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Isolation, Morphological Identification and Invitro Antibacterial Activity of Crude Ink and Mucus of Dolabella Auricularia, Wedge Sea Hare (Light Foot 1786)

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ABSTRACT
The formative research focuses on the isolation, morphological identification and invitro antibacterial properties of ethanolic crude extracts of ink and mucus derived from sea hare *D.auricularia* a marine gastropod against selected human pathogenic bacteria. Using the disk diffusion method, antibacterial activity evaluated and the inhibition zones ranged from 5–10 mm for ink and 6–8 mm for mucus, with *Staphylococcus aureus* showing the highest susceptibility (10 mm). Compared to ciprofloxacin (28–33 mm), *D.auricularia* extracts were less effective. *Bacillus cereus* exhibited resistance to mucus extract. These findings suggest sea hare *D.auricularia* as a potential source of therapeutic agents, requiring further investigation to isolate bioactive compounds for pharmaceutical applications.

KEYWORDS: *D.auricularia*; Invitro; Ethanolic extract; Antibacterial activity.

INTRODUCTION

The sea is a treasure of life, which contains a wealth of varied organisms that yield new chemical compounds. These natural chemicals hold immense value for application in diverse industries like medicine, cosmetics, nutrition, and agriculture. In recent times, researchers have found numerous novel marine-derived compounds possessing strong medicinal properties. Even though only some of the marine-derived products are presently available on the market, some promising compounds are now in the process of being tested through clinical trials to be developed into drugs. While the marine world offers an extremely rich resource for novel compounds, it also represents a great challenge that requires multidisciplinary approach to bring the marine chemical diversity up to its therapeutic potential. Therefore, the marine environment with special reference to invertebrates that rely solely on innate immune mechanisms for host defence, is a spectacular resource for the development of new antimicrobial compounds (Maripandi *et al.,* 2010). The marine environment comprises of complex ecosystem with a plethora of organisms and many of these organisms are known to possess bioactive compounds as a common means of defence. The marine natural products have been investigated predominantly for their antimicrobial, cytotoxic, antitumour and anti-inflammatory properties (Anand *et al.,* 1997).

An increase in the number of people in the world, having health problems leading to various types of cancers, drug-resistant bacteria, parasitic protozoans and fungal infection is a cause for alarm (Singh *et al.,* 2017). Recently, drug discovery programs have directed their attention to unusual sources like marine invertebrates (molluscs, sponges, sea cucumber, etc) hoping to identify more efficacious therapeutic tools with novel chemical structures and unique modes of action (Nocchi *et al.,* 2017). Sea hares are a group of molluscs, Gastropod; shell-free marine opisthobranchs, which includes several genera and many species (Derby, 2007) and recognized as a source of a diverse range of metabolites 5alpha,8alpha-Endoperoxides belong to a group of oxidized sterols commonly found in marine organisms and display several bioactivities, including antimicrobial, anti-tumour, and immunomodulatory properties. (Pereira *et al.,* 2019). A huge number of molecules have been discovered from Aplysia species possess secondary metabolites (Abe Kawsar *et al.,* 2010), such as anti-cancer, anti-tumour and anti-viral compounds which are very useful in the pharmacological industry. In India, sea slugs have been used for the extraction of natural anti-cancer compounds like Cemadotin, Soblidotin, Kahalalide F and Synthadotin/ILX6512. (Sethi, 2019). The internal defence system of mollusks represents an efficient protection against pathogens and parasites, involving several biological immune processes, such as phagocytosis, encapsulation, cytotoxicity and antigenic recognition of self and non-self. (Alesci, 2023).

Therefore, the screening of marine organisms to explore their potential as a source of biologically active products is necessary. A deep understanding of chemical and biological structure of bioactive compounds from marine organisms

will pave way for identification of novel drugs. Based on the above facts the present study has been initiated as preliminary study to test the antibacterial properties from the crude ink and mucus of marine gastropod sea hare, *D.auricularia.*

MATERIALS AND METHODS

COLLECTION OF *SAMPLES*

For the present study, *D. auricularia* (sea hare) samples were collected from Therespuram, located in the Gulf of Mannar coastal region of Tuticorin. The specimen was collected at a depth range of 30 to 50 m (8° 51′ 217″ N and 078°09′ 048″ E) by using a drift gill net in a traditional fishing craft during a biodiversity survey. After collection, the sea hares were immediately washed with seawater at the sampling site to remove adhered sediments and impurities. Once after proper cleaning the samples were then placed in polythene bags for transportation. On the same day, the samples underwent a quick rinse with tap water in the research laboratory to get rid of remaining contaminants, guaranteeing the purity of the specimens for further analysis. (Hassan *et al.*, 2020).

ISOLATION OF WEDGE SEA HARE (*D.auricularia*)

Live specimens, *D.auricularia* were isolated from seawater in order to ensure safe contamination. Following isolation, the organisms underwent morphological identification and processed for the extraction of mucus and ink for antibacterial activity.

MORPHOLOGICAL IDENTIFICATION

Total body length and width varied from 6.1-8.3cm and 3.4-4.5 cm respectively. Total body weight ranged from 140-1840 g. Body is truncated, flattened and sloping posterior, up to 20-40 cm long. Body colour is muddy brown; Aplysia known to be sea hare because of the two-rabbit ear like tentacles on top of their head called rhinophores smaller than cephalic tentacle on the dorsal side. A shell was present on the ventral side which covers ink gland. Body is covered with tubercles, irregular nodules and skin flaps; The animals giving a prickly appearance due numerous distributions of papillae. It can be easily recognized by a flattened disk on the posterior surface of the animal. This species occurs with soft pustules, so it has a quite knob-like appearance. The head is short and blunt. The inner shell is earlike in typical form. It emits purple ink when disturbed or at dangerous situations. The penis is unarmed and located at right side of the head region.

EXTRACTION OF CRUDE INK AND MUCUS

- **INK EXTRACTION:** The ink sac of species was carefully dissected from the specimens, and the ink was extracted under sterile conditions. The crude ink was diluted with sterile distilled water, centrifuged to remove debris, and stored at -20°C until use.
- **MUCUS EXTRACTION:** Mucus of species was obtained by gently stimulating the specimens to secrete their slime. The mucus secretions were collected, centrifuged, and filtered to obtain clear samples.

PREPARATION OF EXTRACT

Wedge sea hare D.auricularia was dissected with caution to obtain its ink and mucus. After dissection, the ink was drained out and kept in a vial, then the mucus was collected and kept in a different vial. For preparing the extracts, the ink and mucus samples were treated with 70% aqueous ethanol. Further, 1 mL of ink was poured into a sterile tube, and 9 mL of ethanol were added to it. The mixture was blended well for even distribution, creating a 10% (w/v) ink extract in ethanol. following the same, 1 mL of mucus was mixed with 9 mL of ethanol in another sterile tube and blended well, creating a 10% (w/v) mucus extract in ethanol. After the preparation, the extracts were filtered through Whatman No. 4 filter paper to ensure removal of contaminants. The resultant purified extracts were then screened for their activities.

ANTIBACTERIAL ACTIVITY ASSAY BY DISC DIFFUSION METHOD (KIRBY& BAUER, 1966)

REFERENCE MICROBES AND CULTURE MEDIA

During this work, three Gram positive Staphylococcus aureus, Bacillus subtilis, Bacillus cereus and two Gram negative bacteria Escherichia coli, Pseudomonas aeruginosa were used as a reference strains. On the other side, Muller-Hinton agar (MHA) media was used to culture the reference strains and to determine the antibacterial activity of ethanolic wedge sea hare extract, whereas Ciprofloxacin was used as standard.

PROCEDURE FOR ANTIBACTERIAL ASSAY

The test bacteria were inoculated in peptone water and incubated for about 3 – 4 hours at 35 ºC. Mueller Hinton agar plates were prepared and poured in sterile Petri plates. 0.1 ml of bacterial culture was inoculated on the surface of Mueller Hinton agar plates and spread by using L-rod. The inoculated plates were allowed to dry for five minutes. The disk loaded with samples concentration 1000 µg/ml was placed on the surface of inoculated Petri plates using sterile technique. The plate was incubated at 37 ºC for 18-24 hours for bacterial growth. After incubation, the plate was examined for inhibitory zone and the zone of inhibition was measured in mm. The size of the zone and concentration of the antibacterial compound was directly proportional. The greater antibacterial activity was indicated by a larger zone. The diameter was measured in millimetres (mm) and also indicated the susceptibility of the test bacteria to the antibacterial compound.

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RESULT AND DISCUSSION

SYSTEMATIC POSITION

Order: Anaspidea
Superfamily: Aplysioidea
Family: Aplysiidae
Genus: *Dolabella*
Species: *auricularia*

ANTIBACTERIAL ACTIVITY

Antibacterial activity was tested against three Gram positive *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and two Gram negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa* with respect to ethanolic extract of ink and mucus of sea hare. Regarding the antibacterial activity of wedge sea hare's ink and mucus, inhibition zone developed against above mentioned bacteria are shown in Table 1.

Table 1: The inhibition zone by ink and mucus of *D.auricularia* against different bacteria and ciprofloxacin as standard.

Bacteria	Inhibition zone in mm		
	Ciprofloxacin A	I - Ink	M- Mucus
Escherichia coli	33	7	6
Staphylococcus aureus	30	10	7
Bacillus subtilis	28	6	5
Bacillus cereus	31	5	--
Pseudomonas aeruginosa	33	7	8

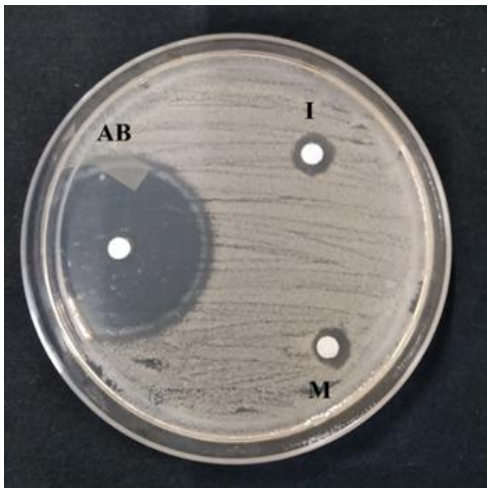

Fig. 1: The inhibition zone formed by ink & mucus against *Escherichia coli*

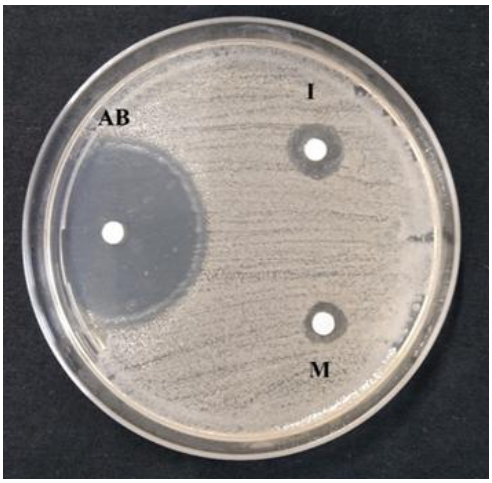

Fig. 2: The inhibition zone formed by ink & mucus against *Staphylococcus aureus*

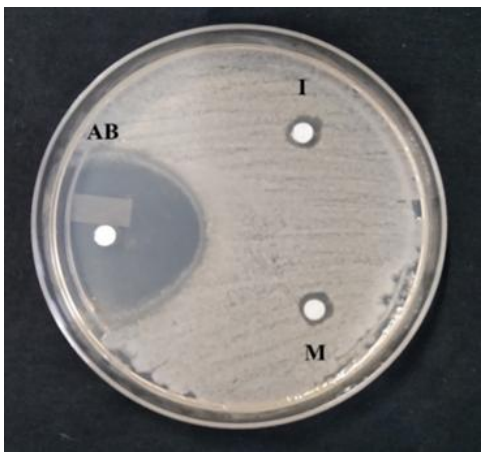

Fig. 1: The inhibition zone formed by ink & mucus against *Bacillus subtilis*

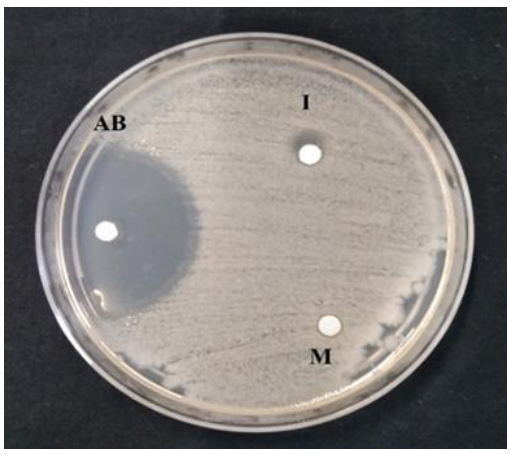

Fig. 2: The inhibition zone formed by ink mucus against *Bacillus cereus*

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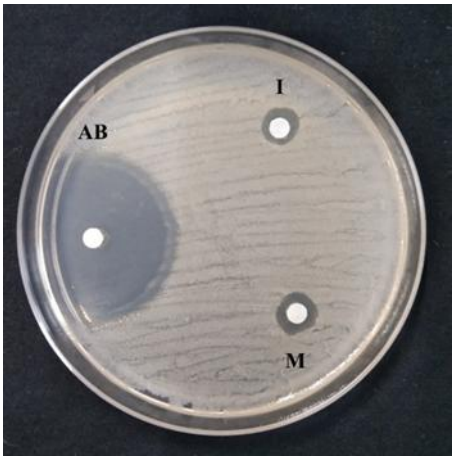

Fig. 5: The inhibition zone formed by ink & mucus against Pseudomonas aeruginosa

The antibacterial activity of ethanolic extract of ink and mucus from the marine gastropod *D.auricularia* against selected human pathogenic bacteria (HPB), *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, and *Pseudomonas aeruginosa*. The zone of inhibition was measured to evaluate the antibacterial activity against these human pathogenic bacteria. To measure the zone of inhibition, Muller-Hinton agar (MHA) was prepared and sterilized by autoclaving at 121°C for 30 minutes. The sterilized MHA was poured into sterile Petri plates and was allowed to solidify. Well cutter was used to create wells and the bacterial culture of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, and *Pseudomonas aeruginosa* was uniformly spread on the Petri plates.

The inhibition activity of ink and mucus was evaluated, additionally Ciprofloxacin was used as standard. The drug used is an effective antibiotic against the growth of bacteria because of its synthetic composition. The chemical name of the antibiotic is onecyclopropyl-6-fluoro-4-oxo-7-(piperazine-1-yl)-1, 4-dihydroquinoline-3-carboxylic acid. Its chemical formula is C17H18FN3O3, and its molecular weight is 331.34 g/mol. Both in vitro and in vivo studies have demonstrated that ciprofloxacin is effective against various gram-positive and gram-negative bacteria isolates. It is frequently used to treat serious digestive, respiratory, and urinary tract infections (Akhtar *et al.,* 2016; Sharma *et al.,* 2017). Data obtained revealed that all bacterial strains were susceptible to ciprofloxacin and the inhibition zones ranged from 28mm to 33mm. Regarding the antibacterial activity of wedge sea hare ink and mucus, inhibition zone of ink ranged from 5mm to 10mm, while mucus ranged from 6mm to 8mm against the tested bacteria. The highest antibacterial activity was detected against *Staphylococcus aureus* (10 mm), followed by *Escherichia coli* (7 mm) and then by *Pseudomonas aeruginosa* (7 mm). Thus, the antibacterial properties of Aplysia's ink and mucus were less effective in comparison to ciprofloxacin. These results are in agreement with the findings of Ibrahim *et al.,* (2020). Melo *et al.,* (1998) findings showed the crude extract inhibited the growth of all species including *Pseudomonas aeruginosa,* our study also showed higher antibacterial activity against the same species. *Bacillus cereus* (5mm), showed high resistant towards ink and no inhibition towards mucus whereas in the study of Ibrahim et al., (2020) *Bacillus cereus* showed no inhibition towards crude extracts of *Aplysia fasciata*. Preliminary study revealed that the Gram-positive bacteria were more resistant than Gram-negative bacteria, denied the findings of Exner *et al.,* (2017). On other hand, antibacterial activity of sea hare, D.*auricularia* ink and mucus was less potent in comparison with ciprofloxacin.

CONCLUSION

In conclusion, the present study indicates, the ethanolic crude ink and mucus of *D.auricularia* showed good spectrum of antibacterial activity. The study proved as therapeutic agents in handling bacterial infections and diseases. However further investigations to separate and elucidate the bioactive compounds and mechanism of actions are needed for development of novel drugs. Moreover, present data of our study can collaborate with the defense mechanism of the genus aplysia.

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