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ANTIMICROBIAL ACTIVITY OF THE BODY FLUID OF *SACCOSTREA CUCULLATA* FROM SUNDERBAN MANGROVE FOREST, NORTH-EAST COAST OF BAY OF BENGAL, INDIA

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ABSTRACT

Oysters are nutritious sea food and an important source for biomedicine products. In this study, antimicrobial activity of body fluid of *Saccostrea cucullata* oyster was investigated against different human pathogens such as gram-positive bacteria *Staphylococcus aureus, Bacillus subtilis* and gram negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and two fungus *Candida albicans* and *Aspergillus flavus*. The body fluid of *Saccostrea cucullata* showed highest antimicrobial activity against *B. subtilis* and *C. albicans*. The MIC value of crude body fluid against *B. subtilis* and *C. albicans* were 5µg/ml and 4mg/ml respectively. From the lyophilized body fluid four different antimicrobial proteins were purified by HPLC and molecular mass were determined by MALDI-TOF spectroscopy as 35.1 kDa, 46kDa, 50.5 kDa and 54.7 kDa. Among them, 50.5kDa proteins showed maximum growth inhibitory activity against *B. subtilis* and *C. albicans.* The research also revealed that it has synergic effect against analyzed selected bacterial and fungal strain. The synergic effect against *B. subtilis* showed maximum inhibitory activity at the concentration of 5µg/ml whereas; *C. albicans* exhibit maximum inhibitory activity at the concentration of 25mg/ml. The results indicate that body fluid of *S. cucullata* is a potential source of antibacterial and antifungal agents used

as ethno-medicinal purpose. Therefore, body fluid of *Saccostrea cucullata* can be an alternative natural bio-product to cure some bacterial infections (e.g., *B. subtilis)* and fungal infections (e.g., *C. albicans*).

KEYWORDS: Oysters, *Saccostrea cucullata*, antimicrobial activity.

INTRODUCTION

Ocean is known as a large biodiversity of fauna and flora.[1] Therefore, the marine environment is an exceptional reservoir of rich diversity of marine organisms such as slugs, whelks, clams, mussels, oyster, scallops, squids and octopus.[2] Many studies reveled that molluscs has antitumor, antileukemic and antiviral activities.[3-5] So, there is good opportunity for the discovery of new bioactive substances from marine and estuarine ecosystems.

Oysters in the oceans are a common sight and are virtually untapped resource of novel compounds. Oysters are bivalve soft bodied molluscan shellfish under class Bivalvia, mostly marine or estuarine in habit. As a sea food, oysters have been introduced and established in food permanently in at least 24 countries.[6] In India, the common oyster species are *Saccostrea cucullata, Crassostrea madrasensis*, *Crassostrea gryphoides, Crassostrea rivularis* and *Crassostrea discoidea*. Out of these five dominant species, *S. cucullata* is the most abundant bivalve in the Hooghly estuary and normally found attached to rock, boulders and several under water structures, submerged branches and trunks of mangroves, concretes, embankments and piles and even on light house bases of Indian Sundarbans ecosystem.[7]

On the other hand, antibiotic resistance of different types of microorganism are the upcoming big problem for the treatment of human disease.[8-10] The increasing tendency of microbial infections, rapid immergence of drug resistant to antibiotics and quick evolution through mutation, poses one of the greatest threats to microbial infection control that has generated the urgency to develop new class of antibiotics. This has led to search of more antimicrobial substances from other natural sources including the aquatic environment.[11] There are about 7500 species of bivalves are identified [12] which contain antibody-like materials that serve as defense from disease- causing organisms.[13] The molecular biological approach has proven more powerful than earlier protein/peptide based technique for the detection of novel conotoxins.[12] The screening of marine organisms, especially marine bivalves for therapeutic drugs are of greater interest now-a-days. There is a vital interest in discovering new

antimicrobial compounds with fewer environmental and toxicological risks and no resistance developed by the pathogens.[14] Therefore, the aim of the present study was to evaluate the antimicrobial activity of the body fluid of *Saccostrea cucullata* against different pathogenic bacterial and fungal strains.

MATERIALS AND METHODS

Materials and reagents

Mueller-Hinton Agar (MHA) and Potato-dextrose agar (PDA) medium were purchased from HiMedia Laboratories Pvt. Ltd., India. Tetracycline and Fluconazole were purchased from Sigma-Aldrich, USA. All analytical grade reagents were purchased from Merck, Germany.

Collection and Extraction of sample

Live specimens of *Saccostrea cucullata* were collected from Frezargaunge river at Bakkhali part of Indian Sundarbans at the apex of the Bay of Bengal (between 21°32'to 22°40' N latitude and 88°85'to 89°00' E longitude) India .The sample were kept in ice sterile bucket and taken to the laboratory for analysis within 3 hours of procurement. First of all, the oysters were rinsed thoroughly by sterilized distilled water then hard shell were punctured laterally and the body fluid was sucked by sterile syringe. The crude body fluid of *S. cucullata* was centrifuged at 15,000 rpm for 30 min at 4^0C, the supernatant was collected and stored at -20^0C. The total soluble protein was estimated by Bradford method.

Microbial Cultures

The bacterial strains such as gram-positive *Staphylococcus aureus, Bacillus subtilis* and gram negative *Escherichia coli*, *Pseudomonas aeruginosa* and two fungus *Candida albicans* and *Aspergillus flavus* of pure culture were gifted from Indian Institute of Technology, Kharagpur (IIT, Kharagpur) and Haldia Institute of Technology in India. All the cultures were maintained and sub cultured on nutrient agar medium.

Antimicrobial assay of *S. cucullata* body fluid against selected pathogens

In vitro antibacterial activities of all aqueous body fluid extracts of *S. cucullata* was determined by standard agar well diffusion assay.[15] Petri dishes (100 mm) containing 25 ml of Mueller–Hinton Agar and potato-Dextrose agar seeded with 100 µl inoculum of bacterial strain and fungal strain (Inoculum size was adjusted so as to deliver a final inoculum of approximately 10^6 CFU/ml for bacteria and $2x10^5$ for fungal spore). Media was allowed to solidify and then individual Petri dishes were marked for the bacteria and fungi inoculated.

Wells were cut into solidified agar media with the help of sterilized cup-borer. 100 µl of each body fluid extract was poured in the respective wells and the plates were incubated at 37°C for bacteria overnight[16] and 30ºC for fungi for 96 hours.[17] Sterilized distilled water was used as negative control. The sensitivity was recorded by measuring the clear zone of growth inhibition on agar surface around the discs.

Determination of Minimum inhibitory concentration (MIC) value of lyophilized Body-fluid

Minimum inhibitory concentration (MIC) was determined by agar and broth dilution methods.[18] A twofold serial dilution (0- 10µg/ml for bacteria and 0-30 mg/ml for fungi) of the body fluid was prepared in Mueller–Hinton Agar (bacteria) or potato-Dextrose agar (fungi). For agar dilution assay, previously prepared sensitivity plates, using serial two-fold dilutions of the body fluid, were spot-inoculated (2 x 10^6 CFU per spot for bacteria and 2x10^5 spores per spot for fungi). The inoculated plates were then incubated at 37°C for 24 h (bacteria) and 30°C for 96 h (fungi). While for broth dilution tests, 0.1 ml of standardized suspension of bacteria (10^6 CFU ml $^{-1}$) or fungal spores (5 x 10^5 spores ml^{-1}) was added to each tube containing extracts (final concentration of 0- 10 µg/ml for bacteria and 0-30 mg/ml for fungi) and incubated with shaking, at 37°C for 24 h (bacteria) or at 30°C for 96 h (fungi). [17] The lowest concentration of the plate or tube which did not show any visible growth after macroscopic evaluation was considered as the MIC.[19]

Determination of MIC value of tetracycline and fluconazole

For this present study, tetracycline antibiotic and fluconazole anti-fungal used for positive control. MIC value of tetracycline and fluconazole were determined by agar and broth dilution methods.[18] A twofold serial dilution (0- 250 µg/ml for bacteria and 0-60 mg/ml for fungi) of tetracycline and fluconazole were prepared in Mueller–Hinton Agar (bacteria) or potato-Dextrose agar (fungi). For agar dilution assay, previously prepared sensitivity plates, using serial two-fold dilutions of the tetracycline and fluconazole, were spot-inoculated (2 x 10^6 CFU per spot for bacteria and 2x10^5 spores per spot for fungi). The inoculated plates were then incubated at 37°C for 24 h (bacteria) and 30°C for 96 h (fungi). While for broth dilution tests, 0.1 ml of standardized suspension of bacteria (10^6 CFU ml $^{-1}$) or fungal spores (5 x 10^5 spores ml^{-1}) was added to each tube containing tetracycline and fluconazole (final concentration of 0- 250µg/ml for bacteria and 0-60 mg/ml for fungi) and incubated with shaking, at 37°C for 24 h (bacteria) or at 30°C for 96 h (fungi). [17] The lowest concentration

of the plate or tube which did not show any visible growth after macroscopic evaluation was considered as the MIC.[19]

Determination of synergic effect of mixed solution against selected pathogens

Standard agar well diffusion assay[15] was applied to determine the combined effect of antibiotics and lyophilized oyster body fluid. Petri dishes (100 mm) containing 25 ml of Mueller–Hinton Agar and potato-Dextrose agar seeded with 100 µl inoculum of bacterial strain and fungal strain (Inoculum size was adjusted so as to deliver a final inoculum of approximately 10^6 CFU/ml for bacteria and $2x10^5$ foe fungal spore).). Media was allowed to solidify and then individual Petri dishes were marked for the bacteria and fungi inoculated. Wells were cut into solidified agar media with the help of sterilized cup-borer. 100 µl of mixed solution (50µl antibiotics and 50µl lyophilized body fluid) was poured in the respective wells and the plates were incubated at 37°C for bacteria overnight [16] and 28^0C for fungi for 96 hours.[17] Sterilized distilled water was used as negative control tetracycline and fluconazole were used as positive control.

Estimation of Molecular weight using SDS-PAGE

The molecular weight of the lyophilized supernent of *S. cucullata*'s body fluid was analyzed by SDS-PAGE (15% gel) by using of Laemmli protocol. After electrophoresis, proteins were stained with Coomassie blue. Then the proteins bands were cut and eluted for further processing.

Analysis of aqueous Body Fluid Extract by HPLC

The collected venom (100ml) sample was centrifuged twice for 10 min at 13,000 rpm to precipitate the residual debris. The supernatant was then lyophilized and re-dissolved in 1ml of 5% (v/v) acetonitrile solution containing 0.01% (v/v) trifluoroacetic acid. The sample was fractioned by reverse phase-HPLC (Agilent 1100 series, USA) with a ZORBAX 300 SB C18 column (4.6mm×150mm, particle size 5µ). The sterile Milli-Q water with 0.1%TFA (solvent A) and 80% acetonitrile with 0.1%TFA (solvent B) were used as mobile phase. The system was operated at 1ml min^{-1} flow rate with linear gradient of solvent B (0-60%) for 50 min and the detection was monitored at 215nm in a diode array detector. Selected peaks of HPLC chromatogram were collected using a fraction collector (Gilson, France) coupled with the system.

Determination of Antimicrobial activity of HPLC- Fractionates

In vitro antibacterial activities of four HPLC-fractions sample of *S. cucullata*'s body fluid were determined by standard agar well diffusion assay.[15] Petri dishes (100 mm) containing 25 ml of Mueller–Hinton Agar and potato-Dextrose agar seeded with 100 µl inoculum of bacterial strain and fungal strain (Inoculum size was adjusted so as to deliver a final inoculum of approximately 10^6 CFU/ml for bacteria and $2x10^5$ for fungal spore). Media was allowed to solidify and then individual Petri dishes were marked for the bacteria and fungi inoculated. Wells were cut into solidified agar media with the help of sterilized cup-borer. 100 µl of 100 µl of each fractionates was poured in the respective wells and the plates were incubated at 37°C for bacteria overnight[16] and 30ºC for fungi for 96 hours.[17] Sterilized distilled water were used as negative control while HPLC-fractionates (1 U strength) was used as positive control. The sensitivity was recorded by measuring the clear zone of growth inhibition on agar surface around the discs.

MALDI-TOF Spectrometry

The HPLC fractionates were determined by Voyger DE ProTM mass spectrometer equipped with 337nm N2 laser (Applied Biosystem, USA). The lyophilized *S. cucullata*'s body fluid was directly applied (non-reduced form) and separately reduced with dithiothreitol (DTT) in 50mM NH_4HCO_3 at 37^0C for 2 hours and followed by acidification with 1% TFA. Two microliter of protein was mixed with 24µl of CHCA (cyano-4-hydroxycinnamic acid) 10 mg ml^{-1} which was used as matrix. Then, 1 µl sample was spotted onto stainless MALDI plate and allowed to be dried prior to the MALDI analysis. The spectra were recorded in the linear, positive mode ion mode with an accelerating voltage 20 KV and average 100 laser shots with a grid voltage of 90%.

Statistical analysis

Significant differences were analyzed using Student's T-test. A p value of < 0.05 is considered as significant.

RESULTS

Antimicrobial activity of body fluid of *S. Cucullata* were investigated against six human pathogens such as gram positive bacteria *Staphylococcus aureus*, *Bacillus subtilis,* gram negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and two fungus *Candida albicans* and *Aspergillus flavus*. From the experiment, it was observed that the body fluid of *S. cucullata* oyster showed highest antimicrobial activity against *B. subtilis* (20 mm)

and *C. albicans* (17 mm). In addition, antimicrobial activity of the body fluid of oyster against *Escherichia coli* was 18 mm, *Staphylococcus aureus* was 15 mm, *and Pseudomonas aeruginosa* was 13 mm and *Aspergillus flavus* 11 mm (Figure 1). In addition, MIC value of lyophilized body fluid of *S. cucullata* exhibit inhibitory activity mostly against bacterial strain *B. subtilis* at 5µg/ml and fungal strain *C. albicans* at 4mg/ml.

Tetracycline is an antibiotic, was used as standard drug against *B. subtilis* and fluconazole was similarly used as an anti-fungal reference drug against *C. albicans*. It was revealed that at 5µg/ml concentration of tetracycline had maximum inhibitory effect against *B. subtilis*, i.e. the inhibition zone was 10 mm. similarly, at 25mg/ml concentration of fluconazole had maximum inhibitory against *C. albicans*, inhibition zone 12 mm. When same concentration i.e. 5µg/ml of lyophilized body fluid was used to test the inhibitory effect against *B. subtilis*, then inhibitory effect was significantly increased , i.e, zone of inhibition was 15 mm. It was found that the synergic effect of tetracycline and body fluid (1:1) had maximum inhibitory effect against *B. subtilis*, zone of inhibition was 21 mm. Similarly at 25mg/ml of lyophilized body fluid and the synergic effect of Fluconazole and body fluid (1:1) had maximum inhibitory effect against *C. albicans*, zone of inhibition was 16 mm and 28 mm respectively (Figure 2). Therefore, body fluid *S. cucullata* had more antimicrobial effect against *B. subtilis* and *C. albicans* compare to tetracycline and fluconazole respectively. In addition, they have also synergic effect against microorganism.

It was found that there were four bands of crude body fluid of oyster which was detected by SDS-PAGE (Figure 3). From HPLC- Chromatogram of lyophilized body fluid of *S. cucullata*, it was found that there mainly 4 type of proteins say as protein 1, protein 2, protein 3,and Protein 4 were present of the body fluid of *S. cucullata* (Figure 4) with a major peak at 3 mint in 215nm. Protein 3 exhibit maximum inhibitory activity against *B. subtilis* and *C. albicans*, zone of inhibition were 19mm and 22mm respectively (Figure 5). In addition, it was shown that the mass of proteins 35KDa, 46KDa, 50.5KDa and 54.7KDa proteins which were determined by MALDI TOF spectrophotometry analysis (Figure 6).Therefore, molecular mas of 50.5 KDa protein presented in body fluid *S. cucullata* which showed the maximum antimicrobial activity.

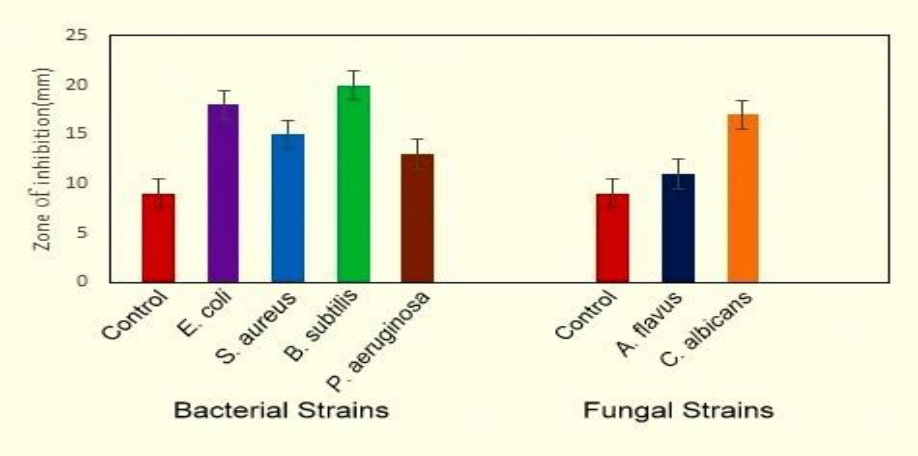

Figure 1: **Anti-microbial activity of body fluid of *S. cucullata* against human pathogens such as gram-positive *Staphylococcus aureus, Bacillus subtilis* and gram negative *Escherichia coli, Pseudomonas aeruginosa* and two fungus *Candida albicans* and *Aspergillus flavus.***

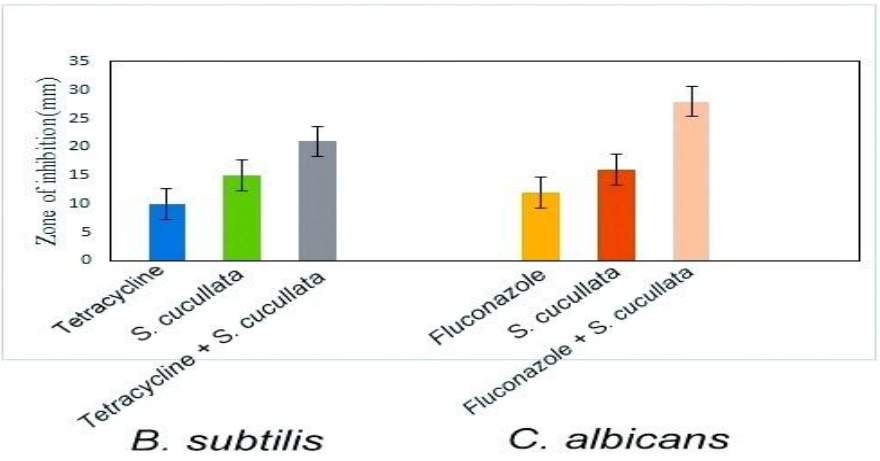

Figure 2: Determination of synergic effect of body fluid against *against Bacillus subtilis* and *C. albicans*. Here Tetracycline and Fluconazole were used as positive control antibacterial and anti-fungal drugs respectively.

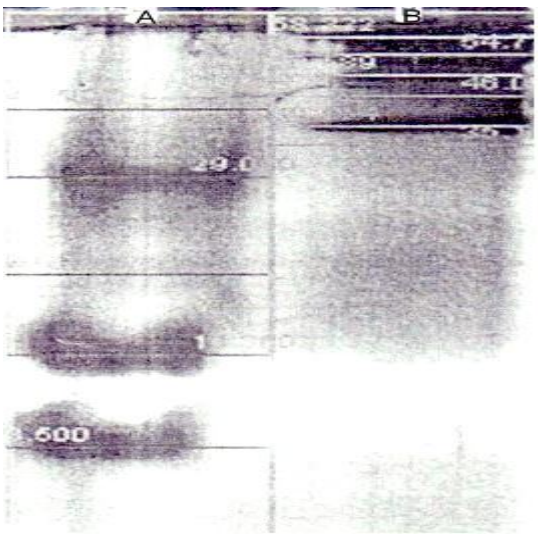

Figure 3. SDS-PAGE of body fluid (lane A and lane B were molecular marker and body fluid respectively).

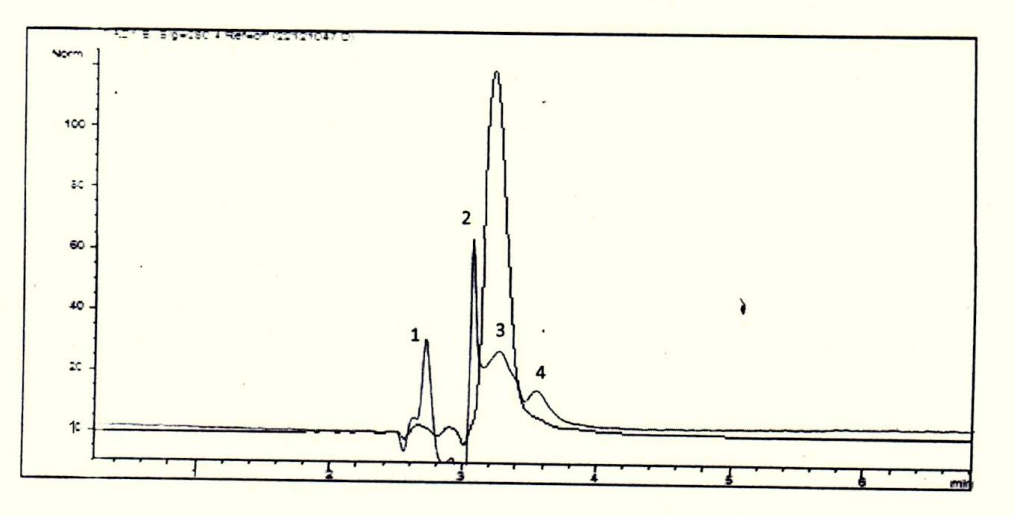

Figure 4: Determination of HPLC- Chromatogram of lyophilized body fluid of *S. cucullata.*

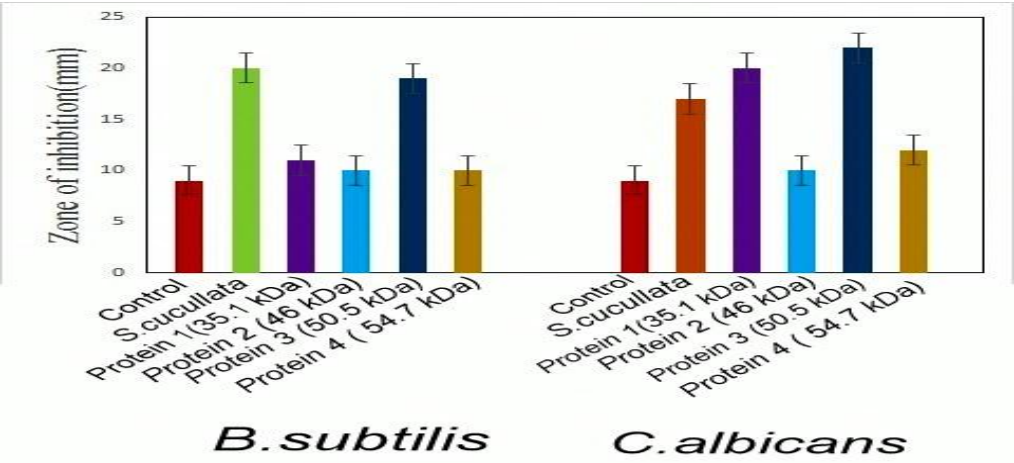

Figure 5: Determination of Antimicrobial activity of HPLC- Fractionates of lyophilized body fluid of *S. cucullata* against *B. subtilis* and *C. albicans.*

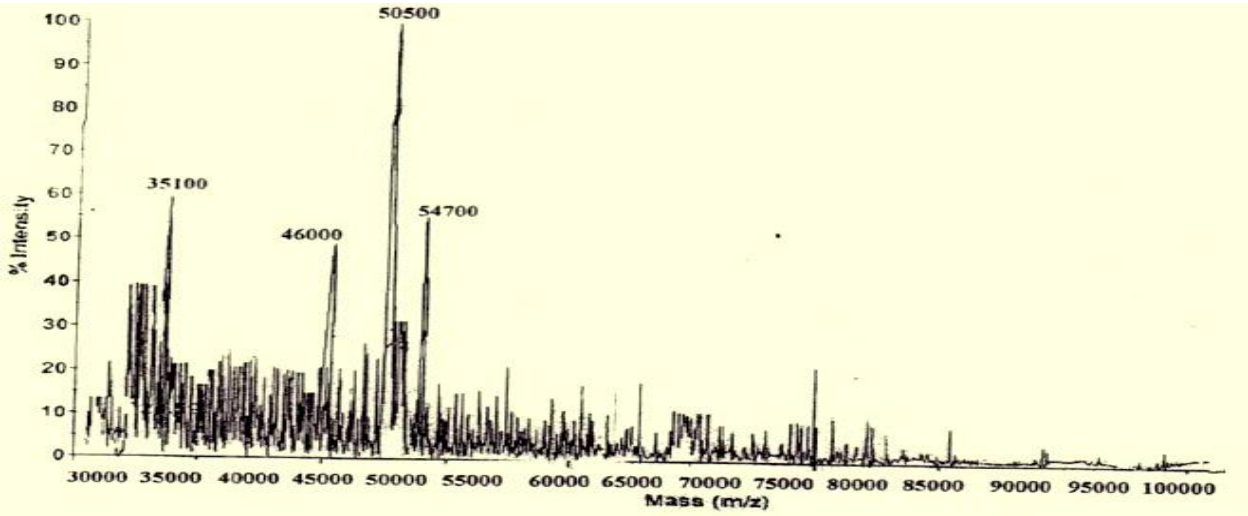

Figure 6. Determination of molecular weight of lyophilized body fluid of *S. cucullata* by MALDI-TOF mass spectrophotometry. The mass of main proteins were 35.1 KDa, 46 KDa, 50.5 KDa and 54.7 KDa.

DISCUSSION

Since antimicrobial resistance is a global public- animal health concern, there is a growing interest in marine ecosystem to find new antimicrobial agents which will be essential drugs for human and animal health and welfare. In the present study, a pronounced antimicrobial activity the body fluid of *S. cucullata* has been observed against some bacterial and fungal strains. Maximum antimicrobial activity was observed against *B. subtilis* and *C. albicans* compare to other microorganism. The MIC value lyophilized body fluid of *S. cucullata* exhibit inhibitory activity mostly against bacterial strain *B. subtilis* and fungal strain *C. albicans*. The study was also showed that the combining effect of *in vitro* antimicrobial activity of aqueous body fluid of *S. cucullata* in association with analyzed antibiotic such as tetracycline and anti-fungal such as fluconazole at their own MIC value concentration exhibit highest zone of inhibition than individual MIC value concentration of body fluid and antibiotics. The research also revealed that it had synergic effect against analyzed selected bacterial and fungal strain. The synergic effect against *B. subtilis* showed maximum inhibitory activity at the concentration of 5µg/ml was 21mm whereas, *C. albicans* exhibit maximum inhibitory activity at the concentration of 25mg/ml was 28mm.The body fluid exhibit most effectiveness as antifungal agent, highest activity against *C. albicans.*

From the study of SDS-PAGE, it showed that four bands were found in body fluid of the species *S. cucullata*, containing molecular weigh about 35KDa, 46 KDa, 50 KDa and 54 kDa which were determined by suitable molecular marker. On the other hand, it also found that four HPLC- Fractionates of body fluid of *S. cucullata*, the molecular mass were 35.1 KDa, 46 KDa, 50.5 KDa and 54.7 KDa. These result was supported the M.W. of observed SDS gel electrophoresis. By the study of HPLC chromatogram, it was found that 50.5 kDa protein which gave peak at 3 mint in 215 nm, had maximum antimicrobial activity against bacterial and fungal strains. The inhibition zone of 50.5 kDa protein was approximately similar of extract of body fluid of S. *cucullata*. Therefore, we concluded that due to presence 50.5 kDa protein, aqueous body fluid of *S. cucullata* have antimicrobial activities against human pathogen *B. subtilis* and *C. albicans*. Synergic effect may be promise as clinically useful antimicrobial drug for treatment of *B. subtilis* and *C. albicans* infection.

CONCLUSION

It is concluded that body fluid of the species *S. cucullata* showed antimicrobial activities against pathogenic microbial tested strain. So, they possess a potential pharmacological

action. It also highlighted on the matter that synergism effect of body fluid and tested antibiotics is more effective than individual action of antibiotic and body fluid along with its nature. However, some novel and uncharacterized mechanisms of action that might ultimately benefit the ongoing global research for clinically useful antimicrobial agents need to be explored to explain the characteristics of antimicrobial activity of *S. cucullata*. But more research need to be carried out to support its therapeutic use. It would first be necessary to investigate the side effects of the bioactive compounds and their possible interactions and to develop more clinical experiments. More research should to determine an optimal dosage range for achieving these effects, to study mechanism of isolated molecules *in vivo* using suitable higher animals to ensure its potentiality and safety.

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