Potential Antibacterial Activity of Marine Ascidian Aplidium multiplicatum From Vizhinjam Coast of India

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Abstract: Ascidians are rich source of bioactive agent which could be used for novel antimicrobial drugs. In the present study a compound ascidia *Aplidium multiplicatum*, collected from Vizhinjam, south west coast of India was assayed for their antibacterial activity against six human bacterial pathogens. The antibacterial activity of crude extract of ascidians showed inhibitory activity against all six species. The crude methanol extract was more active exhibiting a broad spectrum antibacterial activity than the crude ethanol and acetone extract against each of the bacterial species tested. In antibacterial activity the gram negative bacteria *Pseudomonas aeruginosa showed* most sensitive against 12.0533± 0.010116 mm *A.multiplicatum* in crude methanol extract. And the minimum zone of 1.06± 0.121244 mm in *K.pneumoniae* in ethanol extract. One of the six species examined, gram negative were most susceptible after treatment with all fractions. The column purified 80%acetone extract of *A.multiplicatum* exhibited antibacterial activity against Proties mirabilis (12mm). In 100% acetone, 100%chloroform, and 40:60%M: C fractions showed higher activity against. *P. aeruginosa* these results indicated that the ascidian *A.multiplicatum* is found to have remarkable antimicrobial activities against isolated microbes. Further, studies will fulfill for purification and structural elucidation of antimicrobial drugs.

Index terms: Ascidian, Antibacterial activity, crude extract, isolated pathogen, Vizhinjam.

Introduction

Ascidians, commonly called sea squirts (subphylum: Urochordata, Class: Ascidiacea) are a prolific source of diverse bioactive metabolites and also interesting organism from the view point of chemical ecology [1]. The number of natural products isolated from marine organisms increases rapidly and now exceeds with hundreds of new compounds being discovered every year [2] & [3]. A large portion of these natural products have been extracted from marine invertebrates, especially ascidians and some of them are currently in preclinical and clinical trials [2]. The need for discovery of new and novel antibiotics is imperative because evidence suggests that development and spreads of resistance to any new antimicrobial agents is inevitable.

Tunicates have been reported to be rich source of biologically active compounds and ranked third for their overall activities, next to sponges and bryozoans [4]. Although researches on bioactive compounds from ascidians were recently initiated, it is significant that the first marine natural product Didemnin B is entering into human clinical trial and it is an ascidian metabolite. In the last two decades, the incidence of human bacterial and fungal infections has increased dramatically, in parallel with the wide spread of incurable infectious diseases associated with antibiotic –resistant bacteria. Fungal and bacterial diseases have become a growing threat, especially in immunocompromised patients, for whom few or no effective drugs are currently available [5]. Accordingly, a variety of studies have been conducted in an attempt to isolate natural anti-bacterial and anti-fungal substances with potential pharmaceutical utility, and to develop and design new synthetic or semi–synthetic drug[6].

The case of living marine surfaces the colonization process can additionally be affected by organic metabolites produced by the host organisms. These metabolites may affect bacteria in a number of ways, ranging from the induction of chemotactic responses to the inhibition of bacterial growth or cell death. Since they accumulate chemical defences, ascidians have been screened in a variety of pharmacological bioassays. Biological activities which have been frequently observed in ascidian crude extracts include antibiosis against both human microbial pathogens and microorganisms [7]. Hence a broad spectrum screening of ascidians for bioactive compound is necessary. The present study was carried out to investigate the antibacterial activity in crude extracts

of ascidians from Vizhinjam bay, south west coast of India.

MATERIALS AND METHODS

Specimen collection and identification: Ascidians were collected as common and persistent bio foulants from the cement blocks, pilings and pearl oyster cages of Vizhinjam bay (lat 8˚22'35.95" N-76˚59'16.40 E") , by SCUBA diving at the depth ranging from 4 to 6 m between October and November 2011. The samples were thoroughly washed with sea water, cleaned of sand, mud and overgrowing organisms at the site of collection and transported to laboratory and identified by standard study of [8] and [9]

Extraction: The extraction was followed by [10].The freshly collected samples were weighed (20g) each and soaked in methanol; ethanol and acetone for one week and filtering through What man No.1 filter paper and the solvents were concentrated by rotary evaporator with reduced the pressure to give a dark brown gummy mass. The resultant residues were stored at 4˚Cfor further analysis.

Microbial strains used: Antibacterial activity of tissue extract was determined against six different bacterial pathogens, viz., *Klebsiella Pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi* and *Proteus mirabilis*. The clinical strains were obtained from MTCC microbial culture collection, Chandigarh.

Antimicrobial susceptibility assay: The antibacterial activity was carried out by standard disc diffusion method. The extract were applied on to 6mm sterile discs in aliquots of 30µL of solvent, allowed to dry at room temperature and placed on agar plates seeded with microorganisms. The bacteria were maintained on nutrient agar plates and incubated at 37˚C for 24 hrs. Zones of growth inhibition were measured in mm using a scale.

Purification of the active crude extracts

After initial screening the higher activity was shown by methanol extract and it was fractionated by normal phase silica gel column chromatography by employing a step gradient solvent system from low to high polarity. Sequence of 100% chloroform, (40:60%) Methanol: chloroform, (100%) acetone and (80%) acetone where used for elution. thus 4 fractions were collected separately and tested against six bacteria

Statistical analysis: The results were expressed as Mean ±SD of the three independent values

PATHOGENS	METHANOL	ETHANOL	ACETON
Staphylococcus aureus	3.9833±0.035119	3.2733±.24986	2.9667±0.035119
Salmonella typhi	2.1466±0.13081	2.1466±0.1350512	1.97±.03
Klebsiella pneumonia	0	1.06±0.121244	0
Pseudomonas aeruginosa	12.0533±0.10116	10.3533±0.72748	10.143±0.1266
E.coli	5.15833±0.14682	7±0.1	5.9566±0.058595
Proteus mirabilis	4.0733±0.2289	8.0633±0.118462	4.0933±0.17039

RESULTS

Antibacterial activity of crude methanol, ethanol and acetone extract of *A. multiplicatum* against six human pathogenic bacterial strains were presented in Table.1. Among these extracts, methanol and ethanol showed more activity against all pathogens than acetone extract. In the present investigation, methanol extract of *A. multiplicatum* showed high antimicrobial activity against both gram positive and gram negative bacteria. From the bacteria tested *P. aeruginosa* was the most sensitive against methanol extract 12.0533± 0.10116 mm of *A.multiplicatum.* And the minimum zone 10.3533± 0.7274 mm in *P. mirabilis* and minimum zone of 1.06± 0.121249 mm in *K. pneumoniae*. Acetone extract produced of 2.166± 0.13081 mm was observed in *S. typhi*. The corresponding zones of ethanol extract produced a maximum zone of a

maximum zone of 10.143± 0.1266 mm against *P. aereuas* and minimum activity

Table 1. Antibacterial activity of Aplidium multiplicatum against human pathogens

1.97± 0.03 mm against *S. typhi*. There was

no activity was observed in acetone and methanol extract against *K. Pneumoniae*. Both extracts showed a broad spectrum of anti-bacterial activity against, *S. aureus, S. typhi, P. aeruginosa, E. coli* and *P. mirabilis.*

Zone of inhibition* (mm)

*Zone in mm indicates the distance from the border of the disc to the edge of the clear zone

Antibacterial activity of the column purified extracts

The extracts of *A.multiplicatum* were further fractionated to examine their inhibitory effects. The results of an antimicrobial activity of different fractions of the colonial ascidian *A.multiplicatum* were shown in the table (2mm).The 80% acetone fraction of the *a. multiplicatum* revealed a higher antibacterial activity indicated by a zone of inhibition ranging from (5-12mm)in diameter against all bacteria tested and highest activity was observed in *P. mirabilis*(12mm) followed by *P .aeruginosa* (10mm) *S. aureus* (10mm), *S.typhi* (8mm), *K. pneumoniae* (6mm). The lowest activity was found in *E.coli* (5mm).In chloroform100%fraction the activity was found only in *Pseudogeneus* and no activity were found on other bacteria. In this study extracts of *A. multiplicatum* 40:60%methanol and chloroform fraction showed highest activity against *P. aeruginosa* (7),followed by *S. typhi*(5mm).The lowest activity was found on *P.mirabilis*(3mm)and no activity was found on other bacteria .The 100%acetone fraction showed the maximum activity in *S.typhi*(3mm) and *K.pneumoniae* (3mm) *P. aeruginosa* (2mm).And no activity was found on other bacteria .The lowest activity was found on100% acetone fraction against P. *aeruginosa* (2mm).

Table 2. Antibacterial activity of Aplidium multiplicatum against human pathogens

PATHOGENS	Zone of inhibition(mm)			
	Ch 100 %	Me:Ch 40:60	A 100 %	A 80 %
Staphylococcus aureus	_	_	_	10
Salmonella typhi	_	5	3	8
Klebsiella pneumonia	_	_	3	6
Pseudomonas aeruginosa	3	7	2	10
E.coli	_	_	_	5
Proteus mirabilis	_	3	_	12

A: Acetone; Ch: Chloroform; Me: Methanol

DISCUSSION

Marine organisms have been found to produce a great diversity of novel bioactive secondary metabolites and be potential source of drug discovery. Extensive investigations of ascidians

pharmacology research have been undertaken all over the world. Several drug discovery projects have screened ascidians for antibiotic activities. Overall, ascidian extract caused growth inhibition in gram positive and gram negative bacteria, indicating that these extracts do not selectively inhibit one group of microorganisms [11]. Here we examined antibacterial activity of the crude methanol and ethanol extracts of *A. multiplicatum* against gram positive and gram negative bacteria and it was evident that the gram negative strains were more resistant, than gram positive. This study is contrary with the findings of [12] who reported maximum antibacterial activity of the crude methanol extract of the test and mantle bodies of *P. nigra* against the gram positive strains inhibitory zones of (12.3± 0.8 mm) and (8.2± 0.8 mm) respectively.

In the present study *A. multiplicatum* showed promising source of antibacterial activity in crude extracts. It showed high antibacterial activity against six pathogens assayed, from the bacteria tested: *P. aureus* was the most sensitive against methanol extract (12.0533± 0.10116 mm). Minimum zone of inhibition (2.166 ± 0.13081 mm) was observed in *S. typhi* against methanol extract. The crude ethanol extract showed maximum activity against *P. mirabilis* (10.3533± 0.7274 mm) followed by; (8.0633± 0.118462 mm) in *P. aureus* , (7± 0.1 mm) in *E. coli*,(3.2733± 0.24986 mm) in *S. aureus*, and (2.14667± 0.130512 mm) in *S. typhi* respectively. And minimum activity was noticed against (1.06± 0.12124 mm) in *K. pneumoniae*. Acetone extracts showed maximum activity against (10.143± 0.1266 mm) in *P. aereuas,* followed by zone of inhibition (5.9566± 0.058595 mm) in *P. mirabilis*, zone (4.0933± 0.17309 mm) against *E. coli*, (2.9667± 0.03511 mm) in *S. aureus* and minimum zone of (1.97± 03 mm) in *S. typhi*. No activity was observed in both methanol and acetone extract against *K. pneumoniae*. The result of present study similar to that the previous report of [13] who reported that the methanol extract of *A. multiplicatum* exhibited antimicrobial activity against most of the bacterial species studied; no effect was observed in *K.pneumoniae* species.

The crude methanol extract of *Policlinium madrasensis* and *Phallusia arabica* were found to have higher antibacterial activities against *P. aeruginosa* [14]. In this study also *P. aeruginosa* was the most sensitive to methanol extracts of *A. multiplicatum* than ethanol and acetone. [15] Also reported that the *P. aeruginosa* was the most successful bacteria for all fractions of ascidian extracts with maximum zone of 8-

5mm. The bacterial species like *Bacillus* and *Pseudogeneus* species found to have inhibitory effect for the extracts of colonial ascidians with MIC value 200mg/ml (13). [16] reported that the methanol and ethanol extracts of ascidian showed more activity against all pathogens than hexane and butanol extracts .The present study reported that methanol and ethanol extracts of *A. multiplicatum* showed higher activity against microbes than acetone extract .The crude methanol, ethanol and acetone extracts of *A.multiplicatum* was more effective against gram negative bacteria than gram positive bacteria. This study similar to the previous report of [13] reported that the crude ethyl acetate of *A. multiplicatum* was more effective against gram negative bacteria than gram positive bacteria.

Antibacterial activity has previously been detected in methanol and dichloromethane extracts of the ascidians *H. pyriformis* and a mixture of two Styela species where one of the species was *S. rustica* [17]. Prem Anand and [18] reported that comparatively ascidians *D. pasmathodes* seems to be promising source of antibacterial compound. [15] reported that for the crude methanol extract of *D. pasmathodes*, the range of inhibition of bacteria varied from 6 to 10 mm with an average of 7.1 mm. [9] revealed that the preliminary screening of nine species of ascidian indicate, the presence of antibacterial activity of the three different solvent and methylene extract showed maximum activity followed by methanol and hexane. Methanol and methylene chloride extracts of *Aplidium indicam* were active against all pathogens. The test body of *P. nigra* harboured smaller number of total heterotrophic bacteria compared to that of the surrounding water medium [12].

The 80%acetone column purified fraction s found to possess highest activity. The clear zone of 12mm was shown by the 80%acetone column purified fractions of A.multiplicatum against *P. mirabilis* (12mm).But in contrast , the crude extract of *chicoreus virgineus* , after antibacterial assay guided elution , showed activity only in (100%)methanol fraction [19]from the comparatively lesser inhibition by the column fractionated extracts of the crude , it could be obtained that the active compound may have degraded or modified during the fraction process .[20])reported that the minimum inhibitory concentration (MIC)was found to be lesser for the 100%acetone phase of *T.tentorium* (.8mg) for E.coli. in this study also the minimum zone of inhibition was found on 100%acetone fraction against *E.coli* (2mm).of the six strains examined *p.*

aeruginosa was a susceptible bacteria after treatment with all fractions .this study also coincide with the previous report of [21])reported that *P. aeruginosa* was most susceptible bacterium after treatment with all fractions tested.as fraction 4 showed more potent antibacterial activity than the rest of the fractions .further studies are needed to elucidate structure and mechanism of action of these marine ascidian extracts.

The tunicates have the potential to yield novel compounds with ecological, chemical, and biomedical interest [22]. In particular, the cosmopolitan genus Aplidium is renowned for the variability of its metabolites. A large variety of alkaloids have been isolated from this group, such as piperidins, tetracyclic alkaloids and indoles, which display potent bioactivities [23]). Many studies have been conducted to examine the antimicrobial activity of ascidians against bacteria, fungi even tumour cells [24]. The extracts from *D. pasmathodes* showed the promising results against isolated and human pathogens. These results indicate that ascidians exhibits remarkable activity against microbes [25]). The continuing and over whelming contribution of ascidian metabolites to the development of new pharmaceuticals are clearly evident and need to be explored. Antibacterial compounds form natural resources would be alternative to overcome the resistance problems. Thus the current studies revealed the presence of antibacterial activity from ascidians of Vizhinjam bay South west coast of India has much importance in marine secondary metabolites. Further, purification of the actual compounds involved in the activity may lead to the discovery of novel antimicrobial compounds.

CONCLUSION

Activities found in crude extracts showed promising results and with enormous potential for discovery and development and marketing of novel marine bioproducts methods by which these products can be supplied in a way that will not disrupt the ecosystem or deplete the resources. It is worthy to note that the product from nature source is good for health and devoid of side effects. However, further investigations involving application of the extracts as drug for human administration need more research.

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