq *et al*; 2007 Farooq *et al*., 2008; Khan and Akhtar, 2013). The specimen studied differs from *Dorcabune* having more selenodont cusp pattern.

Dentition in PUPC 25/34 is characterized by selenodonty, premolars with very sharp edges, molars with preproto- and premetacristids are longer than the postproto- and postmetacristids, lingual cusps are flat without any prominent bend or rib and lack *Dorcatherium* platform. These characters differentiate it from the genus *Dorcatherium* and help to associate the mandibular fragment with the genus *Siamotragulus*. Table I and Figure 3 show that the dentition in PUPC 25/34 is larger than *Siamotragulus sanyathanai* and the range in the size previously described as *Dorcatherium guntianum* from the Siwaliks. Hence, it became evident that measurements of PUPC 25/34 match with most of the classically described material of Siwalik species *Dorcatherium minus* while the tooth morphology is like that of *Siamotragulus* and size is like that of *guntianum*. Such morphometric analysis is

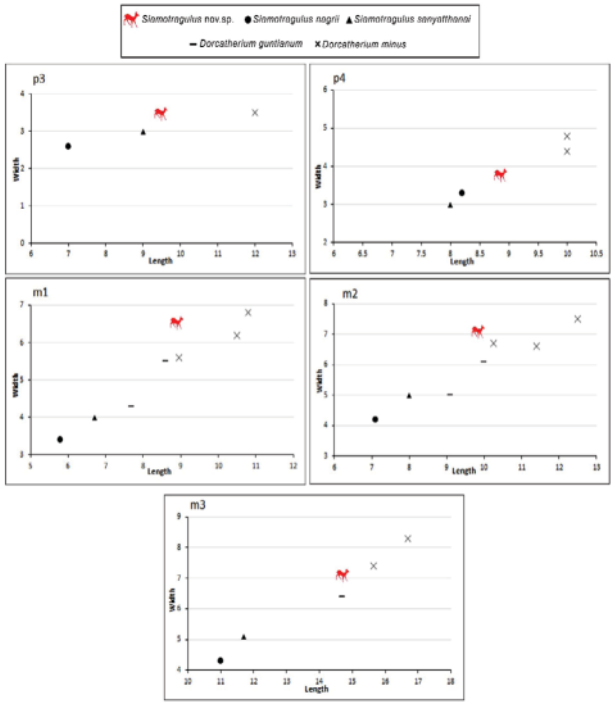

Fig. 3. Graphical representation of size comparison with the help of bivariate chart based on Table I.

convincing enough that PUPC 25/34 can be associated with the genus *Siamotragulus* as well as most of the material previously described as *Dorcatherium guntianum* . Hence, based on its morphology and size, it is referred to as *Siamotragulus* nov. sp. awaiting more material and in-depth study of all the Siwalik tragulid material.

DISCUSSION

The genus *Dorcatherium* was created by Kaup (1833) based on the material from the Germany. Since the wide geographic acceptance of the genus, most of the tragulid material from Miocene to Pliocene deposits was allocated to this genus without any detailed study (Rössner, 2007, Sánchez *et al*., 2010 2015). This led the status of the genus to a potpourri or waste bucket for most of the tragulid material and such a status of this genus was partly compromised by Thomas *et al*. (1990) by the creation of a new genus, *Siamotragulus*, for the tragulid material from Thailand. However, despite this study, the genus *Dorcatherium* remained a primary choice of researchers for the description of their tragulid material until the work of Sánchez *et al*. (2010) who identified the genus *Siamotragulus* from Africa after the indication of (Rössner, 2007). Further work was carried out by Barry (2014) who reported the presence of the genus *Siamotragulus* from the Siwalik Group based on the postcranial material. The next work was done by Sánchez *et al*. (2015) who identified yet another tragulid genus, *Afrotragulus*, from the material that was previously described as member of the genus *Dorcatherium*. This further shows the waste bucket nature of the genus *Dorcatherium*. Finally, such nature is also shown in Barry (2025) who described *Dorcatherium nagrii* as *Siamotragulus nagrii* and *Dorcatherium minimus* as *Siamotragulus minimus* and identified two more species of this genus. Such a nature also applies to the Siwalik tragulids and the description of PUPC 25/34 is an example to demonstrate it.

PUPC 25/34 morphologically shows the characteristics of the genus *Siamotragulus* similar to the specimen AMNH 19365, right mandibular fragment with m_2-m_3. Even in the AMNH 19365, the preprotocristid is longer than the postprotocristid and premetacristid which is longer than the postmetacristid. Even the lingual walls of the metaconid and entoconid are flat and it is also selenodont like PUPC 25/34. However, the illustration of AMNH 19365 shows the *Dorcatherium* platform but this could be due to the illustration bias. Similarly, most of the material described as *Dorcatherium minus* by Khan and Akhtar (2013), Khan *et al*. (2005, 2010, 2012a, b, 2013, 2017), Batool *et al*. (2014), Samiullah *et al*. (2015, 2021), and Draz *et al*. (2020, 2021) shows similar features as in PUPC 25/34 including the measurements. Hence, all such material can be allocated to the genus *Siamotragulus* without any hesitation.

CONCLUSIONS

We are describing a tragulid mandible collected from the Chinji Formation deposits of Dhok Bun Ameer Khatoon, Chakwal, Punjab, Pakistan. The teeth in the mandible show the characteristics of the genus *Siamotragulus* (selenodonty, premolars with very sharp edges, molars with preproto- and premetacristids are longer than the postproto- and postmetacristids, labial cusps are flat without any prominent bend or rib and lack *Dorcatherium* platform) while the measurements are in the range of previously described material as *Dorcatherium guntianum*. Further, most of the material previously described as *Dorcatherium* clearly shows the characteristics of the genus *Siamotragulus*. Hence, we are describing this mandible as *Siamotragulus* sp. nov. until more material is recovered and/or comprehensive study of all the Siwalik tragulid material is done.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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Prevalence of Gastrointestinal Parasites in Pet and Stray Dogs Found in Dir Lower, Pakistan

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ABSTRACT

The fecal material of dogs containing infective stages (eggs, larvae, cysts, and oocytes) of the parasite contaminates the soil and represents a great risk for the human health. Regardless of investigations, there is still a deficiency of information concerning the prevalence and seasonality of the intestinal parasites in pet and street dogs found in district Dir Lower. Therefore, this study has accomplished prevalence of parasites in domestic and street dogs found in Dir Lower, Pakistan and will provide reference point for forthcoming research. During this study fecal samples from 76 domestic and 124 stray dogs (n=200) were collected from different areas of Dir Lower and were examined for the presence of intestinal parasitic infections. Eight helminth species were demonstrated during this study from all dogs. These eight species included six species of nematodes and two species of cestodes. As trematodes parasitic infection in dogs is relatively uncommon therefore no flukes were found. During this study 44 dogs were infected with intestinal parasites showing the overall prevalence of 22%. The most common observed helminths parasites was *Toxocara canis* which was found in the fecal samples of 14 stray dogs and 4 domestic dogs 18 (9%). Other observed helminths parasitic species were *Trichuris* species 09 (4.5%), *Dipylidium caninum* 05 (2.5%), *Strongyloides stercoralis* 05 (2.5%), *Capillaria* spp., 04 (2%), *Taenia* spp., 04 (2%), *Ancylostoma caninum,* 03 (1.5%), and *Toxascaris leonina* 01 (0.5%) respectively. The study highlights the critical need to educate and inform local communities about the significant zoonotic risks posed by parasites. Additionally, the research aims to assess the extent of environmental contamination by dogs and the associated risks to human health.

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Gastrointestinal parasites, Environmental contamination, Zoonotic, Intestinal parasites, Dir Lower, Khyber Pakhtunkhwa

INTRODUCTION

In dogs the most frequent problem is the presence of intestinal parasites such as nematodes predominantly; hookworms and whipworms. Dogs procure these parasites by ingesting parasitic eggs when came in contact with contaminated soil, water, feces, sand and food. Puppy dogs also acquire these worms when they are in the womb or during nursing. In dogs these intestinal parasitic infections mostly cause gastrointestinal disorders. The prevalence of these gastrointestinal parasitic disorders is most common in developing countries (Daryani *et al*., 2009). Dogs have achieved a state of ubiquity, establishing their presence in nearly every corner of the globe where humans reside and, interestingly, in numerous locations where human presence is imprecise. For half of the countries in the world, estimates on the prevalence of dogs are available. It is noteworthy that the global per-capita average of 130 dogs for every 1000 people is a significant measurement, yet it's important to recognize the substantial variations exist in our neighboring countries (Sykes *et al*., 2020).

Various parasitic developmental stages such as ova, larval stages and cysts expelled through dog feces can persist infectious in the atmosphere over an extended period, depending on different conditions. Mostly when the infection is light the intestinal parasites do not show any symptoms. Therefor it is necessary to collect fecal samples after regular intervals and to study these for parasitic infection. When the dogs show clinical signs like weight loss, diarrhea with blood or mucus, swollen abdomen, stunted growth and vomiting then fecal examination is highly recommended. They are a risk factor mutually for humans and animal infections, especially for expecting females, kids, and immunocompromised individuals (Traversa *et al*., 2014). Domestic dogs in Pakistan have

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acknowledged expressively more consideration for deworming than stray dogs. Due to this carelessness, stray dogs are more likely to contract parasites that are extremely harmful to human health. Research on dogs is typically navigated on pet ones, and the outcomes are partial because stray dogs are not included. Insufficient reports are available on the parasitic infection risk transmission from dogs to people living in villages and residential areas in Pakistan (Powell *et al*., 2019). Dogs spread infections between pets and people because they serve as a reservoir host for a variety of parasites. Many parasites have dogs as their definitive hosts, while for some parasites dogs act as intermediate hosts like *Giardia lamblia, T. canis,* hook worms*, E. granulosus, D. caninum,* and protozoan borne toxoplasmosis (Khalifa *et al*., 2023). Humans can become infected by ingesting contaminated food and water having eggs and cysts, touching polluted items, inhaling contaminated dust, or through larvae penetrating their skin. These dogs have frequent contact with other animals, their feces, and a variety of food-stuff contaminated with animal feces and subsequent human exposure. Therefore, to reduce the danger of human infection, revising the epidemiology of the parasites in the canine population is necessary (Yagoob and Bahman, 2014; Khalifa *et al*., 2023).

One published report by Khan *et al*. (2019) is available about the prevalence of gastrointestinal parasites in dogs in Dir Lower in which only simple microscopic examination was used for parasitic infection detection and no other techniques were used. In order to correctly investigate the prevalence of gastrointestinal parasites in pets and stray dogs we used the sedimentation procedure and zinc floatation technique. A comprehensive study was conducted with the objective of to explore the Prevalence and seasonality of intestinal parasitic infections in domesticated and stray dogs found in Dir Lower. Other objective of the study was to identify potential risk factors for transmission of gastrointestinal parasites to human population.

MATERIALS AND METHODS

Background of the study area

Present study was carried out in rural and urban areas of district Dir Lower. Geographically district Dir Lower is situated at 34.9161° N, 71.8097° E, lies to the North of Khyber Pakhtunkhwa (KP), Province in Pakistan. Numerous agroecological zones experience a variety of temperatures, with the maximum temperature of 40 °C in Timergara and the lowest temperature of 1°C in Shahi. The district has a total area of 1583 km^2.

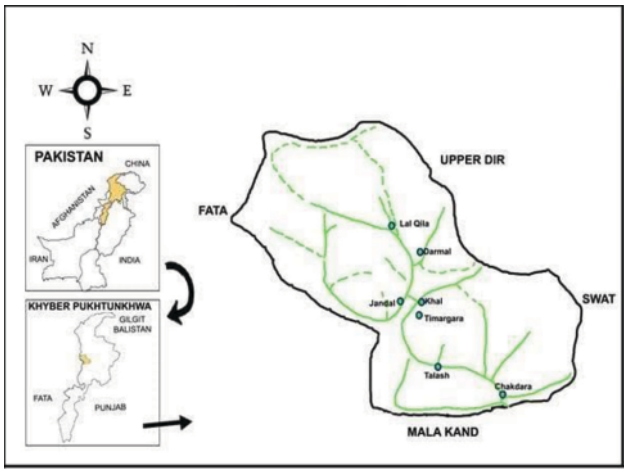

Fig. 1. Map of Dir lower.

Sampling sites

Pets and stray dogs are present in large numbers in Lower Dir. Sampling was done from most villages in Lower Dir. The area is mostly covered with mountain ranges, and the majority of the population lives in hilly areas, where people keep pet dogs for hunting and security purposes. Additionally, some people live in plain areas bordering districts Upper Dir, Bajaur, Swat, and Malakand, where the stray dog population is more prevalent.

Sampling method

Fecal samples from 76 domestic and 124 stray dogs (n= 200) were collected from different areas of Dir Lower from July 2023 to March 2024 and maintained in sterilized disposable labeled plastic bottles. The name and date of collection of the respective sample were written on each plastic bottle and immersed in 10% formalin. Important information was collected from all dog's owners for pet dogs in various villages of Dir Lower through pre-designed study questionnaire. The questionnaire was constructed on information associated to dog's gender, age, variety, anthelmintic practices and feeding procedures.

Parasites isolation and identification techniques

Sedimentation technique

10g of fecal sample was placed in a sterile Petri dish and diluted with 10 mL of distilled water. In order to remove extra plant materials and large debris using sterilized mesh the sample was strained. The filtrate was then poured into a centrifuge tube. The wash sample was centrifuged at a speed of 3000 revolution per minute (rpm) for 5 minutes. After centrifugation, the final supernatant was then discarded and the residue was placed on a clean

glass slide. For each tube, six slides (three direct and three Lugol's iodine smears) were prepared to increase the chance of parasite detection. The slide was then covered with cover slip and examined under the microscope at 100x and 400x objectives of compound microscope for detection and identification of possible parasitic ova, cysts and larvae.

Zinc sulphate floatation technique

For this technique, Zinc sulphate 331 g was dissolved in 900 mL warm distilled water and some additional distilled water was added until the entire solution weighed 1180 g (specific gravity of 1.18). The solution was stirred gently for mixing and then specific gravity was checked by using hydrometer. The residues in the above technique were mixed with zinc sulphate solution. Flotation procedure was used (as revealed previously) for the finding of helminth eggs, larvae and protozoa cysts. The sediments were mixed with zinc sulfate solution and poured into tubes and the solution was left overnight. Then, using a dropper, only the solution from the above the fluid (floatation fluid) was transferred to a clean glass slide. Lugol's iodine stain was used and unstained smears were examined for the parasite stages. For microscopy the mixture was applied onto a freshly clean slide, and a cover slip was gently being placed to evade air froths and overflowing.

The morphological keys currently in use were used to identify any parasite that is found. We used the morphological key reprinted by WHO (2019) with corrections. A dog was considered positive if parasite species was found in the fecal sample.

Data arrangement and analysis

Data regarding pets and stray dog's fecal samples was transferred to excel sheet and was arranged according to locality, host age, seasons and sex. The *p*-value was computed individually for each variable, and a significance level of 0.05 or less was employed to determine statistical significance.

RESULTS

A total of 200 fecal samples from two breeds of dogs (households=76 and stray=124) were examined for the presence of intestinal parasitic infections. Eight helminth species were demonstrated during this study from all dogs. During this study 44 dogs were infected with intestinal parasites showing the overall prevalence of 22%. The most common observed helminths parasites were *Toxocara canis* which were found in the fecal samples of 14 stray dogs and 4 domestic dogs, (n=18 9%). Other observed helminths parasitic species were *Trichuris* species 09 (4.5%), *Dipylidium caninum* 05 (2.5%), *Strongyloides stercoralis* 05 (2.5%), *Capillaria* spp. 04 (2%), *Taenia* spp. 04 (2%), *Ancylostoma caninum,* 03 (1.5%), and *Toxascaris leonina* 01 (0.5%), respectively. The prevalence and intensity of di*erent gastrointestinal parasites in two breeds of dogs is presented in Table I.

Table I. Overall prevalence of gastrointestinal parasites in pets and stray dog's fecal samples collected from district Dir lower, Pakistan.

Parameters	Stray dogs	Pet dogs	Total (%)
Total samples examined	124	76	200
Samples positive	31(25%)	13(17.1%)	44 (22%)
Total samples negative	93 (75%)	63(82.9%)	156 (78%)

Diversity of parasites

In total 200 fecal samples of stray and pet's dogs eight species of intestinal helminths were observed. These eight species included six species of nematodes and two species of cestodes. As trematodes parasitic infection in dogs is relatively uncommon therefore no flukes were found. The six species of nematodes included *Toxocara canis*, *Trichuris* spp., *Strongyloides stercoralis, Capillaria* spp*., Ancylostoma caninum,* and *Toxascaris leonina.* The two species of cestodes were *Dipylidium caninum* and *Taenia* spp. The prevalence of these zoonotic parasites is shown in Table II.

Table II. Parasite species found in fecal samples of pets and stray dogs collected from district Dir Lower, Pakistan.

Parasite	Prevalence in stray dogs	Prevalence in Pet dogs	Overall Prevalence (%)
Toxocara canis	14 (11.3%)	4 (5.26%)	18 (9%)
Trichuris spp.	05 (4.03%)	4 (5.26%)	09 (4.5%)
Dipylidium caninum	05 (4.03%)	0	05 (2.5%)
Strongyloides stercoralis	05 (4.03%)	0	05 (2.5%)
Capillaria spp.	02 (1.61%)	2 (2.63%)	04 (2%)
Taenia spp.	03 (2.41%)	1 (1.31%)	04 (2%)
Ancylostoma caninum	01 (0.80%)	2 (2.63%)	03 (1.5%)
Toxascaris leonina	01 (0.80%)	0	01 (0.5%)
Total parasites found	36	13	49 (24.5%)
Total dogs infected	31	13	44 (22%)
Total examined	124	76	200

Prevalence of parasites: alone or in combination

A total of 49 parasitic infections were detected in 44 dogs out of 200. In fecal samples of 27 stray dogs and 13 household dogs single parasitic infection was found (Table III). The single parasitic infection found in the fecal samples of stray dogs were *Toxocara canis, Trichuris vulpis, Capillaria* spp., *Strongyloides stercoralis, Ancylostoma caninum, Dipylidium caninum* and *Taenia* spp. Similarly, in pet dogs *Toxocara canis, Trichuris vulpis, Capillaria* spp., *Ancylostoma caninum* and *Taenia* spp. were found as monoparasites. In pets dogs no polyparasitism was observed. In fecal samples of 4 stray dog's polyparasitism was observed. In fecal sample of 2 stray dogs *Toxocara canis* and *Trichuris vulpis* were present together. Similarly, *Toxocara canis* was present along with *Toxascaris leonina* in the fecal sample of same stray dog. In the fecal sample of another stray dogs three parasitic species eggs were found *i.e., Toxocara canis, Capillaria* spp and *Trichuris vulpis* (Fig. 2). *Toxocara canis* was also found in the fecal sample of a stray dog (Fig. 3D).

Table III. Prevalence of parasites alone or in combination with pets and stray dogs of district Dir Lower.

Parasite	Species	Stray dogs	Pet dogs	Total
Parasites found alone				
	Toxocara canis,	10	4	14
	Trichuris vulpis	2	4	6
	Capillaria spp. *Strongy-*	1	2	3
	loides stercoralis	5	0	5
	Ancylostoma caninum	1	2	3
	Dipylidium caninum	5	0	5
	Taenia spp.	3	1	4
	Total cases	27	13	40
Parasites found in combinations				
2 species	*Toxocara canis*+ *Trichuris vulpis*	2	0	2
	Toxocara canis+ *Toxascaris leonina*	1	0	1
3 species	*Toxocara canis* + *Capillaria* spp + *Trichuris vulpis*	1	0	1
	Total	4	0	4

Relevance of gastrointestinal parasites to host age, season and sex

Fecal samples from domesticated and stray dogs (*Canis lupus familiaris*) were collected and examined for the presence of parasitic infections. Majority of owners are unaware of the use of anthelminthic drugs. Among 76 pet dogs only one owner gave Ivermectin to his two dogs. In the study area the stray dogs were more infected (25%) with gastrointestinal parasites than pet dogs (17.1%) (Table IV). The high prevalence of parasites in stray dogs is due to poor management and unhygienic habits of stray dogs. The adult dogs were more affected by the parasites than the puppies. The prevalence of gastrointestinal parasites in adult pet dogs were recorded as 18.96% and in puppies it was 11.11%. There is no significant variation in the prevalence of gastrointestinal parasites relative to the sex of host dogs (Table IV).

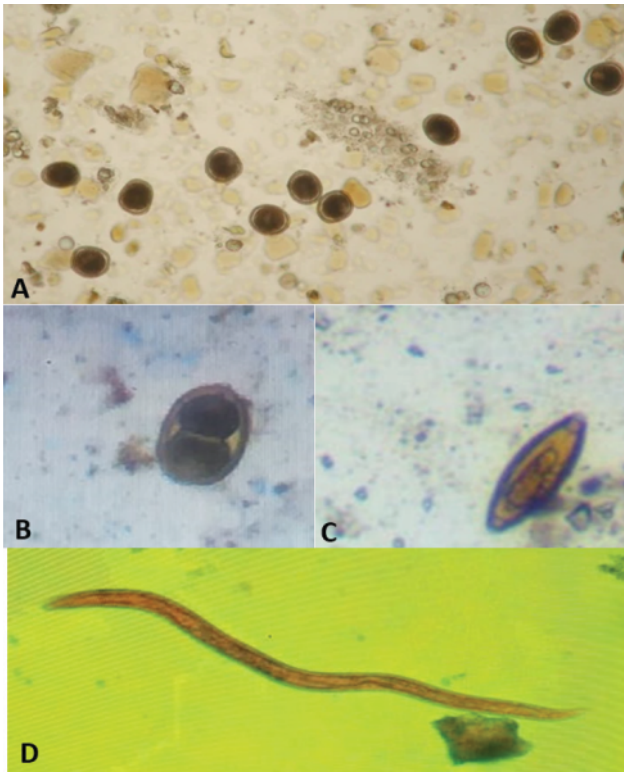

Fig. 2. Gastrointestinal parasitic eggs found in fecal samples of stray and households' dogs: A, *Toxocara canis;* B, *Strongyloides stercoralis;* C, *Trichuris vulpis*; D, *Toxacara canis*.

Table IV. Prevalence of gastrointestinal parasites in domesticated dogs relative to host age and sex.

Category	Number examined	Number infected	%
Domestic dogs	76	13	17.1
Adults	58	11	18.96
Puppies	18	2	11.11
Male	40	7	17.5
Female	36	6	16.66

The prevalence of gastrointestinal parasites shows great variation in different seasons. This study was carried out from July 2023 to March-2024. From July to September the weather was very hot in the study area and the prevalence of parasites was comparatively high in these months (29%). The prevalence was very low during October-March as compared to the previous three months. From this study it was concluded that the humidity, temperature and climatic conditions have great impact on the prevalence of gastrointestinal parasites in stray and pet dogs in district Dir lower (Table V).

Table V. Prevalence of gastrointestinal parasites in household and stray dogs relative to different months.

Months of survey	Number examined		Number infected		Prevalence (%)	
	Pet dogs	Stray dogs	Pet dogs	Stray dogs	Pet dogs	Stray dogs
Jul to Sep 2023	24	55	7	16	29.16	29.09
Oct to Dec 2023	27	40	3	9	11.11	22.50
Jan to Mar 2024	25	29	3	6	12.00	20.68
Total	76	124	13	31	17.10	25

DISCUSSION

This research holds significant value for veterinarians, parasitology researchers, and the general public, offering insights into the prevalence rates of parasites in dogs and aiding in parasite control measures. The findings also hold clinical relevance for human healthcare providers, as there are potentially dangerous zoonotic diseases linked with these parasites, posing a constant threat to human health. Insu*cient awareness about these parasites exacerba tes the risk, as individuals may inadvertently contract and transmit diseases. As data of this sort is limited in Pakistan and there is very little information available on the prevalence of gastrointestinal parasites in dogs with which to compare this data. It's important to note that relying solely on fecal examinations may underestimate parasite prevalence. Studies have demonstrated significant variations when combining fecal examinations with other techniques such as post-mortem examinations or more sensitive diagnostic methods (Barutzki and Schaper, 2011).

Gastrointestinal helminthiasis stands out as the most commonly encountered disease affecting dogs worldwide, including Pakistan. With its diverse climatic zones, ranging from cold alpine to wet tropical regions, Pakistan provides a fertile breeding ground for a diverse range of parasites and pathogens of both medical and veterinary significance. The transmission and distribution of these organisms are largely dictated by geographical, climatic, cultural, and socio-economic variables. Dogs, whether owned or stray, play a pivotal role in the transmission dynamics, representing a potential public health hazard due to their close association with humans. This symbiotic relationship, while offering numerous benefits, also presents a conduit for the transmission of parasitic infections from dogs to humans, either directly or indirectly through environmental avenues. While the specific contribution of each canine population segment to transmission dynamics remains somewhat nebulous, all types of dogs are implicated in this process (Dubná *et al*., 2007).

Questionnaire was used for the owners of pet dogs. The questionnaire contain questions about dogs name, breed, sex, age, approximate weight, number of dogs in home, history of dogs visit to pets clinic, symptoms of gastrointestinal parasites in dogs and treatment measures etc. The results of questionnaire show that in Dir Lower of total 76 pet dog owners no owner lick the face of their dogs. The dogs are not allowed the bedroom of the owners and also dogs have no access to reach the kitchen. Similarly no owner likes to sleep with their pet dogs. Pet's dogs in the study area were mostly stayed indoors. But the dogs defecate on open soil and no litter box was used for defecation. About 60% of the owners remove the feces daily. About 30% owners clean the dog territory twice a week and remaining owners never clean up feces of their dogs. Out of 76 pets dogs only a single owner dewormed his two dogs with Ivermectin. Other owners were mostly unaware from gastrointestinal parasites and deworming. But still the prevalence of gastrointestinal parasites in domesticated dogs is lower than stray dogs which may be due to geographic location and also stray dogs are roaming freely which have more chances of parasitic infection. In the present study the overall prevalence of helminths in both stray and household dogs was 22%. Similar finding was also obtained in various studies conducted in di*erent countries of the world including Pakistan which range from 4.44% to 100% (Swai *et al*., 2010).

Khan *et al*. (2019) studied the prevalence of gastrointestinal parasites in stray and domesticated dogs of district Dir Lower and reported prevalence of gastrointestinal parasites as 26.8%. The most detected eggs were of *D. caninum* (11.8%), followed by *Toxocara canis* (10.5%), *Taenia* spp. (6.57%), *Ancylostoma caninum* (3.94%), *Capillaria* spp. and *Trichuris vulpis* (1.31% each) . The wandering dogs were infected (34.4%) than domesticated ones (16.1%). This study supports our data that after 5 years the situation is still unchanged.

The prevalence of gastrointestinal helminth parasites among dogs in this study (22%) was notably lower as compared to a previous study conducted by Olufemi and

Bobade (1979). over five decades ago, which reported a prevalence of 77.4% among dogs from veterinary clinics at the University of Ibadan and Oyo State Veterinary Clinic. Similar results were obtained by Onyenwe and Ikpegbu (2004), who reported a prevalence of 39.2% among 148 dogs presented to the University of Nigeria Veterinary Teaching Hospital (UNVTH), Nsukka Nigeria. This study also shows lower rate of prevalence than study conducted by Ramirez *et al*. (2004) who present 614 dogs to the veterinary clinic of the University of Zulia, Venezuela and obtained prevalence of 35.5%.

In neighboring Iran Beiromvand *et al*. (2013) conducted research on the prevalence of zoonotic intestinal parasites in domestic and stray dogs in the Chenaran County, Razavi Khorasan Province. From November 2009 to January 2010 they carried out random sampling from 17 villages and obtained the prevalence rate of 66%. This study showed much higher prevalence than our reported results.

In this study interestingly the prevalence of gastrointestinal parasites in pets and stray dogs is lower than some studies conducted in developed countries where the dogs are treated with special care and anti-parasitic programs are regularly carried out to deworm the dogs.

In this study the most common observed helminths parasites were *Toxocara canis* which are found in the fecal samples of 14 stray dogs and 4 household dogs. This prevalence of *T.canis* is less than study conducted by Schwartz *et al*. (2022) who studied 306 fecal samples in which 147 (48%) samples were positive for *T. canis* and reported prevalence of *T.canis* as 16.35%. Conde *et al*. (2022) reported 64.44% prevalence of *T. canis* in dogs and their environmental contamination in Baybay city, Leyte, Philippines. The prevalence of *T. canis* in this study was lower in Lahore Pakistan.

So all the above reports present higher prevalence from the present study. The observed disparities can be ascribed to differences in sampling size and type, geographic location, the rate of parasite infection of hosts in each area and sampling methodologies. Various laboratory techniques could also be involved in a role in retrieving different parasites since some procedures can either float or sediment the parasites. But still there are some studies in which the prevalence of *Toxocara canis* is less than the current study. Nijsse *et al*. (2015) reported 4.6% prevalence of *T.canis* in pet dogs. Another study was conducted in Northern Belgium by Claerebout *et al*. (2009) who tested 1159 fresh fecal samples and report prevalence of 4.4% for *T. canis.*

CONCLUSION

The current study aimed to assess the prevalence of intestinal parasites in dogs in district Dir Lower, Khyber Pakhtunkhwa Pakistan. Parasitic infections are still problem in the study area. Stray and domesticated dogs, are infected with different types of intestinal parasites that may root numerous common diseases to human being. The study highlights the critical need to educate and inform local communities about the significant zoonotic risks posed by parasites. Additionally, the research aims to assess the extent of environmental contamination by dogs and the associated risks to human health.

DECLARATIONS

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Ethical statement and IRB approval

Approval for collection of dogs faeces was obtained from dogs owners & Qurtuba University IRB and Ethical board.

Generative AI or AI-assisted technology statement

The data presented in this article are original and research work performs solely by the authors, without the use of AI or automated data generation tools.

Statement of conflict of interest

The authors have declared no conflict of interest.

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Review Article

Fundamentals of Fish Farming: Overview of Key Considerations for Fish Culture

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ABSTRACT

In aquaculture, understanding the specific feed requirements of fish at various developmental stages and the appropriate feeding frequency is essential for successful production. Feeding systems in aquaculture are generally categorized based on feed type and availability into extensive (no feed provided), semi-intensive, and intensive production methods. Fertilization and hatching of fish eggs and milt, whether from cultured or wild sources, can achieve success rates of up to 97%. However, the survival and growth of fry and larvae heavily depend on appropriate feeding strategies, as improper feeding can result in significant losses. Fish feeding behavior and e*ciency are influenced by a range of factors classified as external and internal stressors. External factors include changes in water quality, feed quality, and environmental disturbances, while internal factors relate to the physiological status of fish, such as reproductive cycles. A thorough understanding of fish behavior, ecology (the interaction between fish and their environment), nutritional needs, energy metabolism, and feed characteristics (type and particle size) is vital for optimizing aquaculture outcomes. Feed utilization in fish metabolism is a complex but critical process. Metabolic activities begin with the ingestion of feed, supplying energy necessary for maintaining vital functions, compensating for energy loss through excretion and wear, and supporting growth. Metabolism concludes with the excretion of nitrogenous wastes via urine and feces. Metabolic rates vary with activity: basal metabolism during rest, routine metabolism during regular swimming and foraging, and active metabolism during intense physical exertion. For optimal growth, fish should be maintained in low-stress environments with optimal water conditions and high-quality feed formulations that promote e*cient feed conversion and weight gain. In conclusion, sustainable aquaculture relies on the integration of optimal nutrition, environmental stewardship, species-specific practices, and continued technological advancement. This review summarizes essential considerations in fish farming, focusing on feeding strategies, metabolism, and environmental management, supported by a broad literature base. It presents practical recommendations for improving growth performance, feed e*ciency, and environmental sustainability.

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INTRODUCTION

Aquaculture plays a vital role in the global economy by providing employment opportunities, generating income, and contributing significantly to food security. With ongoing investments, technological advancements, and the adoption of sustainable practices, the aquaculture sector is expected to experience substantial growth in the coming years (Yanik, 2022). Fish farming, a major component of aquaculture, has become a pivotal industry in global food production, supplying an increasing share of the world's seafood to meet rising consumer demand (FAO, 2022). As wild fisheries face pressures from overfishing and environmental degradation, aquaculture emerges as a sustainable alternative capable of supporting food security and fostering the economic development of coastal and rural communities (Pradeepkiran, 2019). However, achieving long-term success and environmental sustainability in fish farming requires the optimization of biological, technological, and environmental management factors.

Despite its potential, fish farming is confronted by environmental challenges such as pollution, overuse of antibiotics, and disease outbreaks (Arshad *et al*., 2024). These issues have stimulated growing interest in sustainable aquaculture approaches, including integrated multi-trophic aquaculture (IMTA) systems and the development of novel feeds such as plant-based or synthetic alternatives to reduce dependence on fishmeal and fish oil (John and Okuthe, 2024).

In regions with favorable geographical conditions and abundant water resources, there is an urgent need to intensify support for small-scale rural fish farming.

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Sustainable production in these areas depends on establishing standardized guidelines that are adaptable regardless of water characteristics, fish species, or regional differences. The successful integration of fish farming into rural economies hinges on developing a critical mass of well-trained fish farmers who can provide expertise and services to smaller producers, thereby ensuring sustainability without ongoing institutional or governmental intervention. Implementing minimum acceptable standards across all regions could significantly increase production levels. Moreover, simplifying and clarifying the concept of fish farming is essential for improving public acceptance. Prior to making financial investments in aquaculture, presenting a clear, straightforward mission to all stakeholders is crucial (Yanik, 2022; Shankar and Yanik, 2024).

Key factors in effective fish farming include water quality management, efficient feeding regimes, selective breeding programs, disease control, and waste management. Maintaining optimal water parameters such as temperature, salinity, and dissolved oxygen is fundamental to ensuring healthy fish stocks (Jana and Sarkar, 2005). Advances in nutrition and the formulation of specialized feeds have contributed to improved growth rates and enhanced disease resistance in cultured species (Rombenso *et al*., 2022). Furthermore, genetic improvement programs focusing on breeding for disease resistance and productivity have become widely adopted, promoting more resilient and sustainable aquaculture practices (Gjedrem and Baranski, 2009).

This overview aims to discuss essential considerations in fish farming, emphasizing the scientific principles that underpin sustainable practices and address current challenges within the sector.

ESSENTIAL CONSIDERATIONS OF FISH FARMING

Both cultured and wild fish eggs and milt can achieve hatching success rates of up to 97%. However, improper feeding of fry and larvae may result in the loss of the entire batch, highlighting the critical importance of understanding the feeding process in fish culture. A thorough knowledge of key factors such as fish behavior, nutrient requirements, energy metabolism, and the type and size of feed is essential for successful aquaculture (Volkoff, 2016).

The coordinated act of food searching and ingestion involves complex interactions between the nervous and endocrine systems (Kuz'mina, 2019). Control of feeding behavior is multifaceted, involving communication between central brain structures and peripheral signals (Volkoff *et al*., 2009). Metabolic sensors in the central nervous system enable the hypothalamus to monitor the nutritional status of fish, providing qualitative regulation of food intake (Conde-Sieira *et al*., 2010). Neural effectors from the hypothalamus integrate hunger and satiety signals to regulate the amount of food consumed (Lin *et al*., 2000). Hunger signals include peptides such as apelin and neuropeptide Y (Aldegunde and Mancebo, 2006), whereas satiety signals involve amylin and the cocaine- and amphetamine-regulated transcript (Thavanathan and Volkoff, 2006; Volkoff and Peter, 2001). Additionally, tachykinins may exert an anorectic effect in fish. The hypothalamic region of the brain is linked to the control of food intake, although areas beyond the hypothalamus also play a role in this process (Lin *et al*., 2000). Understanding these regulatory mechanisms is particularly relevant to fisheries and aquaculture, as manipulation of feeding behavior and endocrine responses can optimize fish growth and reproduction.

The nutritional requirements of fish vary depending on species, size, age, reproductive status, and environmental conditions. Nonetheless, all fish require essential nutrients, including proteins, lipids (fats), carbohydrates, vitamins, minerals, essential amino acids, dietary fiber, antioxidants, and essential fatty acids. Balancing these nutrients in the diet is crucial to support optimal growth, health, and reproductive success. Commercial fish feeds are formulated to provide a balanced nutrient profile tailored to the specific needs of different species and life stages.

Photoperiod and light regimes also influence biological functions in fish (Biswas and Takeuchi, 2003). Studies across various species have demonstrated that the duration of light exposure can significantly affect feeding behavior, growth, and survival rates (Nwosu and Holzlöhner, 2000). Additionally, photoperiod plays a key role in regulating the overall well-being and daily rhythms of fish (Adewolu *et al*., 2008).

Proper feeding practices are essential for fish welfare. Since dietary needs differ between species, understanding the specific nutritional requirements of the cultured fish is vital. In aquaculture, the choice of feed type and size depends on factors such as species, life stage, nutritional demands, feeding behavior, and production objectives. Selecting appropriate feed types and sizes is critical to maximize feed conversion efficiency, growth performance, and profitability. Moreover, formulated feeds must meet the nutritional needs of the target species while being managed carefully to minimize waste and reduce environmental impacts. Feed type and particle size, determined by ingredient origin and processing method are crucial to feed acceptance and utilization. Feeds are classified by moisture content (wet, moist, dry) and processing technique (compressed, expanded, extruded

pellets). Physical characteristics such as pellet density, size, shape, color, and texture influence feeding efficiency.

Research shows optimal pellet size correlates with approximately 25–50% of fish mouth width (Wankowski and Thorpe, 1979; Tabachek, 1988; Tucker, 1998). Larger pellets attract quicker responses but may increase handling time, as seen in Arctic charr, where intermediate-sized pellets minimized rejection and handling time (Linnér and Brännäs, 1994).

KNOWLEDGE OF FEEDING BEHAVIOR OF FISH FOR EFFECTIVE MANAGEMENT STRATEGIES

Feeding behavior

The feeding behavior of fish is governed by the interpretation of both external and internal environmental signals via receptors and signaling molecules. This behavior is influenced by various internal and external stressors, such as changes in water and food quality, reproductive status, photoperiod, temperature, water flow, weather conditions, salinity, oxygen levels, CO_2 concentration, ammonia levels, pH, food availability, composition, and social interactions. The hypothalamus plays a central role in this regulatory process, alongside other brain regions (Volkoff, 2016; Kuz'mina, 2019; Assan *et al*., 2021). Feeding acts as one of the most influential external factors that stimulate growth and modulate feeding behavior (Conde-Sieira *et al*., 2017). The availability and composition of food significantly impact these processes by affecting hormone levels that regulate appetite and metabolism (Bertucci *et al*., 2019). Even a single meal can trigger fluctuations in the expression and secretion of appetite-regulating hormones in both the brain and peripheral tissues (Wall and Volkoff, 2013).

A comprehensive understanding of fish feeding behavior is essential for aquaculturists, anglers, ecologists, and fisheries managers. Such knowledge provides valuable insights into dietary preferences, habitat utilization, trophic interactions, and overall ecosystem dynamics. By studying feeding behavior, researchers can better understand the ecological roles of different fish species and develop effective strategies for their conservation and sustainable management.

Fish feeding behavior exhibits significant diversity across species, habitats, and environmental conditions. Common feeding strategies include carnivory, herbivory, and omnivory, alongside variations in feeding frequency, feeding zones, social and territorial behaviors, selective feeding, and adaptive learning processes. Extensive research on feeding behavior in both wild and cultured populations has enhanced our ecological understanding of these processes (Houlihan *et al*., 2001; Gerking, 2014).

Fish responses to feeding stimuli are influenced by factors such as feeding techniques, habits, regularity, feed detection mechanisms, and preferences (Lall and Tibbetts, 2009). Changes in dietary behavior and appetite often correspond to alterations in gene expression and protein levels of appetite-regulating hormones or their receptors. Such shifts in mRNA and protein expression, triggered by starvation or feeding, illuminate the physiological roles of these hormones in regulating feed intake. However, feeding regulation is multifactorial; compensatory mechanisms may modulate behavior, and fluctuations in hormone levels do not always directly correlate with changes in feed consumption (Volkoff *et al*., 2010).

PROVISION OF OPTIMAL LIVING ENVIRONMENT

Providing an optimal living environment is crucial for fish health and well-being, whether in captivity or the wild. Key factors influencing this environment include water quality, temperature, stocking density, habitat complexity (decorations and hiding places), lighting, diet, species compatibility, water movement, and maintenance practices. Proper management of these variables significantly contributes to fish health and longevity.

Water quality plays a pivotal role in fish growth and development, with chemical parameters such as hardness, pH, oxygen concentration, and salinity, as well as physical characteristics like temperature and color, requiring regular monitoring. For example, cold-water species such as salmon thrive at 14–16°C, while warm-water species like channel catfish prefer approximately 30°C (Jiang *et al*., 2021). Deviations from these optimal temperatures can increase nutrient demands without yielding growth benefits, as temperature directly influences metabolic processes essential to health and development.

Lighting and photoperiod regimes also profoundly affect fish biology. Species-specific requirements vary with developmental stages, and manipulating photoperiod can enhance aquaculture productivity and sustainability (Nwosu and Holzlöhner, 2000). Studies on species such as *Clarias gariepinus* demonstrated that 24 h of darkness yielded the highest feed intake and feed conversion ratios (Adewolu *et al*., 2008). Similar findings with *Lophiosilurus alexandri* and *Diplodus puntazzo* reinforce the critical role of photoperiod in regulating feeding behavior and growth (Kitagawa *et al*., 2015; Vera *et al*., 2006). These findings underscore the necessity of further research into photoperiod effects across diverse species.

Fish exhibit species-specific feeding times aligned with circadian rhythms; some feed diurnally (e.g., Atlantic

salmon, redbelly tilapia, rohu, common carp), while others are nocturnal feeders (e.g., European catfish, zebrafish) (Isorna *et al*., 2017; Boujard, 1995; del Pozo *et al*., 2011). Feeding schedules aligned with natural rhythms improve feed efficiency and growth, as demonstrated in rainbow trout and goldfish (Noeske and Spieler, 1984; Gelineu *et al*., 1998).

The processes of starvation, craving, and satiation describe the physiological states governing food intake. Starvation elicits active searching and hunger behaviors, whereas satiation signals fullness after feeding. Appetite or craving is influenced by sensory stimuli such as food appearance, odor, and taste (Hoskins and Volkoff, 2012). Environmental factors, food availability, and fish physiological status modulate these behaviors.

NUTRIENT REQUIREMENTS OF FISH

Fish growth depends on the quantity and quality of feed consumed and efficiently metabolized. Maintaining optimal environmental conditions including water quality and temperature is fundamental for maximizing growth potential. A balanced diet tailored to species-specific nutrient needs is essential.

Nutrient requirements vary significantly among species, ages, sizes, and environmental contexts. Protein is the most critical nutrient supporting growth and physiological functions such as immunity and reproduction. However, excessive feeding of high-energy diets can cause undesirable fat deposition, compromising fish health and marketability. Thus, promoting lean, protein-rich muscle development is paramount. The majority of herbivorous and omnivorous fish studied thus far necessitate a diet containing 25 to 35 percent crude protein; in contrast, carnivorous species may need 40 to 50 % crude protein (Wilson, 2003).

Proteins are the major organic material in fish tissue, making up about 65–75% of the total on a dry-weight basis. Fish consume protein to obtain amino acids. A regular intake of protein or amino acids is required because amino acids are used continually by the fish, either to build new proteins (as during growth and reproduction) or to replace existing proteins (maintenance) (Wilson, 2003). Fish primarily use protein as an energy source, though fats and carbohydrates also contribute according to their caloric content. Vitamins and minerals are indispensable micronutrients regulating metabolism, skeletal integrity, and physiological processes. Deficiencies in any essential nutrient can limit growth, illustrating the law of the minimum, whereby a single nutrient shortage restricts overall performance. The protein needs for various fish species vary between 28 and 56 percent of their dry diets. It appears that carnivorous species from both marine and freshwater environments necessitate 40-55 percent of dietary protein, whereas the majority of freshwater omnivorous and herbivorous species require 30-40% of their dry diet to consist of protein. Similar to finfish, most crustaceans examined thus far exhibit relatively high protein requirements, ranging from 30 to 60 % of the dry diet (Hasan, 2001).

In summary, aquaculture success hinges not only on feed quantity but also on feed quality and composition, tailored to the nutritional requirements of target species. Properly balanced diets optimize growth, health, and fish product quality.

UNDERSTANDING METABOLISM OF FISH

Understanding fish metabolism is vital for optimizing feeding regimens, ensuring fish welfare, and promoting sustainable aquaculture. Fish metabolism encompasses the biochemical processes converting feed into energy and essential compounds for growth, maintenance, and reproduction. Influential factors include water quality, diet composition, feeding frequency, and environmental conditions.

In intensive aquaculture, fish are typically fed high-protein, energy-dense diets in controlled environments. Feed utilization efficiency is influenced by temperature, physiological condition, stress, and water parameters (pollution, pH, oxygen). Metabolic processes initiate with feed consumption, providing energy for basal maintenance, activity, growth, and culminate in excretion of nitrogenous waste via urine and feces.

Metabolic rate varies with activity level: basal metabolism during rest, routine metabolism during swimming and feeding, and active metabolism during heightened activity (Treberg *et al*., 2026). Energy use is quantified through measurements such as digestible energy (feed energy minus fecal energy), metabolic energy (digestible energy minus endogenous nitrogen energy), gross energy, and metabolizable energy, using methods like bomb calorimetry and respirometry (Dickson and Kramer, 1971). The energy consumed through food incurs energetic expenses known as specific dynamic action (SDA), and a portion of this energy will be lost by the animal through egestion (indigestible materials and unassimilated carbon) or nitrogenous excretion (Fig. 1). The remaining energy is utilized to cover the costs of living, which include basal expenses such as the maintenance of ion gradients and the repair of proteins and DNA. Any energy that exceeds these basal requirements is directed towards growth and storage, locomotion, physical labor, or reproduction, which may manifest as the production of gametes or be retained as gonadal investment (Treberg *et al*., 2016).

Fish size, temperature, oxygen concentration, salinity, activity, food intake, and fasting affect metabolism. Being ectothermic, fish do not regulate body temperature internally; thus, external temperature significantly influences metabolic rates. Smaller fish have higher metabolic activity relative to larger individuals, with metabolic rates decreasing approximately 90% from 10 mg fry to 1 kg adults.

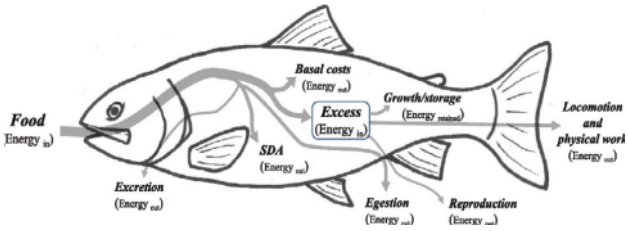

Fig. 1. Energy budget in a fish (Treberg *et al*., 2016).

Optimal growth temperatures vary by species, with salmonids favoring 14–16°C. Metabolic scope, defined as the difference between active and standard metabolic rates, reflects oxygen consumption during activity. Studies on salmon and rainbow trout illustrate how metabolic rates correspond with swimming velocity and feeding state (Brett, 1965; Albrecht, 1974).

CHALLENGES

The aquaculture industry faces significant challenges due to the limited availability of fish meal and fish oil, resources increasingly controlled by a small number of entities. Although alternative sources of amino acids are abundant, they often exhibit imbalanced nutritional profiles and lack desirable flavor characteristics. Furthermore, reliance on alternative proteins may adversely affect the productivity and resilience of farmed fish. The supply of essential fatty acids is also constrained; substitution with alternative lipids can reduce the nutritional quality of fish fillets and impair reproductive performance, posing additional challenges for sustainable aquaculture development. Major challenges include limited fishmeal and oil supply, imbalances in alternative protein sources, and reduced fillet quality or reproductive performance from suboptimal lipid sources (Paspatis *et al*., 1999; John and Okuthe, 2024).

CONCLUSIONS AND RECOMMENDATIONS

Aquaculture is currently the fastest-growing food production sector worldwide, driven by the expansion of both intensive and semi-intensive farming systems. Recent advancements have concentrated on sustainability, genetic improvement, alternative feed development, traceability, certification, and technological innovation all aiming to optimize fish production and ensure sector resilience.

The future of aquaculture is promising, with its capacity to provide a reliable protein source for a growing global population. To meet increasing demand, however, the industry must enhance production efficiency, minimize environmental impacts, and improve resource use. Key trends expected to shape the sector include continued technological innovation, heightened emphasis on sustainability, and increased attention to social and environmental responsibility.

Moreover, external influences such as rising energy costs, evolving government regulations, societal acceptance, and climate change will significantly affect aquaculture's trajectory over the next fifty years. Both domestic policies and international market forces are propelling global aquaculture growth, underscoring its critical role in feeding future generations.

DECLARATIONS

Generative AI and AI-assisted technology statement

The authors have declared that no generative AI or AI-assisted technologies were used to create this manuscript.

Statement of conflict of interest

The author has declared no conflict of interest.

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Evaluation of Synthetic Drugs vs. Plant Extracts as Sustainable Antibacterial Agents against Milk-Borne Pathogens: A Greener Approach to Antibacterial Activity

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ABSTRACT

Milk, rich in proteins, vitamins, and minerals like calcium, is a key nutritional source but prone to contamination by pathogens, especially in regions lacking robust pasteurization. Multidrug-resistant bacteria, driven by antibiotic overuse, heighten health risks. This study evaluates *Azadirachta indica* (Neem) and *Avicennia marina* (Mangrove) extracts against milk-borne pathogens *Staphylococcus aureus*, *Escherichia coli*, and *Enterobacter aerogenes* to address antimicrobial resistance (AMR). Extraction from 50 g dried plant material using methanol, chloroform, and hexane yielded 2.56–8.74%, with methanol extracts most e*cient (8.74% for *A. indica*, 4.38% for *A. marina*). Using the disc di*usion method, *A. indica* methanol extract (M1) showed superior antibacterial activity, with zones of inhibition of 38 mm (*S. aureus*) and 35 mm (*E. aerogenes*) at 400 mg/mL, outperforming ciprofloxacin (7 mm) and levofloxacin (14 mm) at 500 mg/ml. Minimum inhibitory concentrations (MICs) confirmed M1's potency (12.5 mg/ml for *S. aureus*, 25.0 mg/mL for *E. aerogenes*), linked to azadirachtin and flavonoids. *A. marina* chloroform extract (C2) was e*ective against S. aureus (MIC: 25.0 mg/ml) but less so at higher concentrati ons, possibly due to phytochemical antagonism. Both plants showed minimal activity against MDR *E. coli*, indicating Gram-negative resistance. Methanol's e*cacy in extracting bioactive compounds supports its use. These results advocate *A. indica* and *A. marina* extracts as sustainable, multi-targ*et al*ternatives to combat antimicrobial resistance in dairy safety.

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Key words
***Azadirachta indica*, *Avicennia marina*, milk-borne pathogens, antibacterial activity, antimicrobial resistance**

INTRODUCTION

Milk, a staple in human nutrition due to its rich composition of proteins, lipids, vitamins, and minerals, is susceptible to contamination by pathogenic microorganisms during production, handling, and storage, thereby posing substantial risks to public health (Oliver *et al*., 2005). Milk-borne pathogens, such as *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, and *Listeria monocytogenes*, are frequently implicated in foodborne outbreaks, with raw or unpasteurized milk serving as a primary reservoir for these bacteria (Claeys *et al*., 2013). The isolation and identification of such microorganisms from milk samples are essential steps in epidemiological surveillance and quality control, typically involving culture-based techniques, biochemical assays, and molecular methods like polymerase chain reaction (PCR) to detect virulence and resistance determinants (Amagliani *et al*., 2012).

The escalating challenge of antimicrobial resistance (AMR) among milk-associated pathogens exacerbates the public health threat, as evidenced by the detection of resistance genes conferring tolerance to beta-lactams, aminoglycosides, and tetracyclines in retail milk products (Verraes *et al*., 2014). This resistance is often linked to the overuse of synthetic antibiotics in dairy farming for mastitis treatment and growth promotion, leading to the selection and dissemination of multidrug-resistant strains through the food chain (Oliver *et al*., 2011). Consequently, conventional synthetic drugs, while historically e*ective, are increasingly inadequate, necessitating the exploration of alternative antimicrobial agents that minimize ecological impact and resistance development.

Plant extracts, derived from medicinal herbs rich

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in phytochemicals such as alkaloids, flavonoids, and terpenoids, represent a greener paradigm in antibacterial therapy, exhibiting broad-spectrum activity against bacterial pathogens through mechanisms including cell membrane disruption, efflux pump inhibition, and biofilm interference (Cowan, 1999; Savoia, 2012). *Azadirachta indica* (neem), thriving in arid and saline environments, contains potent phytochemicals like azadirachtin, nimbin, and quercetin, which exhibit antibacterial, antifungal, and anti-inflammatory properties, attributed to their ability to disrupt bacterial cell walls and inhibit protein synthesis (Biswas *et al*., 2002; Alzohairy, 2016). Similarly, *Avicennia marina* (grey mangrove), adapted to harsh coastal and saline conditions, produces tannins, flavonoids, and triterpenoids, which confer antimicrobial activity by altering membrane permeability and inhibiting enzymatic functions (Nayak *et al*., 2014). The resilience of both plants to extreme conditions enhances their phytochemical diversity, making them ideal candidates for extracting robust antimicrobial compounds.

Comparative evaluations have demonstrated that these natural compounds can rival or surpass synthetic antibiotics in efficacy, particularly against resistant isolates, while offering advantages in sustainability, reduced toxicity, and lower propensity for inducing resistance (Ncube *et al*., 2008; Gyawali and Ibrahim, 2014). For instance, extracts from plants like *Aloe vera*, *Curcuma longa*, and *Ocimum basilicum* have shown potent inhibitory effects on food spoilage and pathogenic bacteria, positioning them as viable alternatives in food preservation and therapeutic applications (Mostafa *et al*., 2018).

The present study addresses this gap by a comparative assessment of synthetic antibiotics versus selected plant extracts against bacterial strain isolated from milk sample. This approach not only elucidates the microbial burden in milk but also advocates for eco-friendly strategies to combat AMR, aligning with global efforts toward sustainable antimicrobial stewardship.

MATERIALS AND METHODS

Plants extraction preparation

Plant material of two plant species included in this study was collected. *A. indica* (neem) leaves were collected from the premises of Jinnah University for Women, Karachi, Pakistan and *Avicennia marina* (grey mangrove) leaves were collected from sandpit backwaters. Plant samples were washed, sterilized, rinsed with distilled water, and shade-dried. Dried material was ground into fine powder and sieved through a 100-mesh screen for extraction. Three different solvents (methanol, hexane and chloroform) were used for extraction separately for each plant. 50g of fine powder was soaked in 200 ml methanol, hexane and chloroform separately with stirring 24 h, filter through whatman filter paper no. 41 to obtain filtrate. The residues were re-extracted with same solvents separately for 24 h. Combine both the filtrates of same solvents and evaporated to dry under reduced pressure using rotatory evaporator. Total three extracts were prepared from *A. indica* (neem) leaves, including methanol extract (M1), hexane extract (H1) and chloroform extract (C1) while another three extracts were prepared from *Avicennia marina* (grey mangrove) leaves, methanol extract (M2), hexane extract (H2) and chloroform extract (C2). Extract yields were weighed, stored in clean glass vials at 5°C, and percentage yields calculated using the formula: extract yield % (R/S) × 100, where R is the weight of extracted plant residue and S is the weight of the raw plant sample.

Bacterial strains

The antimicrobial efficacy of each plant extract was assessed against three bacterial strains associated with foodborne illnesses. Three bacterial strains were used for antibacterial activity one strain of Gram positive MRSA (*Staphylococcus aureus* JUW-IB2323) and two strains Gram negative (*Enterobacter aerogenes* (JUW-IB2442)) and *Escherichia coli* (JUW-IB2552) clinical isolates obtained from cow milk at the Department of Zoology, Jinnah University for Women.

Disc diBusion assay

Initial antibacterial activity was screened using the disc diffusion method. Different concentrations (200mg/ml, 300mg/ml and 400mg/ml) of both plant extracts were prepared in 10% DMSO. The bacterial colonies were dissolved in normal saline, and suspensions were standardized to a 0.5 McFarland turbidity, equivalent to ~1.5 × 10^8 CFU/ml (Macfarland standards: 9.95 ml of 10% H_2SO_4 in destilled water +0.05 ml of 1 % $BaCl_2$ in distilled water) and spread on Mueller-Hinton agar plates. Sterile Whatman No. 1 filter paper discs were soaked with 10 %:l of varying plant extract concentrations,10% DMSO (solvent control), and placed on inoculated plates with ciprofloxacin (500 mg/ml) of (10 %:l) used as the positive control for the above Gram negative bacteria and levofloxinoxin 500mg/ml of (10 µl) as the positive control for Gram positive MRSA pathogenic bacteria. The plates were incubated at 35°C ± 2°C for 24 h. Inhibition zone diameters were measured with a vernier caliper to assess the antibacterial activity of the extracts.

Determination of minimum inhibitory concentration (MICs) of the effective plant extract

The MIC of *A. indica* methanol (M1) and chloroform

(C1) extracts, effective against *Staphylococcus aureus* and *Enterobacter aerogenes*, was evaluated using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) protocols (CLSI, 2018). Overnight cultures in Mueller-Hinton broth (MHB) were adjusted to a 0.5 McFarland standard (~1.5 × 10^8 CFU/ml) at 630 nm and diluted to ~1.5 × 10^6 CFU/ml. Stock solutions of M1 and C1 (400 mg/ml in 10% DMSO) were serially diluted two-fold in MHB within 96-well microtiter plates (400–0.781 mg/ml). Each well contained 100 µl extract and 100 %:l inoculum, yielding final concentrations of 200–0.391 mg/ml. Positive (bacteria in MHB), negative (MHB alone), and solvent (10% DMSO) controls were included, with ciprofloxacin (500 mg/ml) as the reference. Plates were incubated at 37°C for 24 h. MIC was the lowest concentration preventing visible turbidity. Non-turbid wells were subcultured (10 µl) on Mueller-Hinton agar to determine the minimum bactericidal concentration (MBC), defined as no colony growth after 24 hours at 37°C. Experiments were conducted in triplicate, with MIC values reported as the mean.

RESULTS AND DISCUSSION

Plant extract yield

The ethnobotanical data of *Azadirachta indica* (neem) and *A. marina* (mangrove) using three solvents (methanol, chloroform, and hexane) are presented in Table I. Extraction was performed using 50g of dried plant material for each plant, with yields calculated as the percentage dried extract residue. The extract yields ranged from 2.56 to 8.74% across the solvents. For *A. indica*, methanol extraction yielded the highest residue (8.74%), followed by chloroform and hexane. For *A. marina*, methanol extraction also produced the highest yield (4.38%), followed by chloroform and hexane extract, respectively. Yields are reported as mean ± standard deviation from triplicate extractions. Methanol consistently provided the highest extraction efficiency for both plants, likely due to its polarity, which effectively solubilizes bioactive compounds such as flavonoids and alkaloids (Sultana *et al*., 2007).

Antibacterial assays

The antibacterial efficacy of organic extracts from *A. indica* (neem) and *Avicennia marina* (grey mangrove) was evaluated using the disc diffusion method, a standard technique for assessing zone of inhibition (ZOI) as an indicator of antimicrobial potency. Extracts were prepared in methanol (M1 for *A. indica*, M2 for *A. marina*), chloroform (C1 and C2), and hexane (H1 and H2) at concentrations of 200, 300, and 400 mg/mL, and tested against *Enterobacter aerogenes*, *Escherichia coli*, and *Staphylococcus aureus*. Standard antibiotics (ciprofloxacin [S] and levofloxacin [L] at 500 mg/mL) and DMSO (control) were included for comparison. Data are presented in Tables II, III, IV, with ZOI values reported as mean ± standard deviation (mm).

Table I. Extract yields (%) leaves of selected plant species.

Plant species	Extraction solvent	Extract yield (%)
Azadirachta indica	Methanol (M1)	8.74
	Hexane (H1)	3.54
	Chloroform (C1)	4.26
Avicennia marina	Methanol (M2)	4.38
	Hexane (H2)	2.56
	Chloroform (C2)	3.75

Table II. Antibacterial activity of *A. indica* and *A. marina* against *Enterobacter aerogenes*.

Plant species	Extracts	Zone of inhibition (mm) at concentration		
		200 mg/ml	300 mg/ml	400 mg/ml
Azadirachta indica (Neem tree)	M1	16± 0.1	25± 0.04	35± 0.2
	C1	07± 0.2	2± 0.5	25± 0.2
	H1	1± 0.3	14± 0.03	21± 0.2
Avicenia marina (Mangrove plant)	M2	03± 0.01	04± 0.2	05± 0.2
	C2	04± 0.04	02± 0.3	04± 0.2
	H2	02± 0.3	03± 0.3	05± 0.1
Standard	S 500mg/ml	16± 0.5		
	L 500mg/ml	06± 0.1		
DMSO	Control	0		

S, Ciprofloxacin; L, Levofloxacin.

The methanol extract of *A. indica* (M1) exhibited significant concentration dependent inhibitory activity against *E. aerogenes* and *S. aureus*, with ZOI increasing concentration-dependently (16–35 mm for *E. aerogenes*; 26–38 mm for *S. aureus*), surpassing the standard drugs at 500mg/ml concentrations. *A. indica* methanol extract (M1) exhibited the lowest MIC (12.5 mg/ml) and MBC (25.0 mg/ml) against *S. aureus*, indicating superior potency, likely due to bioactive compounds like azadirachtin and flavonoids (Biswas *et al*., 2002). For *E. aerogenes*, M1 showed an MIC of 25.0 mg/mL and MBC of 50.0 mg/ml (Table V). This aligns with studies demonstrating that *A. indica* contains bioactive compounds such as azadirachtin, nimbin, and flavonoids, which disrupt bacterial cell

membranes and inhibit enzymatic activity (Biswas *et al*., 2002; Alzohairy, 2016).

Table III. Antibacterial activity of *A. indica* and *A. marina* against *Escherichia coli.*

Plant species	Extracts	Zone of inhibition (mm) at concentration		
		200 mg/ml	300 mg/ml	400 mg/ml
Azadirachta indica (neem tree)	M1	01± 0.2	01± 0.02	02± 0.1
	C1	00± 0.3	00± 0.02	01± 0.1
	H1	00± 0.1	00± 0.2	01± 0.1
Avicenia marina (mangrove plant)	M2	00± 0.00	01± 0.1	00± 0.0
	C2	01± 0.2	01± 0.1	01± 0.1
	H2	00± 0.02	01± 0.2	01± 0.2
Standard	S 500mg/ml	05± 0.4		
	L 500mg/ml	17± 0.1		
DMSO	Control	0		

S, Ciprofloxacin; L, Levofloxacin.

Table IV. Antibacterial activity of *A. indica* and *A. marina* against *Staphylococcus aureus.*

Plant species	Extracts	Zone of inhibition (mm) at concentration		
		200 mg/ml	300 mg/ml	400 mg/ml
Azadirachta indica (neem tree)	M1	26± 0.1	30± 0.1	38± 0.2
	C1	23± 0.2	23± 0.04	29± 0.2
	H1	16± 0.3	19± 0.4	25± 0.2
Avicenia marina (mangrove)	M2	18± 0.3	15± 0.1	17± 0.2
	C2	18± 0.4	22± 0.2	07± 0.2
	H2	05± 0.3	05± 0.4	15± 0.3
Standard	S 500mg/ml	07± 0.1		
	L 500mg/ml	14± 0.1		
DMSO	Control	0		

S, Ciprofloxacin; L, Levofloxacin.

The chloroform extract (C1) demonstrated high efficacy against *S. aureus* (23–29 mm) particullarly at 200 mg/ml, consistent with reports that chloroform enhances extraction of lipophilic antimicrobials (Sultana *et al*., 2007). The chloroform extract (C1) required higher concentrations (MIC: 25.0–50.0 mg/ml; MBC: 50.0–100.0 mg/ml) (Table V), aligning with studies noting methanol's efficacy in extracting polar antimicrobials (Alzohairy, 2016).

Hexane extract (H1) was notably active against *S. aureus* (16–25 mm) at lower concentrations and *E. aerogenes* (1–21 mm) at higher ones (Table II), corroborating findings that non-polar solvents extract terpenoids with antibacterial properties (Cowan, 1999). However, all *A. indica* extracts displayed minimal activity against *E. coli* (0–2 mm), likely due to the outer membrane barrier and efflux pumps in Gram-negative bacteria, which limit penetration of phytochemicals (Nikaido, 2003). Organic solvents, particularly methanol and chloroform, enhanced extraction efficiency for bioactive compounds compared to aqueous methods, likely due to better solubility of lipophilic antimicrobials (Zhang *et al*., 2018).

Table V. MIC's of the most eNective plant extract against *S. aureus* and *E. aerogenes.*

Plant species	Ex-tracts	Bacterial Strain	MIC (mg/mL)	MBC (mg/mL)
Azadirachta indica	M1	*S. aureus*	12.5 ± 0.2	25.0 ± 0.3
		E. aerogenes	25.0 ± 0.3	50.0 ± 0.4
	C1	*S. aureus*	25.0 ± 0.3	50.0 ± 0.4
		E. aerogenes	50.0 ± 0.4	100.0 ± 0.5
Avicennia marina	M2	*S. aureus*	50.0 ± 0.4	100.0 ± 0.5
		E. aerogenes	200.0 ± 0.6	400.0 ± 0.7
	C2	*S. aureus*	25.0 ± 0.3	50.0 ± 0.4
		E. aerogenes	100.0 ± 0.5	200.0 ± 0.6

For *A. marina* extracts, chloroform (C2) and methanol (M2) showed selective inhibition against *S. aureus.* Methanol (M2) and chloroform (C2) extracts showed ZOI of 15–18 mm and 7–22 mm, respectively, at lower concentrations (Table IV). Chloroform extract (C2) showed notable MIC activity against *S. aureus* (MIC: 25.0 mg/ml; MBC: 50.0 mg/ml), while methanol (M2) less potent (MIC: 50.0–200.0 mg/ml; MBC: 100.0–400.0 mg/ml) (Table V). For *E. aerogenes*, all *A. marina* extracts had higher MICs (100.0–200.0 mg/ml), reflecting Gram-negative resistance due to outer membrane barriers (Nikaido, 2003). However, the e*cacy declined at high concentration 400 mg/ml against *S. aureus.* (e.g., C2: 7 mm). The reduction may result from antagonistic interactions among bioactive compounds, precipitation of active compounds, or microbial defense mechanisms such as efflux pumps and biofilm formation as reported in high-concentration scenarios (Savoia, 2012; Gyawali and Ibrahim, 2014). High concentrations may lead to aggregation of phenolics or flavonoids, reducing bioavailability and promoting oxidative degradation. Both extracts were inactive against MDR *E. coli* and *E. aerogenes* (Fig. 1A, B). Hexane extract (H2) was less effective (5–15 mm against *S. aureus*) with negligible

activity against *E. aerogenes* and *E. coli*. These findings are consistent with studies indicating that *A. marina* contains tannins and terpenoids with selective efficacy against Gram-positive bacteria due to their simpler cell wall structure (Vadlapudi *et al*., 2010). The lack of activity against *E. coli* and *E. aerogenes* aligns with literature highlighting Gram-negative resistance to plant extracts due to lipopolysaccharides and efflux systems (Tegos *et al*., 2002) (Fig. 1C).

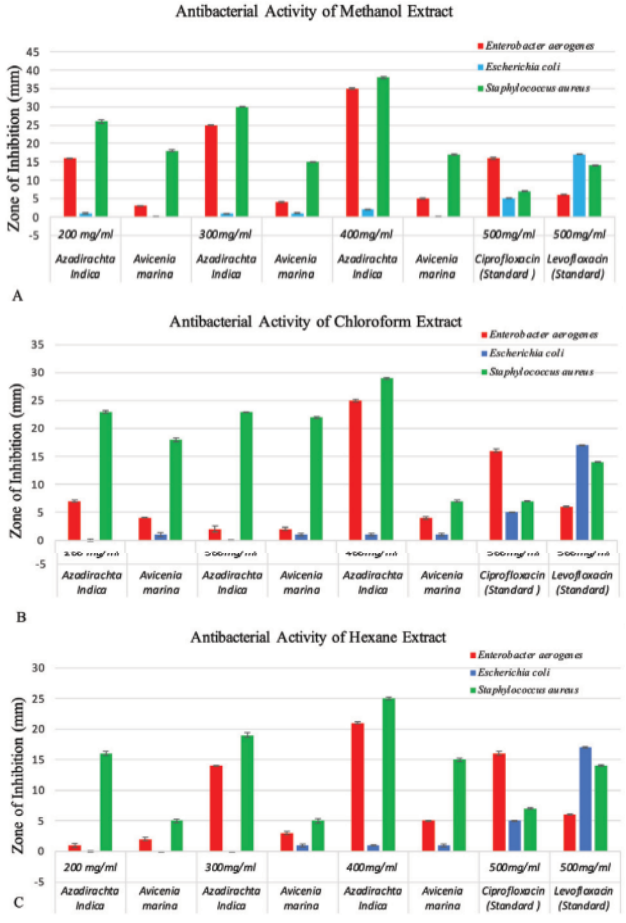

Fig. 1. Comparative antibacterial activity of *A. indica* and *A. marina* methanol (A), chloroform (B), and hexane (C) extracts.

The superior performance of *A. indica* extracts, particularly M1, against *S. aureus* (38 mm at 400 mg/mL vs. ciprofloxacin 7 mm and levofloxacin 14mm) and *E. aerogenes* (35 mm vs. ciprofloxacin 16 mm and levofloxacin 6mm) (Fig. 1A) underscores the potential of plant-based efficacy at low concentrations suggests potential as eco-friendly alternatives (Savoia, 2012). DMSO controls showed no activity, confirming extract-specific effects. Bactericidal activity supports therapeutic potential (Pankey and Sabath, 2004). These results corroborate studies showing that plant extracts target multiple bacterial pathways (e.g., membrane disruption, enzyme inhibition), reducing the likelihood of resistance compared to single-target antibiotics (Cowan, 1999; Mostafa et al., 2018). The limited efficacy against MDR *E. coli* reflects the challenge of overcoming Gram-negative resistance, as noted in prior research (Nikaido, 2003; Li *et al*., 2015). The observed decline in *A. marina* activity at higher concentrations supports reports of concentration-dependent antagonism or compound precipitation, which may reduce bioavailability (Savoia, 2012).

Organic solvents (methanol, chloroform) outperformed hexane in extracting bioactive compounds, consistent with findings that polar solvents efficiently extract phenolics and flavonoids with antimicrobial properties (Tuney *et al*., 2006). The synergistic potential of combining *A. indica* extracts with antibiotics, as suggested by enhanced efficacy at lower doses, aligns with studies demonstrating reduced antibiotic doses and resistance when paired with phytochemicals (Hemaiswarya *et al*., 2008). Environmentally, plant-based drugs provide sustainable options with lower ecological footprints through renewable cultivation, contrasting chemical-intensive antibiotic synthesis (Ncube *et al*., 2008). However, challenges such as variability in extract potency due to cultivation or extraction methods necessitate standardization, as emphasized in prior studies. These findings advocate plant extracts as greener adjuncts or alternatives in antimicrobial stewardship (Ncube *et al*., 2008).

CONCLUSION

The study revealed that A. indica and A. marina exibit significant antibacterial activity, especially in methanol and chloroform extracts. The methanol extract of *A. indica* showed the strongest inhibition against *S. aureus* and *E. aerogenes,* attributed to its efficient extraction of bioactive compounds such as flavonoids and alkaloids. *A. marina* extracts were selectively active against *S. aureus* but less effective against Gram-negative bacteria due to membrane barriers. Overall, methanol proved the most effective solvent. These findings highlight A. indica as a promising ecofriendly antimicrobial source and support further studies on bioactive compounds isolation and synergistic application with antibiotics.

DECLARATIONS

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Ethical statement

This study was conducted in accordance with Ethical research standards, ensuring integrity, transparency, and responsible data handling.

Generative AI and AI-assisted technology statement

The data presented in this article are original and research work performs solely by the authors, without the use of AI or automated data generation tools.

Statemenet of conflict of interest

The authors have declared no conflict of interest.

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Existence of Abnormal Sea Stars of Two Species, *Astropecten indicus* and *Astropecten polycanthus* Found in the Coastal Waters of Pakistan

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ABSTRACT

Starfish (*Astropecten*) are the most important and well-known marine invertebrates. Starfish are remarkably diverse and mostly found in submerged habitats. It proved difficult to identify *Astropecten* species, even though many species are flourishing as planktotrophs. They have symmetrically extended long or short rays from a center disk and are regular pentagonal, flattened, free-living stars. It has also been discovered that asteroids contain abnormalities in the shape of their bodies, such as an odd number of arms in normally pentamerous species but sometime predator damage recovery is usually the cause of these oddities. The present study provides the first report on the evidence of aberrant *A. indicus* and *A. polycanthus* species that were collected from Karachi's Clifton Sea view coast during the continuing study's collection attempts.

INTRODUCTION

Sea stars are members of the class Asteroidea, phylum Echinodermata, Kingdom Animalia. Asteroids usually have five arms that radiate from the central disks and every one of them has tube feet or podia that protrude along the arm's underside grooves (Fenner, 2009). They are widely distributed in the Antarctic, Pacific, Atlantic, and Indian Oceans, where they primarily inhabit wave-exposed intertidal zones of coastal waters, sea stars are most diverse in coastal locations (Freeman, 2005). According to Christensen (1970), they are most common in tropical and subtropical areas, particularly in the Indo-Pacific.

There are roughly 370 genera and 1890 species among the 36 families that make up the group of sea stars or starfishes belongs to the class Asteroidea (Mah and Blake, 2012). Sea stars are usually pentagonal and flattened. These are free-living, symmetrically radiating creatures with long or short arms that originate from a central disc, having a mouth at the lower or ventral surface is referred as the oral surface, and the upper surface, where the anus is present, is known as the aboral surface. Orally, a notable groove known as ambulacral groove that runs the length of each arm reaching from the area around the mouth to the arms tip (Chamundeeswari *et al*., 2013). Sea stars play important ecological roles as predators, scavengers, and prey items in both the intertidal and subtidal zones of the marine ecosystem (Menge, 1986). Sea stars are noticeable and prosperous animals and they can go months without eating and consume nearly every kind of marine life that they come across. The genus *Astropecten* of seastars feeds on a broad range of food, including fish, sipunculids, pennatulids, polychaetes, crabs, and sediments (Jangoux and Lawrence, 1982; Wells and Lalli, 2003). Thus, they can be significant predators of other invertebrates, such as clams, barnacles, and other associated marine life, which makes them ecologically significant. Sea stars are reported to be preyed upon by a variety of echinoderms, including fish, birds, and crabs. Because their calcified bodies are tough to chew and not very nutritious, they often feed on the tips of their arms (Fell, 1962).

In principal echinoderms are pentamerous, the majority of asteroids only have five limbs. While some asteroids, such as *Acanthaster planci* and *Luidia maculate*, invariably have more than five arms (James 1999). Some asteroids do, however, have more than five arms. A typical formation during development may result in fewer or more arms than five in some asteroids, such as *Pentaceraster regulus* and *Protoreaster linckii.* Asteroids have also been found to exhibit anomalies in body shape, such as an unusual arm count in species that are typically pentamerous. Hotchkiss (1979) asserts that these anomalies are typically the result of predator damage regeneration. The current

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study generates the initial report on the evidence about the existence of aberrant *A. indicus* and *A. polycanthus* species that were gathered from the Clifton Sea view coast of Karachi during the collection attempts of ongoing study.

MATERIALS AND METHODS

Study area

Sea view, another name for Clifton Beach, is situated along the Arabian Sea in Clifton, Karachi, Pakistan. It is a well-liked leisure area in Pakistan that extends from Karachi to Ormara in Balochistan. The beach is a component of the Karachi South district's broader Clifton neighborhood. Similar to other coastal locations, this area features a diverse range of habitats for different animal species. Clifton sea view is an important site for marine and coastal species because of its closeness to the Arabian Sea. Though these habitats are impacted by the area's urban development, they nevertheless play a vital role in Karachi's coastal ecosystem.

Sample collection

The study was conducted from October 2022 to November 2023 and specimens were collected by hand picking, once in a month at regular intervals from the exposed area during the low tide. Collected samples were brought back into the laboratory, where the specimens were washed with filter sea water to remove the attached mud and other particles, then samples were freezed at -4 ^{o}C for species identification and further analysis. Specimen identifications were done by the study of morphological characteristics like surface of body, shape of arms and shape of madreporite under the Olympus microscope through available taxonomic Keys (Tahera, 1996; Adeli *et al*., 2022). In each collection the lengths of the each arm R (from the mouth center to the tip of arm in mm), Disc diameter (from one end of arm base to the other end of interradius) by using Vernier Caliper and weight of each Sea star were measured in g by electronic balance.

RESULTS AND DISCUSSION

The abnormal forms of two species of *Astropecten (Astropecten indicus* (N=7) and *Astropecten polyacanthus* (N=5) were observed from the collection (Fig. 1). A. indicus and *A. polycanthus* are the common sea stars found in the sandy and muddy intertidal region of tropical coastal areas mainly in Indian Ocean. Tahera (1996) was the first to describe two species of *Astropecten* along the coast of Pakistan*, A. indicus* and *A. polyacanthus*, commonly found in Pakistan's coastal waters, particularly in the Northern Arabian Sea and during present study both species were collected in the subtidal area of Karachi's, sandy Clifton Sea view waterfront.

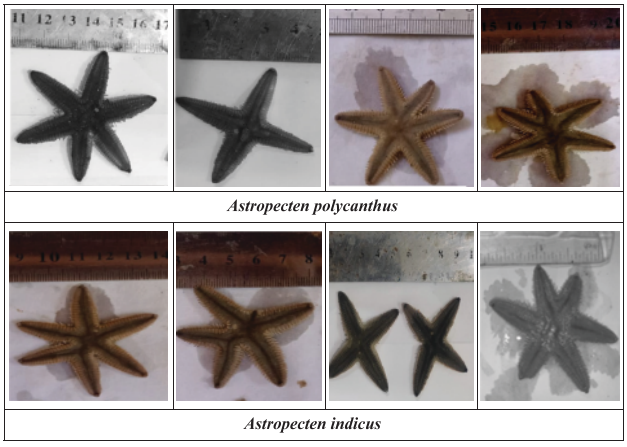

Fig. 1. Abnormal forms of *Astropecten indicus* and *Astropecten polycanthus* found during the Oct. 2023 to Nov. 2023 from Clifton sea view Karachi.

According to taxonomic feature; *A. indicus* has closely packed small, regular arranged Paxillae/inner superomarginal spine and outer supero marginal spine on superomarginal plates/ whereas in *A. polycanthus* had one row consecutive supero marginal spine without any gap. The supero marginal spines were reduced after the ones on proximal supero marginal plates. In *A. indicus* adambulacral plates had two series of spines with same length and width whereas in *A. polycanthus* Adambulacral spines were larger than the furrow spines and middle furrow spines were larger than others (Tahera, 1996; Adeli *et al*., 2022).

Because the both species of sea star typically abides by the pentamerism rule, the interradial area of a normal animal bears a L shape (90°). The coding method was used to number each asteroidal arm; for example, arm one is the arm left side of the madreporite, and then next counts clockwise in serial order. The interradial region of the four-armed species had a V shape rather than a L; this was less evident in the six armed specimens (Fig. 1). Teratological incomplete development is the cause of the four or three arms that arise during the metamorphosis. This is typically in line with the findings of Clark and Rowe (1971) on *Patiria miniata,* Marsh *et al*. (1986) on *Echinaster spinulosus*, Allain (1972) on *Asterina gibbosa* and *A. indicus.* However, Watts *et al*. (1983) provide evidence that excessive salinity during early growth may be the origin of ray number aberrations in asteroids. Both injury and autonomy, particularly when disturbed, were the causes of the odd or atypical forms that were

displayed. *A. indicus* and *A. polycanthus* usually have five arms but both with six arms (Fig. 1) were found in the present observation.

The both species having six arms have extra arms that regenerate from any normal arm. Most abnormalities in the morphometry from the medriporide to the right side of the ram have been observed in the second aram. It was also observed that although none of the aberrant specimens showed abnormality in the first arm of all the specimens of both species as located first left to madreporite, but in three specimens having abnormal situation in the fifth arm, that noticed carefully as significant due to having madreporite between them. The unusual or abnormal forms with additional arm were display (Y) shape in structure of ambulacra groove. However, all the originated/additional arms were observed smaller than their originator arm (Table I).

Table I. Arms length, disc diameter and weight of abnormal sea stars of two species (*A. indicus* and *A. polycanthus)* found during the collection from the Karachi, Clifton Beach.

S. No	Weight (g)	Disc dia (mm)	Arm length				
			First (cm)	Second (cm)	Third (cm)	Four (cm)	Fifth (cm)
A. indicus							
1	4	2.3	2.3	1	2.3	2	2.5 (2*)
2	3.5	2.4	2	2.3 (1.7*)	2.1	2.5	2.3
3	3.2	2.1	1.8	1.3	2 (1.5*)	1.5	2
4	4	2.5	1.9	2 (1.4*)	2.5	2.2	1.6
5	2.5	2.3	1.7	1.7	1.9 (1.8*)	1.9	1.8
6	3.4	2.4	2.3	2.1	2.3	2	
7	3.2	2.2	1.8	1.5	1.8	1.9	
A. polycanthus							
1	2.8	2.4	2.1	2.2	2.1	2.3	2.1 (2.0*)
2	4	2.5	1.9	2 (1.4*)	2.5	2.2	1.6
3	2.5	2.3	1.7	1.7	1.8	1.9 (1.9*)	1.8
4	3.1	2.2	1.7	1.5	1.9	1.8	
5	3.3	2.4	2.2	2.1	2.3	2.1	

Only a small number of observations of the aberrant forms of sea stars have been made worldwide (Hotchkiss, 2000). *A. indicus,* with six-armed starfish, and a few more unusual sea stars from the Gulf of Mannar region have been also reported by James (1999), and Chamundeeswari *et al*. (2013).

The idea that pentamerism has rigid control is supported by the huge number of strictly pentamerous starfish families, which can only produce five arms, however occasionally an uneven arm number is also seen. This author experimentally observed selective breeding in aberrant ray numbers and produced only five-rayed offspring, not four-rayed ones, as demonstrated by Lawerence and Kumatsu (1990) demonstrated that the control of ray number is quite accurate in five-rayed species. This is evident from the ambulacral grooves that the basic plan is pentamarous but the few studies support the anomalous ambulacral grooves and rays, but there is no appropriate literature regarding asteroids' abnormalities. According to Hyman (1955) distally forked arm is typically the consequence of a twofold out growth when an arm is split vertically. Although partial disc incisions usually heal, occasionally one or even two arms may protrude from the wound closure site (King, 1900). According to Hotchkiss (2000), the five hydrocoel during metamorphosis do not cause the double ambulacral groove. As the basic pentamerous structure can be seen in all six arm specimens, the current results indicate that deviations from pentamerism are not a heritable characteristic. The presence of four arms indicates abnormalities during the larval developments, while the presence of more than five arms may be the result of environmental perturbations on the metamorphosis of larvae. Further study is needed to understand the reason behind the abnormality in sea stars.

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Generative AI and AI-assisted technology statement

The authors have declared that no genarative AI or AI-assisted technology was used to creat this mansucript.

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Evaluation of Bait Formulations of Coumatetralyl (Racumin) for Rodent Control in Wheat Crop in Pothwar Zone

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ABSTRACT

Rodents pose a significant threat to wheat crop, leading to substantial yield loss in agricultural systems. Therefore, there is a need of effective methods for the control of field rats by using chronic poisons. This study aimed to evaluate the preferred bait base at each stage of wheat crop growth (initial, heading and maturity) and its effectiveness by adding chronic poison. Overall, screening of 4 bait bases (wheat, rice, maize and millet), all in cracked form were carried out in multi-choice feed preference tests. Millet was used as control while millet+maize, millet+wheat and millet+rice in 50:50 ratio were used as treatments. Screening of three bait additives (peanut butter, egg-shell and cooking oil) was carried out by adding in the top ranked bait base in the 4% ratio. Evaluation of poison bait was carried out by adding developed bait at each stage and millet (control) with coumatetralyl (0.0375%). Bait base and additives test were carried out at each stage while poison test was carried out at maturity stage of wheat crop. Results showed that millet, wheat and peanut butter were more preferred for poison baiting at initial stage of wheat crop while millet, rice, peanut butter and racumin are the e*ective combinations for controlling field rats at maturity stage of wheat crop. It is recommended that rodent control strategies should be adapted based on the growth stage of the wheat crop, with bait base preferences shifting from millet and wheat to millet and rice as the crop matures.

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US designed and conducted the research trials. US and IA wrote the manuscript. IA conceptualized the study. ZP and IA equally contributed in field work. AAK selected the site, review and edited final version of manuscript.

Key words
Rodents, Control, Rodenticides, Coumatetralyl, Bait formulations, Crop stages

INTRODUCTION

Rodents can cause significant damage to field crops in developing countries, as well as to stored produce and infrastructure, affecting food security and income of small-holder farmers. Wheat (*Triticum aestivum*) and groundnuts (*Arachis hypogea*) are important cash crops for local farmers in Pakistan. Damage levels in crops can vary from insignificant to very significant (>30%) or to almost complete crop loss. In Pakistan, rodent damage to wheat crop, in majority of cases, has been studied in Central Punjab. Among the field rats *Bandicota bengalensis* has been studied as the most abundant and damaging rodent pest in wheat fields followed by *Nesokia indica, Millardia meltada* and *Mus musculus* (Beg *et al*., 1980). Beg and Khan (1977) calculated 7.5% damage to wheat in Faisalabad District. Beg *et al*. (1978) conducted a study in four districts of central Punjab and estimated 5 % reduction potential of yield. On a much larger scale Fulk *et al*. (1980) estimated rodent damage to 1978 and 1979 in Punjab, Sindh and NWFP and found that 5 % of the farms sampled had more than 10 % of yield loss due to rodent damage. This study also indicated that the losses were severe in rain-fed areas and where wheat is grown after rice harvest. As an example of the severe levels of rodent damage to crops was reported in 1956 in the district of Sialkot which revealed that the quantity of grains (rice and wheat) lost due to rodents was enough to feed the population of the district (0.3 million at that time) for ten years.

Five species of field rodents (*Bandicota bengalensis, Nesokia indica, Tatera indica, Golunda ellioti* and *Mus musculus*) have been reported in the agriculture fields of Pothwar region (Hussain *et al*., 2003). The lesser bandicoot rat (*B. bengalensis*) is one of the most abundant rodent pests in field crops of Pakistan. It is also a serious problem of wheat throughout southern and Southeastern Asia. Pakistan has two isolated populations of this rat, one throughout central and northern Punjab and in the southern part of Khyber Pakhtunkhwa province, and the other in the

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southern Sindh (Smiet *et al*., 1978).

The majority of the rural communities of the Pothwar are dependent on farming (i.e., wheat, groundnut, millet/ maize and sorghum) where 90% of their farm sizes are less than two hectares and the majority of farming is conducted by small-holder farmers. Rodents are fossorial animals (those both foraging and sheltering underground). They feed primarily on root systems of the plants and cut them from lowest part to eat the sweet and milky part of the stem and on aboveground consume herbaceous plant parts (Sarwar, 2016). Management of rodents is generally most effective when utilizing an integrated pest management (IPM) approach that incorporates multiple techniques. Of these potential management tools, rodenticide application is generally the preferred option by pest control professionals and land managers which is the quickest and least expensive management tool available for rodents (Baldwin *et al*., 2016).

Rodenticide baits consist of a nontoxic food base, flavor, attractants, additional additives (such as bittering, moisture-resistant, coloring and preservative agents) and toxic active substance. Food composition plays a key aspect for bait efficacy as it ensures its palatability. Baits may be based on acute (Henry *et al*., 2022) and anticoagulant active ingredients (McGee *et al*., 2020). Anticoagulants belong to the chronic rodenticides with the delayed mode of action. They stop the blood coagulation process in the liver via interruption of the vitamin K cycle, which results in a fatal haemorrhage after several days (typically 4-10 days). This delay prevents rodents from connecting the signs of toxicity with the consumed food, which favours anticoagulants over the acute rodenticides. Although mode of action in terms of mortality is not instant, they may inhibit food acceptance quite rapidly (Frankova *et al*., 2017).

The cumulative rodent population abundance indices in wheat crop in all growth stages were *B. bengalensis* (21%), *N. indica* (11.1%), *T. indica* (7.77%) and *G. ellioti* (2.22%) (Munawar *et al*., 2022). In wheat, an analysis between damage levels at different crop stages showed significant differences, the overall rodent damage was highest at maturity with 10.74% followed by flowering (6.4%) and tillering (3.5%) (Munawar *et al*., 2022).

Mostly, the limited bait consumption is a serious issue in many control campaigns against pest rodent species, worldwide. So, comparatively more preferred food baits are needed to attract rodent species in the area (Berry and Alleva, 2010). The bait may become more palatable by adding certain food additives in the plain bait in an optimum concentration, so that the target species consumes a lethal dose of the rodenticide.

A number of studies have shown the enhancement of the bait consumption by adding a variety of additives for different small rodent species, such as *Rattus norvegicus, Rattus rattus, Mus musculus* and *B. bengalensis* (Singla and Kanwar, 2014). Shafi *et al*. (1990) role of some taste additives to enhancer poison bait acceptance in the black rat, *Rattus rattus*. The literature reveals the use of cereals in whole-some, cracked form as well as mixed with additives such as vegetable oil, egg-shell, egg yolk, minced meat, sugar, spices and flavours (Pervez *et al*., 2003). In another study, feed preference of rodents was carried out for locally available and palatable food grains viz. millet (whole), wheat (cracked) and rice (broken) and taste additives namely whole egg (5%), egg-shell (5%), peanut cracked (5%) and yeast (2%) that were offered mixed in millet-wheat (50:50 body wt.) bait (Shahwar *et al*., 2024). In rice and wheat, coumatetralyl bait usage either inside the burrows or in bait stations is the two better delivery methods (Hussain *et al*., 2002).

The main aim of this study was to evaluate the preferred bait base and additives at each stage of wheat crop growth and its effectiveness by adding chronic poison.

MATERIALS AND METHODS

Study site

The study area was selected under wheat cultivation at National Agricultural Research Centre NARC, during the year 2024. The total area of NARC is about 600 ha (approximately 1500 acre). The geographical location of NARC is (longitude: 33.69 “N and latitude: 73.03’E). A variety of crops grown at NARC which includes di*erent agronomical crops such as rice, sugarcane, wheat, maize fodder, pulses and horticultural crops such as orchards of citrus, grapes, and guava etc. Infestation of rodents at NARC campus has been reported for the last many years (Brooks *et al*., 1987; Shahid and Ahmed, 2013).

Survey and selection of treatment sites

A thorough survey of wheat field area was carried out to see level of rodent infestation. Treatment sites were selected after thorough survey of field area where sufficient number of active burrows system and fresh droppings were found. Six number of burrow systems were selected and each treated as a single replication. A minimum of 340 meters distance was maintained between each burrow system.

Design of experiment

The feeding test was carried out at three different stages of wheat crop i.e., initial, heading and maturity stage. At each stage screening of both bait bases and bait

additives were carried out but poison baiting was carried out at maturity stage of the crop only.

Screening of bait bases in multi-choice feeding tests were carried out to identify the most preferred cereal grain as a bait base by taking average daily intake (ADI) with 06 replications. A total of 4 treatments were used and prepared in following ratios:

(i) Millet+Wheat 1:1; (ii) Millet+Rice 1:1; (iii) Millet+Maize 1:1 and (iv) Millet.

Screening of bait additives/enhancers was carried out by adding in the top ranked bait base in 4% proportion along with bait base alone (control) in multi-choice feeding tests with 06 replications. Peanut butter, cooking oil and egg shell in 4% proportion were incorporated as taste additives (bait enhancers). Candidate food consumption data were recorded by taking ADI for 6 consecutive days.

Evaluation of poison bait was carried out by adding developed bait at each stage and millet (control) with coumatetralyl (0.0375%).

Bait application methodology

Each candidate bait was presented in environmentally safe PVC bait stations measuring 30 cm long and 08 cm in diameter keeping both ends open for free accessibility of rats and to protect the bait consumption from non-target animals and other climatic factors. Bait stations were deliberately placed near active burrow openings of the bandicoot rat. In each test 100 g of each candidate food was offered each day in each bait station. Food consumption was recorded daily; taking spillage into account i.e., left over and spillage were calculated after 24 h interval and weighed to calculate ADI using electronic balance with accuracy to 0.1gram of each candidate bait.

Bait was replenished on daily basis and position of the bait stations was changed daily to avoid place preference trend. At least 5 days lag period was maintained between multi-choice feed preference test of bait bases and bait additives.

Statistical analysis

Descriptive statistics were performed to compute means and standard errors (±) of the data. Mean food consumption data was analysed by one way ANOVA with test foods as main factors. In order to assess the significant differences among means, least significant difference (LSD) test was applied. LSD test demonstrated the significant differences between the mean values of different treatments at 5% level of significance. Percentage preference values for different baits were calculated by dividing test food by total food consumption and then multiplying by 100.

RESULTS

Evaluation of cereals in multi-choice feeding test

To see the preference of different cereal grains and bait additives by bandicoot rat at different stages of wheat crop, experiments were carried out at NARC campus fields. Feed preference of cereal grains were carried out by offering these grains, simultaneously in a multi-choice food preference tests to identify most preferred bait base at initial, heading and maturity stage of wheat crop. Mean daily consumption and percentage preference of cereals at each stage is given in Table I.

Consumption of bait bases at initial stage revealed that combination of millet+wheat was most preferred (39.81%) followed by millet+maize (23.48%), millet (22.98%), and millet+rice (13.70%) (Table I). Analysis of variance revealed that there was significant difference in the consumption between different grain baits ($F = 7.70$, $P=0.001$).

Consumption of bait bases at heading/flowering stage showed more consumption of each cereal grain as compared to initial stage. But the most preferred grain bait was millet+rice (28.18%) which revealed a shift of bait preference at flowering stage. Bait consumption pattern of other cereal grains were millet+maize (24.96%), millet+wheat (23.6%), and millet (23.24%). Analysis of variance revealed that there was significant difference in the consumption between different grain baits ($F= 7.51$, $P=0.001$).

At maturity stage same pattern was observed as millet+rice was the most preferred grain bait i.e., 37.16%. Bait consumption pattern of other cereal grains were millet (32.58%), millet+maize (16.12%), and millet +wheat (14.12%). Analysis of variance revealed that there was significant difference in the consumption between different grain baits ($F= 38.54$, $P=0.00$). The analysis of daily consumption of all foods showed that *B. bengalensis* sampled each food daily; none of the food was totally rejected in spite of daily rotation of the foods.

Evaluation of additives in multi-choice feeding test

Taste of food plays a significant role in food preference trend. Bait shyness problems may be overcome by using attracting palatable bait. The most preferred combination of bait base of each stage was further evaluated with the addition of different additives at 4% concentration. Mean daily consumption and percentage preference of additives at each stage is given in Table II. It was observed that most preferred bait combination at initial stage was millet+wheat+peanut butter i.e., 31.53%. While bait consumption pattern of other baits were millet+wheat+cooking oil (24.46%), millet+wheat+egg-

shell (21.89%) and millet+wheat (20.10%). Analysis of variance revealed that there was significant difference in the consumption between different bait additives (F= 4.28, P=0.017).

Table I. Mean daily consumption (g Mean ±SEM) and percentage preference of bait bases at diNerent growth stages.

Bait bases offered			
Millet	**Millet+Wheat**	**Millet+Rice**	**Millet+Maize**
Crop growth stage: Initial			
38.00 ± 7.29 (22.98%)	65.83 ± 4.82 (39.81%)	22.67 ± 5.01 (13.70%)	38.83 ± 19.61 (23.48%)
Crop growth stage: Heading			
354.8 ± 9.71 (23.24%)	360.3 ± 9.27 (23.6%)	430.1 ± 14.81 (28.18%)	381.0 ± 15.04 (24.96%)
Crop growth stage: Maturity			
84.16 ± 4.49 (32.58%)	36.50 ± 8.98 (14.12%)	96.00 ± 5.88 (37.16%)	41.66± 4.95 (16.12%)

Consumption of bait was higher at heading/flowering stage same as cereal bait consumption. But the most preferred bait was millet+rice+peanut butter (28.70%) which showed peanut butter as the most preferred bait additive. Bait consumption pattern of other bait additives were millet+rice+cooking oil (25.79%), millet+rice (23.45%), and millet+rice+egg-shell (22.04%). Analysis of variance revealed that there was significant difference in the consumption between different bait additives (F= 2.84, P=0.064).

Consumption of bait additives at maturity stage revealed that combination of millet+rice+peanut butter was mostly consumed i.e 70.40% followed by millet+rice+cooking oil (17.31%), millet+rice+egg-shell (6.26%), and millet+rice (6.0%). Analysis of variance revealed that there was significant difference in the consumption between different grain baits (F= 12.12, P=0.00).

Evaluation of preferred bait along with poison at maturity stage

The bait base combination along with 3 types of additives were evaluated at each stage and the most preferred bait of each stage was further evaluated for the control of bandicoot rat using chronic poison, coumatetralyl (0.0375%). Results of the offered baits on daily basis for a span of 6 days revealed that highly significant preference was given to millet+rice+peanut butter+ racumin (63.87%) (Table III). Mean daily consumption and percentage preference of preferred bait along with poison at maturity is given in Table III.

The consumption rate tended to be decreased abruptly and little or no bait consumption was recorded on 5th day. Bait consumption pattern of other baits were 26.56% for millet+wheat+peanut butter+racumin and 9.56% for millet+racumin. The values of analysis of variance were (F=4.046, P=0.039).

Table II. Mean daily consumption (g) and preference (%) of bait base with additives at different growth stages of wheat crop.

Bait additives offered			
Mil-let+Wheat	**Millet+Wheat +Peanut butter**	**Millet+Wheat+ Eggshell**	**Millet+Wheat+ Cooking oil**
Crop growth stage: Initial			
85.83 ± 7.44 (20.10%)	134.7 ± 10.58 (31.53%)	93.50 ± 7.62 (21.89%)	113.0 ± 14.81 (24.46%)
Crop growth stage: Heading			
235.8±19.42 (23.45%)	288.7 ± 15.02 (28.70%)	221.7 ± 14.19 (22.04%)	259.3 ± 20.14 (25.79%)
Crop growth stage: Maturity			
11.50 ± 4.75 (6.00%)	134.8 ± 29.19 (70.40%)	12.00 ± 2.54 (6.26%)	33.16 ± 16.13 (17.31)

Table III. Mean daily consumption and percentage preference of preferred bait along with poison at maturity stage of wheat crop.

Bait bases offered	Mean consumption (g)
Millet+Racumin	13.50 ± 5.45 (9.56%)
Millet+Rice+Peanut Butter+Racumin	90.16 ± 27.17 (63.87%)
Millet+Wheat+Peanut Butter +Racumin	37.50 ± 19.28 (26.56%)

DISCUSSION

Rodent populations fluctuate with different crop seasons and growth stages in the agricultural systems. They are known to damaging seedlings and mature crops (Sarwar, 2015). Crop stage is an important factor influencing damage levels. In the present study, the highest damage was observed during maturity stages in wheat fields. Similar findings were observed by Miller *et al*. (2008), for *Rattus tanezumi* in the rice terraces of Banaue, in northern Luzon, Philippines. Research trials in the present study were conducted at three different growth stages of wheat crop to evaluate the preferred bait base at each stage. Munawar et al. (2020) collected data from a

wheat crop at three different growth stages to determine the mean intake of the formulated bait.

Experimental findings of the present study proposed that mean per day consumption of millet+wheat (as a bait base) and millet+wheat+peanut butter (as additive bait) was recorded to be highly preferred at initial stage of wheat crop. Bandicoot rat might be attracted to wheat initially due to the distinctive aroma from the wheat field. This natural scent could serve as a strong attractant, influencing the feeding behaviour of the rats during this critical crop growth stage. At other growth stages i.e., flowering and maturity stage, the bait base preference was shifted to the combination of millet+rice. In another study of Naeem *et al*. (2011), same grains were evaluated under captivity which revealed the highest consumption of millet (2.67±0.40), followed by maize (1.75±0.39), rice (1.12±0.33), and wheat (0.65±0.18), respectively.

Taste of food plays a significant role in food consumption. Bait shyness problems may be overcome by using attracting palatable bait. Several studies have been conducted to enhance existing rodent control strategies, aiming to improve their effectiveness by incorporating various locally available palatable foods or bait additives (Pervez et al., 2005; Naeem *et al*., 2011). In this experiment peanut butter, cooking oil and egg shell in 4% proportion were incorporated as taste additives (bait enhancers). Peanut butter in 4% concentration significantly enhanced the consumption of bait at each stage of crop. Another study of Munawar et al. (2020), confirmed that mixing egg shells and peanut butter with zinc phosphide and brodifacoum improved bait acceptance, and there was minimal bait shyness for rodent control. The variation observed could be attributed to differences in the base materials used in the bait. From previous and present studies it can be assumed that use of peanut oil at 4% concentration may enhance bait intake as compared to using egg shell, cooking oil or plain bait.

Poison baiting was not carried out during the initial and flowering stages of the wheat crop to prevent a significant reduction in the bandicoot rat population at the established baiting points. Since the study required consistent rodent activity across all crop stages, early poison baiting could have compromised the continuity of data collection. To ensure reliability and comparability, all baiting trials were conducted at the same pre-established points throughout the wheat crop.

According to Hussain *et al*. (2003), there was a 92% reduction in live burrow activity with the use of zinc phosphide (2%), and a similar reduction of 92.2% was observed with coumatetralyl (0.0375%). These results support the current study in which we observed gradual decrease in consumption rate and no bait consumption was recorded on 5th day. The analyses of daily consumption of all foods showed that *B. bengalensis* sampled each food daily; none of the food was totally rejected in spite of daily rotation of the bait stations under test.

CONCLUSION

It can be concluded from current study that millet ± wheat in (50:50) ratio and peanut butter in (4%) concentration as an additive found most effective for poison baiting at initial stage of wheat crop while millet ± rice in (50:50) and peanut butter in (4%) concentration and racumin (0.0375%) are an effective combination for controlling field rats at maturity stage of wheat crop. Rodent control strategies should be adapted based on the growth stage of the wheat crop, with bait base preferences shifting from millet and wheat to millet and rice as the crop matures. Further studies should be performed to evaluate different bait base combinations and other bait additives/ enhancers at different plant growth stages across a variety of crops, including wheat, rice, maize, groundnut, pulses, and horticultural crops.

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Ethical statement

The authors complied with all the laws and regulations that apply to science and profession during the period of studies.

Generative AI and AI-assisted technology statement

The authors have declared that no generative AI or AI-assisted technologies were used to create this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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