Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(2): 1352-1355

Received: 08-01-2019
Accepted: 12-02-2019

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A comparative study on antibacterial activity of integumentary extract of selected freshwater fish Species and Neem extracts against gram-positive and Gram-Negative bacteria

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Abstract
The present investigation was conducted to assess the antibacterial activity of integumentary extract of freshwater fish species *viz.*, Singhi (*Heteropneustes fossilis*) and Mrigal (*Cirrhinus mrigala*) and neem (*Azadirachta indica*) extracts (leaves and bark) against the selected microbes namely *Aeromonas hyprophila* and *Staphylococcus aureus*. Disc diffusion method was employed to determine the antibacterial activity of different extracts. In this study *Heteropneutes fossilis* integumentary secretions extract demonstrated greater inhibitory ability than *Cirrhinus mrigala*. It was also observed that *Heteropneutes fossilis* integumentary extract have more inhibitory effect against *Aeromonas hyprophila* (22±2.05) than *Staphylococcus aureus* (18±1.56). While, the ethanol extract of neem leaves was observed to possess higher antibacterial activity than that of neem bark extract. Interestingly, the neem leaves extract had shown more inhibitory activity against the *Staphylococcus aureus* (13±1.76) as compared to *Aeromonas hyprophila* (12±1.88). The study highlights the importance of fish integumentary and neem extracts as potential antibacterial agents.

Keywords: Antibacterial activity, freshwater fish species, integumentary secretions, Neem extracts

1. Introduction
Several microbes both of pathogenic or non-pathogenic nature have inhabited together in the aquatic environment. In response of such threats, fish used their innate immune systems that have secreted specific compounds against the microbes infected on them. However, identification and characterization of such innate immune compounds need to be carried out. Since, global fisheries and aquaculture sector has been constantly a source for food production as well as nutritional security. In India, fisheries sector is contributing around 6.3 per cent of the global fish production and creating a work place for about 14 million people (NFDB, 2018) [1]. Although, with the changing climate along with the increasing accumulation of unwanted substances in the water body with due course of time has put the developmental process of the fishes become limited and creates several problems. It is believed that several organisms generally of pathogenic nature have affected the fish health system (Balasubramanian *et al*., 2012) [2]. This has resulted diseases in fish and cause the major economic losses in the field of aquaculture (Faruk and Anka, 2017) [3]. In response of these unwanted results, several chemicals are often used to treat diseases of fish and continuous use of these chemicals would cause multi resistant microbial strains found inside the water body (Idowu *et al*., 2017; Subhashini *et al*., 2013; Subramanian *et al*., 2007) [4, 5, 6]. In this context, the understanding of the fish innate immune system is highly desirable. Heavy or continuous application of chemical antibiotic to microbial diseases of fishes has created a resistant to multi strains of microbes. Moreover, most of chemical which are used for the treatment of diseases have restricted use due to their toxicity and persistence in the environment. Therefore, the development of new drugs (either inhibit the growth of pathogen or kill them and having no or least quantum of side effect) from natural substances have an immense role in preventing the major economic losses of fishes on one side and improving the aquatic environment by restricting the use of chemicals on the other side. In this situation, limited studies have been carried out in North-eastern states of India on this aspect and hence the present study was undertaken to assess the antibacterial activity of neem extract and selected fresh water fish species.

2. Materials and Methods

2.1 Collection of fish

For assessing the antibacterial activity of integumentary fish extract, Singhi (*Heteropneustes fossilis*) and Mrigal (*Cirrhinus mrigala*) were collected from Fish farm complex of the institute. The collected fish samples (n=10) were acclimatized to laboratory condition and maintained in the wet laboratory for 5 days by providing artificial feeding.

2.2 Preparation of integumentary extract

Fish integumentary extract were prepared by scrapping the fish mucus from the body surface. The extracted mucus was kept in the sterile condition. The collected extract were then stored in 4°C for future use.

2.3 Preparation of Neem leaves and bark extracts

The collected mature neem (*Azadirachta indica*) leaves and bark samples were initially washed with tap water followed by distilled water. The leaves and bark were then kept for air drying. The air-dried materials were later pulverized and made into powder. Ethanol extracts of neem leaves and bark were carried out by following standard Soxhlet extraction technique (AOAC, 2005) [7]. A ratio of 1:10 was retained inside the round bottom flask by taking 50 g each of dried powdered leaves and bark along with 500ml of the extracting solvent (ethanol). The extraction was carried out for about 60-70 cycles (4-6h) under reflux condition. Finally, the concentrated extracts were transferred into clean petri plates for incubation at 37°C for 48 hrs.

2.4 Selection of bacterial strains

To evaluate the anti-bacterial activity of integumentary secretion of fish and neem extracts, two bacterial strain *viz*., *Staphylococcus aureus* (Gram + ve) and *Aeromonas hyprophila* (Gram-ve) were selected. The selected bacterial strains *Staphylococcus aureus* (ATCC 25923) and *Aeromonas hyprophila* (ATCC 7966) were procured from Hi Media Laboratories.

2.5 Antibacterial activity assessment

Antibacterial activity was determined following the agar diffusion method (Brumfitt *et al.,* 1990) [8]. The inoculated bacterial strains (0.1 ml) was cultured in Mueller Hinton Agar medium (20 ml) and kept for 24 hrs at 37°C. Following this, paper disc were impregnated on the surface of the plate by using sterile forceps and then loaded with 10 µl of each integumentary extract. These prepared plates were then incubated for 24 hrs at 37°C. Finally, the antibacterial activity was assessed by observing the zone of inhibition (mm). The antibiotic Ciprofloxacin was used as positive control.

2.6 Statistical Analysis

To elucidate the antibacterial activity of selected freshwater fish species and neem extracts, data were expressed in Mean and standard error of mean (S.D). Two-tailed paired sample t test at $p<0.05$ have been performed by using SPSS 17.0 (SPSS Inc., Chicago, USA) windows version package.

3. Results

Based on the average zone of inhibition (mm), the study demonstrated that *Heteropneutes fossilis* integumentary extract have more interference performance against the growth of selected bacterial strains *Aeromonas hyprophila* and *Staphylococcus aureus* as compared to *Cirrhinus mrigala* integumentary extract (Table 1). It was observed that *Heteropneutes fossilis* integumentary extract showed marked significant ($p<0.05$) inhibition effect against *Aeromonas hyprophila* (22 mm) and *Staphylococcus aureus* (18 mm) in comparison with the performance of *Cirrhinus mrigala* integumentary extract (20 mm and 15 mm). In contrast, the performance of positive control Ciprofloxacin (20 mm) was found to be similar in both the selected bacterial strains (Figure 1). Overall, the results have shown that *Heteropneutes fossilis* integumentary extract can perform better than the positive control antibiotic Cirpofloxacin against *Aeromonas hyprophila.* However, it indicated a lesser inhibition ability against the *Staphylococcus aureus.*

The ethanol extract of neem leaves and bark against *Aeromonas hyprophila* and *Staphylococcus aureus* have revealed higher inhibition ability in the leaves extract as compared to bark extract (Table 2). Furthermore, the ethanol extract of Neem leaves and bark did not show significant difference against *Staphylococcus aureus*; however, leaves extract possessed maximum antibacterial activity (13 mm) than bark extract (12 mm). In contrast, there was a marked significant variation in inhibitory activity between neem leaves (12 mm) and bark extract (10mm) against *Aeromonas hyprophila* (Figure 2). In spite of showing significant results against *Aeromonas hyprophila*, the level of inhibitory activity was greater against *Staphylococcus aureus.*

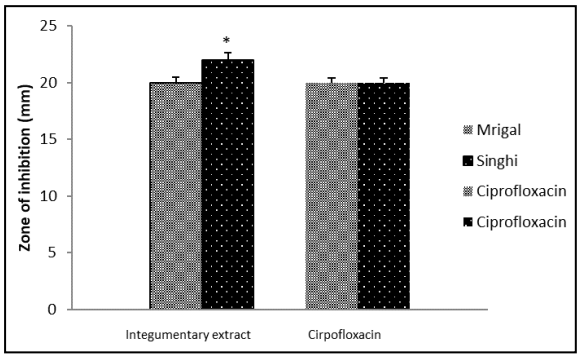
Table 1: Zone of inhibition (mm) of integumentary mucus of *Cirrhinus mrigala* and *Heteropneutes fossilis* against selected bacterial strains.

Fish species	Bacterial strains	Zone of inhibition (mm)	Positive control (mm)
Cirrhinus mrigala	*Aeromonas hyprophila*	20±1.63	20±1.24
Heteropneutes fossilis	*Aeromonas hyprophila*	22±2.05	20±1.41
Cirrhinus mrigala	*Staphylococcus aureus*	15±1.33	20±1.56
Heteropneutes fossilis	*Staphylococcus aureus*	18±1.56	20±1.76

Table 2: Zone of inhibition (mm) of ethanol extract of neem leaves and barks against selected bacterial strains.

Neem Extract	Bacterial strains	Zone of inhibition (mm)
Leaves	*Staphylococcus aureus*	13±1.76
Bark	*Staphylococcus aureus*	12±1.33
Leaves	*Aeromonas hyprophila*	12±1.88
Bark	*Aeromonas hyprophila*	10±1.41

Aeromonas hyprophila



Staphylococcus aureus

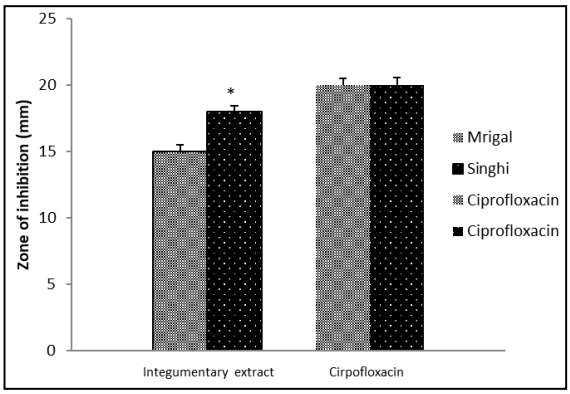


Fig 1: Antibacterial activity comparative performance of integumentary extract and control Ciprofloxacin

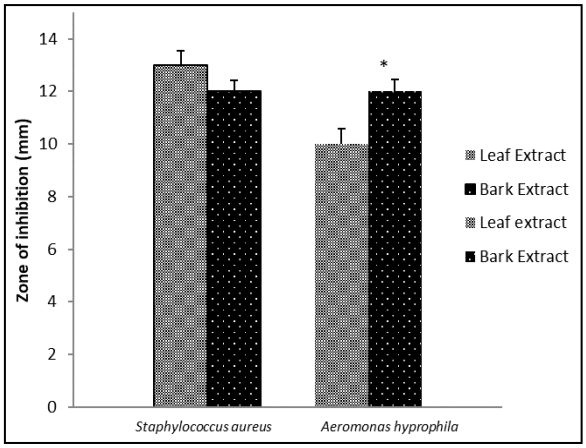


Fig 2: Antibacterial activity of Neem Extract (Leaves and bark) against *Staphylococcus aureus* and *Aeromonas hyprophila*

3.1 Discussion

In this study, two freshwater fish species *viz*., Singhi (*Heteropneustes fossilis*) and Mrigal (*Cirrhinus mrigala*) were selected based on the presence or absence of body scales. Moreover, both the species have high economic value in the Northeast India. The selected bacterial strains *Aeromonas hyprophila* and *Staphylococcus aureus* have significant importance in terms of causing economic losses due to its widespread presence in aquatic environment. The variation demonstrated in antibacterial activity among the fish integumentary extracts may be due to difference in the concentration level of various enzymes present in fish mucus (Subramanian *et al*., 2007) [6, 21]. Fish integumentary, which is generally consists of skin, have a mechanical protective importance against external injuries. The mucus layer present on the skin is believed to be key innate immune secreting compound and considered to be one of the important components of first line of defense against infectious pathogens (Anbuchezhian *et al*., 2011; Subramanian *et al*., 2008) [9, 10]. It is probable that the amount and rate of mucus secretion depends on the environment in which they are exposed such as quantum of microbes inhabited, quality of water, perturbation, etc (Ellis, 2001) [11]. Integumentary extract from *Cirrhinus mrigala and Heteropneutes fossilis* demonstrated bacterial inhibitory property with more effect against gram negative bacteria. However, Haniffa *et al*. (2014) [12] had claimed that more antimicrobial effect of *Heteropneustes fossilis* was achieved against the gram-positive bacteria as compared to gram negative bacteria. Interestingly, more antibacterial activity was observed in the integumentary extract of *Heteropneustes fossilis* (22 mm) than *Cirrhinus mrigala* (20 mm) against the *Aeromonas hyprophila* as compared to *Staphylococcus aureus*. In this present investigation, *Cirrhinus mrigala* extract (20 mm and 15 mm) showed lesser antibacterial activity against *Staphylococcus aureus*. However, the previous work of Kuppulakshmi *et al*. (2008) [13] had found a higher level of antibacterial activity in *Cirrhinus mrigala* extract (23 mm) as compared to *Staphylococcus aureus.*

Azadirachta indica (neem) is considered a multipurpose tree species and it is widely used as an antimicrobial agent. Identification of natural origin substance which is having the potential to derive bioactive compounds in order to develop new pharmaceutical drugs for addressing the issue of antibiotic multi resistant is highly essential. Evaluations of neem leaves and bark extracts antimicrobial activity against *Staphylococcus aureus* have been studied by many workers (Kumar *et al*., 2018; Francine *et al*., 2015; Gajendrasinh *et al*., 2012) [14, 15, 16]. In this study it was found that Neem leaves extract had a higher inhibition ability than bark extract. This may be due to presence more bioactive compounds in the leaves which are responsible for producing greater antibacterial activity than bark extract. Our result supports the works of Reddy *et al*. (2013) [17] and Subapriya and Nagini (2005) [18]. The gram-positive bacteria *Staphylococcus aureus* was observed to possess higher antibacterial activity as compared to gram negative bacteria *Aeromonas hyprophila.* This finding is also consistent with the previous works of Lin *et al.* (1999) [19] and Raut *et al* (2014) [20].

4. Conclusion

The integumentary extracts fish species Singhi and Mrigal possesses bactericidal properties which may help in preventing the fish against bacterial disease. In the present study, it was found that Singhi integumentary extract have more antibacterial property as compared to Mrigal. Likewise, neem leaves and barks ethanol extracts also showed antibacterial activity against *Staphylococcus aureus* and *Aeromonas hyprophila* with leaves extract causing more inhibitory action. Thus, the present study has great importance in identifying the natural origin substance which is having the potential to derive bioactive compounds in order to develop new pharmaceutical drugs for addressing the issue of antibiotic multi resistant.

5. Acknowledgement

We are thankful to Director, ICAR Research Complex for NEH Region, Umiam, Meghalaya for providing necessary facilities to carry out this research work under the project

"Antimicrobial activity of integumentary secretion from selected cultivable fishes" PIMS Project Code: IXX13390.

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