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## Bioactive proteins from pipefishes

E. Rethna Priya, S. Ravichandran\*, R. Ezhilmathi

Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai–608 502, India

### PEER REVIEW

#### Peer reviewer

Dr. Thiru. Vi. Ka., Associate Professor,  
Govt. Arts College, Tiruvarur, India.  
Tel: 0091–9441811123  
E-mail: [aldobiotech@yahoo.com](mailto:aldobiotech@yahoo.com)

#### Comments

This study mainly deals with the antimicrobial properties of the two pipefishes. These results may lead to the use of these biologically active proteins that can be used for human welfare to treat various microbial diseases.

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### ABSTRACT

**Objective:** To screen antimicrobial potency of some pipefish species collected from Tuticorin coastal environment.

**Methods:** Antimicrobial activity of pipefishes in methanol extract was investigated against 10 bacterial and 10 fungal human pathogenic strains.

**Results:** Among the tested strains, in *Centriscus scutatus*, pipefish showed maximum zone of inhibition against *Vibrio cholerae* (8 mm) and minimum in the sample of *Hippichthys cyanospilos* against *Klebseilla pneumoniae* (2 mm). In positive control, maximum zone of inhibition was recorded in *Vibrio cholerae* (9 mm) and minimum in *Klebseilla pneumoniae*, and *Salmonella paratyphi* (5 mm). Chemical investigation indicated the presence of peptides as evidenced by ninhydrin positive spots on thin layer chromatography and presence of peptide. In SDS PAGE, in *Centriscus scutatus*, four bands were detected in the gel that represented the presence of proteins in the range nearly 25.8–75 kDa. In *Hippichthys cyanospilos*, five bands were detected in the gel that represented the presence of proteins in the range nearly 20.5–78 kDa. The result of FT-IR spectrum revealed that the pipe fishes extracts comprised to have peptide derivatives as their predominant chemical groups.

**Conclusions:** It can be conclude that this present investigation suggests the tested pipe fishes will be a potential source of natural bioactive compounds.

### KEYWORDS

Antimicrobial, Bioactive compound, Pipefishes, SDS PAGE, Thin Layer Chromatography, FTIR

## 1. Introduction

Marine organisms represent an excellent source for bioactive compounds. Fishes are one of the organisms that have managed to survive in a milieu of pathogenic organisms. Fishes live in intimate contact with an environment containing both saprophytic and pathogenic microbes capable of digesting and degrading fish tissues[1,2]. The slow adaptive immune response of fish makes innate immunity, which is fast acting and temperature independent predominant system of fish host defense[1]. This innate

immune response is essential for the survival of this whole class of animals. The defense includes many elements such as antimicrobial peptides[3], antimicrobial lipid[4], and polypeptides[5]. Non-classical complement activation leads to release of cytokines, inflammation and phagocytosis[1,6]. Concisely, fish have evolved a number of innate immune responses to defend themselves against infection. Seahorses are used as a traditional medicine and invigorant for the treatment of erectile dysfunction (ED) due to the compellent activity. However, antimicrobial effects of pipefishes are not yet studied. Hence the present study is an attempt to screen

\*Corresponding author: S. Ravichandran, Senior Lecturer, Ph.D, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai–608 502, India.

Tel: +91–9443909137

E-mail: [sravicass@gmail.com](mailto:sravicass@gmail.com)

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antimicrobial potency of some pipefish species collected from Tuticorin coastal environment.

## 2. Materials and methods

### 2.1. Collection and Identification

Two pipefish species of *Hippichthys cyanospilos* (*H. cyanospilos*) and *Centriscus scutatus* (*C. scutatus*) were collected as a trash resource from the Tuticorin coastal environment (Lat 8.8100 °N, Long 78.1400 °E) southeast coast of India. Pipe fishes were identified based on the skeletal characteristics (size and shape) and external morphology. Collected pipe fishes were stored at -80 °C before extraction.

### 2.2. Extraction

The frozen samples were cut into small pieces and then homogenized a mortar and pestle. The minced sample was exhaustively extracted with methanol. The combined organic extract was evaporated at reduced pressure at 35 °C on a rotavapor and then used for further study.

### 2.3. Antimicrobial activity

The spectrum of antibacterial and antifungal activity was studied against 20 pathogenic strains viz., 10 bacterial *Klebsiella pneumonia* (*K. pneumoniae*), *Proteus mirabilis*, *Salmonella typhi* (*S. typhi*), *Escherichia coli*, *Staphylococcus aureus*, *Vibrio parahemolyticus* (*V. parahemolyticus*), *Pseudomonas aeruginosa*, *Vibrio cholerae* (*V. cholerae*), *Salmonella paratyphi* (*S. paratyphi*), *Klebsiella oxytoca* and ten fungal *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus alternaria*, *Candida albicans*, *Epidermophyton floccosum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Pencillium* sp., *Rhizopus* sp., *Mucor* sp. human pathogens. These pathogens strains were obtained from the Department of Medical Microbiology (Raja Muthiyah Medical College and Hospital) Annamalai University, Annamalai nagar. Positive controls were tetracycline for bacteria and flucanazole for fungal. Antibacterial and antifungal activity was observed as zones of inhibition.

### 2.4. Thin layer chromatography (TLC)

TLC plate was first developed in a butanol: acetic acid: water (B:A:W) solvent system, dried at room temperature, sprayed with ninhydrin and heated at 100 °C in oven for few minutes till the colored spots were visible. Pink spots were checked for the presence of proteins.

### 2.5. SDS-PAGE

SDS-PAGE is used to find out the molecular weight active fractions of the sample. SDS-PAGE was performed in 12% separating gels, according to the method described by Laemmli[7].

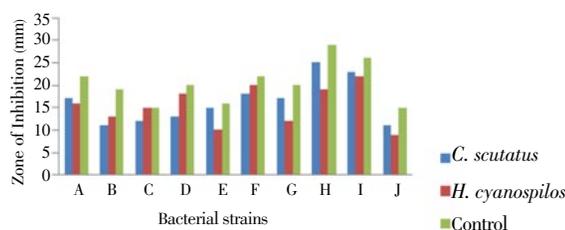
### 2.6. Fourier transform-infrared spectroscopy (FT-IR)

FT-IR was used to identify the chemical identities in a wide range of compounds. Infrared spectroscopy was a useful analytical technique for detection of functional groups in organic compounds. Sample of 10 mg was mixed with 100 mg of dried Pottasium bromide (KBr) was compressed to prepare as a salt disc (10 mm dia) for spectrometer reading further. The infrared absorption bands identified molecular components and structures.

## 3. Results

### 3.1. Antimicrobial activity

Antimicrobial activity of methanol was investigated against 10 bacterial and 10 fungal human pathogenic strains. Among the tested strains in *C. scutatus*, maximum activity (zone of inhibition) was recorded in *V. cholerae* (8 mm) and minimum in *K. pneumoniae*, and *S. typhi* (3 mm), and in *H. cyanospilos*, maximum zone of inhibition was recorded against *V. parahemolyticus* (7 mm) and minimum in *K. pneumoniae* (2 mm). In positive control, maximum zone of inhibition was recorded in *V. cholerae* (9 mm) and minimum in *K. pneumoniae*, and *S. paratyphi* (5 mm) (Figure 1). But none of the fungal strains were showing notable activity in both the pipefish samples.



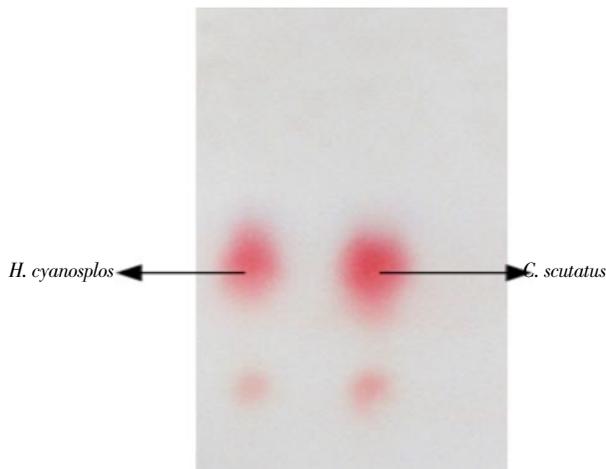
**Figure 1.** Antibacterial activity of 10 bacterial strains.

A: *S. aureus*; B: *S. typhi*; C: *S. paratyphi*; D: *K. oxytoca*; E: *P. aeruginosa*; F: *E. coli*; G: *P. mirabilis*; H: *V. cholerae*; I: *V. parahemolyticus*; J: *K. pneumoniae*.

### 3.2. TLC

TLC profiling was done for the samples of the *C. scutatus* and *H. cyanospilos* in solvent system which was a combination of butanol, acetic acid and water (B:A:W) in proportions of 5:1:4. The plates developed in the solvent

systems showed light pink spots, while sprayed in ninhydrin showing pink spots which indicated the presence of amino acids and peptides (Figure 2).

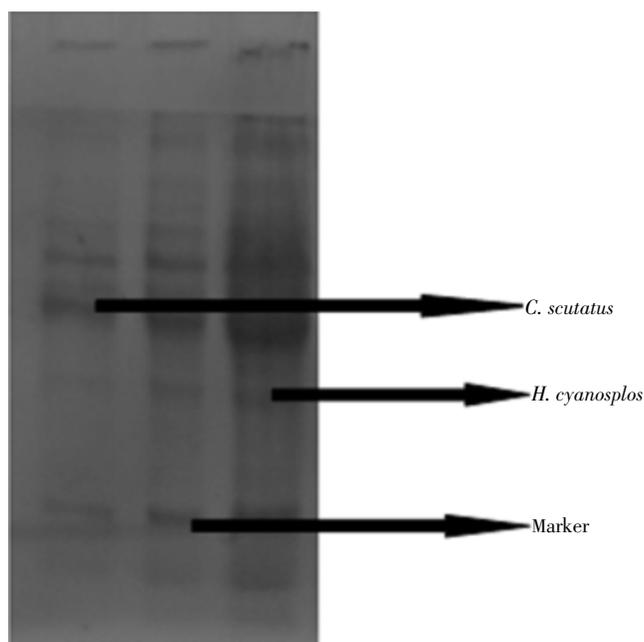


**Figure 2.** Thin-Layer Chromatography.

### 3.3. Determination of molecular weight

The samples *C. scutatus* and *H. cyanospilos* showed antibacterial activity was subjected to SDS-PAGE to estimate the molecular weight of active proteins present in it. Different standard were used to determine the molecular weight of proteins. The stained gel revealed that the contained a simple population of proteins. There was different molecular weight markers that were used.

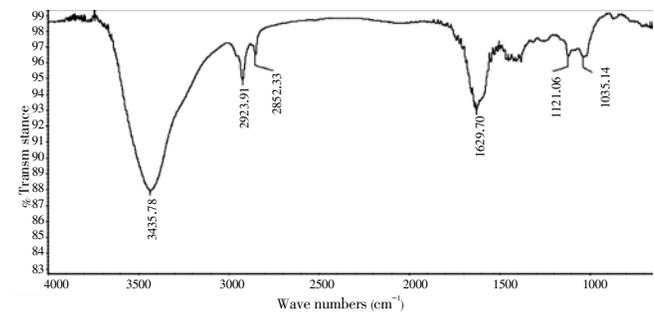
In *C. scutatus*, four bands were detected in the gel that represented the presence of proteins in the range nearly 25.8–75 kDa. In *H. cyanospilos*, five bands were detected in the gel that represented the presence of proteins in the range nearly 20.5–78 kDa (Figure 3).



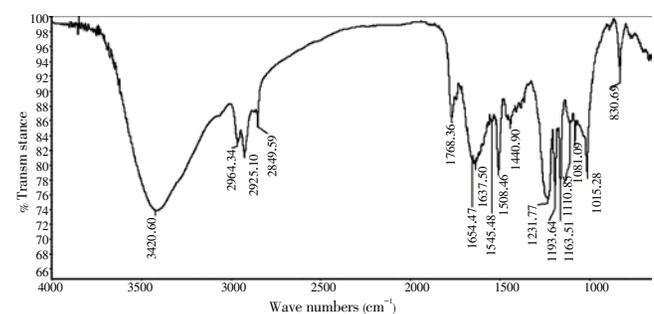
**Figure 3.** SDS-PAGE.

### 3.4. FT-IR

As a preliminary analytical screening, three species of pipe fishes exhibited very good antimicrobial potency and were subjected for FT-IR spectral studies for screening of basic functional compounds present in it. The results of FT-IR spectrum revealed that the pipe fishes extracts comprises to have peptide and phenolic derivatives as their predominant chemical groups which is shown in Figures 4–5.



**Figure 4.** FT-IR of *C. scutatus*.



**Figure 5.** FT-IR of *H. cyanospilos*.

## 4. Discussion

Endogenous antimicrobial peptides in marine organisms are exciting candidates for the development of new antimicrobial compounds, due to their broad activity spectrum and the difficulty for bacteria to develop resistance to them[8]. In the present study, *C. scutatus* and *H. cyanospilos* showed antimicrobial activity against a range of both Gram-positive and negative pathogenic bacterial strains. In recent years, great attention has been paid to the studies of the bioactivity of natural products and their potential pharmacological utilization. The rationale of searching for drugs from marine environment from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition, predation, surface fouling and reproduction. Many of these organisms have been found to possess antimicrobial properties, although most of the antibacterial agents that have been isolated from marine sources have not

been active enough to compete with classical antimicrobials obtained from microorganisms[9].

However, majority of marine organisms are yet to be screened for discovering useful antibiotics. Seahorses are proved to possess an enormous bioactive potential like antioxidant and anti–arthritis compounds[10–12]. Comparative antimicrobial chart of antibacterial activity have previously detected in soft corals[13]. In the present study, the maximum antibacterial effect was observed in the pipefish of *C. scutatus* against *V. cholerae* and minimum in *K. pneumoniae*, and *S. typhi* in the case of *H. cyanospilos*, maximum zone of inhibition was noticed against *V. parahemolyticus* and *K. pneumoniae*. In control, maximum zone of inhibition was recorded in *V. cholera* and minimum in *K. pneumoniae*, and *S. paratyphi*. Similar result was observed with the puffer fish *Arothron immaculatus* skin and liver extracts against clinical pathogens[14]. Number of  $\alpha$ –helical antimicrobial peptides such as pardaxins, misgurin, pleurocidins, parasin, oncorhyncin II and III, chrysopsin and Hagfish Intestinal Antimicrobial Peptides (HFIAP) have been isolated from different species of fishes[15–17]. But antimicrobial activities of the present study are not reported earlier.

The pipefish samples that showed antibacterial activity were subjected to SDS–PAGE. Clear bands were detected in the gel which represented proteins of molecular weight between 20–56 kDa which is similar to the antibacterial peptides in the hemolymph of the range of 1–100 kDa reported from *C. sapidus*[18]. Although there were several reports on antibacterial activity in seminal plasma and few antibacterial peptides have been reported in *Scylla serrata* [19–21].

Out of the two species studied maximum activity was found in *Callinectes sapidus* than the *H. cyanospilos*. This shows that the pipefishes are extremely strange to the bacterial contact and its mediated infection. There is growing evidence that pathogens have significant impacts on marine systems[22]. Fungal pathogens are present in reef communities and emerge when conditions favor them, and when hosts are stressed and their defenses compromised[23]. Changing water quality can affect the host resistance to pathogens, which is an important factor in mediating host pathogen interactions [24].

The antimicrobial property of the seahorse extracts reveals that they are high enough to bring the effect against fungal pathogens than the bacterial. It may be due to the incidence of bacterial presence in their habitat induce the seahorses to produce the antimicrobial compounds. Meager antimicrobial effect towards fungus may be of due to their rare presence in their habitat. Majority of the positive

results have been recorded in the methanolic fraction and it shows that it is a good solvent system to for the solubility of bioactive compounds present in the pipefishes. Seahorses are confirmed to have a phthalate derived compounds in it to bring an effective cathepsin inhibitory activity[25].

The future prospective of this research mission is facing towards the analysis of possible organic compounds present in the pipefish extract and compound purification and isolation will be attempted in order to bring the exact target moiety, which is responsible for bringing the antimicrobial effect against the tested pathogenic organisms.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

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### Comments

#### Background

Marine organisms represent an excellent source for bioactive compounds. Fishes are one of the organisms that have managed to survive in a milieu of pathogenic organisms. Pipe fishes are one of the important candidate organisms which have been used in Chinese traditional medicine from time immemorial.

#### Research frontiers

Seahorses and Pipe fishes are used in Chinese medicine treat various diseases. In the present study, authors have demonstrated the antimicrobial activity of *C. scutatus* and *H. cyanospilos*

#### Related reports

The antimicrobial property of the Pipe fish's extracts reveals that they are high enough to bring the effect against fungal pathogens than the bacterial. It may be due to the incidence of bacterial presence in their habitat induce the Pipe fishes to produce the antimicrobial compounds. Majority of the positive results have been recorded in the methanolic fraction and it shows that it is a good solvent system to for the solubility of bioactive compounds present

in the pipefishes and confirmed to have a phthalate derived compounds in it to bring an effective cathepsin inhibitory activity.

### Innovations and breakthroughs

In this paper, authors have isolated the biologically active proteins which can be useful to human welfare.

### Applications

The isolated bioactive proteins may have the application in the field of biotechnology to treat various microbial diseases.

### Peer review

This study mainly deals with the antimicrobial properties of the two pipefishes. These results may lead to the use of these biologically active proteins which can be used for human welfare to treat various microbial diseases.

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