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SCREENING OF ANTIMICROBIAL ACTIVITY OF COLONIAL ASCIDIAN *Aplidium multiplicatum* FROMVIZHINJAM BAY, SOUTH WEST COAST OF INDIA

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ABSTRACT

Ascidians are rich source of bioactive agent which could be used for novel antimicrobial drugs. Ascidians are belongs to phylum chordata and class ascidiacea. 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. In the present study *A. multiplicatum* extract has showed promising source of antibacterial activity. It showed high and moderate antibacterial activity against six pathogens assayed. From the tested bacteria, *P. aureus* was most sensitive against methanol extract (12.05±0.10mm). Minimum zone of inhibition (2.16±0.13 mm) was observed in *S. typhi* against methanol extract. After initial screening, the higher activity was shown by methanol extract. It was fractionated by silica gel column chromatography and it was assayed for antimicrobial activity. 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. *Pseudogeneus aeruginosa* (10mm). One of the six species examined, gram negative were most susceptible after treatment with all fractions. Further, studies will fulfill for purification and structural elucidation of antimicrobial drugs.

KEYWORDS: Ascidian, Antibacterial Activity, Methanol Extract, Isolated Pathogen, Methanol, Ethanol, Acetone

Ocean has potent bioactive compounds present in marine organisms which are used mainly as food source. A large portion of natural compounds have been extracted from marine organisms especially from ascidians, bryozoans, sponges and molluscs. These natural products are currently used in clinical trials (Proksch *et al.,* 2002). Bioactive compounds exhibiting antitumor, anti-bacterial and antiviral activity has reported worldwide. Ascidians, commonly called sea squirts (subphylum: Urochordata, Class: Ascidiacea) are a prolific source of diverse bioactive metabolites and also interesting organisms from the view point of chemical ecology (Hongwei *et al*., 2004). A large portion of these natural products have been extracted from marine invertebrates, especially from colonial ascidians and some of them are currently in preclinical and clinical trials (Proksch *et al.,* 2002). Ascidians give rise to a great array of structurally diverse amino acid derived metabolites in which the precursor in phenylalanine, tyrosine or both (Bradford, 1976).

Tunicates have been reported to be rich source of biologically active compounds and ranked third for their overall activities, next to sponges and bryozoans (Davis and Bremner, 1999). Although research 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. The antibacterial activity of the compounds isolated from the colonial ascidian *Didemnum psammathodes* was reported by Ramasamy and Murugan (2003). An investigation was carried out by Ali *et al*. (2008) to analyse the bioactive compounds of *P. nigra* and *H. pallida* and also their possible antagonistic effects against several bacterial pathogens. *Halocynthia roretzi* is a solitary ascidian that produces tetra peptide antibiotics against microorganisms. Natarajan *et al*. (2010) studied the antitumor activity of methanolic extract of *Polyclinum madrasensis*, collected from eastern coast of Tamil-Nadu, India. Already various ascidians such as *Botryllu*s species and *Didemnum* species were proved for producing anti-cancer drugs (Jain *et al*., 2008).

Kumaran *et al*. (2012) have been evaluated the biological properties from the biofouling ascidian *Lissoclinum fragile*, collected from the Tuticorin coast of India. Bragadeeswaran *et al*. (2010) studied the pharmacological properties of the biofoulent ascidian *P. madrasensis* from south east coast of India. Methanolic extract of ascidians were evaluated and well documented as potent antibacterial activity (Anbuselvi *et al*., 2009). Hence a broad spectrum screening of ascidians for bioactive compounds is necessary. The present study was carried out to investigate the antibacterial activity in crude extracts of ascidian *Aplidium multiplicatum* collected from Vizhinjam bay, south west coast of India.

MATERIALS AND METHODS

Specimen Collection and Identification

Ascidians were collected from the cement blocks, pilings and pearl oyster cages of Vizhinjam Bay (lat 8˚22'35.95"N-76˚59'16.40E"), by SCUBA diving at depth ranging from 4 to 6 m between June to November 2011. The samples were thoroughly washed with sea water,

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cleaned of sand, mud and overgrowing organisms at the site of collection and transported to laboratory and identified by standard keys of Kott (1985, 1989, and 2002).

Extraction

The freshly collected samples (20g) were weighed and soaked in methanol, ethanol and acetone for one week and filtering through Whatman No.1 filter paper and the solvents were concentrated by rotary evaporator with reduced pressure to give a dark brown gummy mass. The resultant residues were stored at 4˚C for further analysis. The extraction process was carried out by the method of Chellaram *et al*. (2004).

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 of crude extract was carried out by standard disc diffusion method. The crude extracts 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.

The antibacterial activity of the crude extract at concentrations of 25, 50, 75 and 100 mg/ml) was done using disk diffusion method (Kirby and Bauer, 2000). Diameter of zone of inhibition was measured for determining the antibacterial activity. Streptomycin was used as a control (Sri Kumaran *et al*., 2011) and the extracts were tested in triplicate.

Purification of the Active Crude Extracts

In the screening of the antibacterial activity the highest activity was shown by methanol extract and it was fractionated by normal phase silica gel column chromatography by employing a step gradient solvent system with increasing polarity. Sequence of chloroform (100%), Methanol: chloroform (40:60%), acetone (100%) and acetone (80%) were used for eluting the fractions. Thus fractions were collected separately and tested against six bacteria.

Statistical Analysis

The results are expressed as mean ±SD of the three independent values.

RESULTS

Antibacterial activity of crude extract of *A. multiplicatum* against six human pathogenic bacterial strains was presented in Table-1.Among these extracts, methanol and ethanol showed more antibacterial activity against all tested 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. Among the tested bacteria *P. aeruginosa* (12.05±0.10mm) was the most sensitive bacteria against methanol extract of *A. multiplicatum* and minimum zone of inhibition (2.16±0.13mm) was observed against *S. typhi.* The corresponding zones of ethanol extract produced a maximum inhibition zone of 10.35±0.72mm in *P. mirabilis* and minimum zone of 1.06± 0.12 mm was observed in *K. pneumoniae.* Acetone extract inhibited a maximum zone of inhibition (10.14±0.12mm) against *P. aeruginosa* and minimum (1.97±0.03mm) antibacterial activity was reported against *S. typhi*. Acetone and methanol extract has not showed any antibacterial activity against *K. pneumoniae*. But both acetone and methanol extract showed a broad spectrum of antibacterial activity against *S. aureus, S. typhi, P. aeruginosa, E. coli* and *P. mirabilis.*

In the present investigation, the antibacterial activity of methanol, ethanol and acetone extracts at different concentrations of *A. multiplicatum* against gram negative and gram positive bacteria was shown in Tables and represented as figures 1, 2, 3 and 4. High concentration of methanol extract of *A. multiplicatum* showed strong antimicrobial activity against tested pathogens. Methanol extract at 100mg /ml concentration produced a maximum zone of 24±0.035mm against *P. aeruginosa* and minimum zone of inhibition was exhibited by *S. typhi* (10.21±0.008mm) at a concentration of 25mg/ml. In ethanol extract maximum zone of inhibition (18.05±0.028 mm) was exhibited by *P. mirabilis* at a concentration of 100mg/ml and minimum zone of inhibition (8±0.057mm*)* was observed in *S. aureus* at low concentration of 25mg/ml. In the case of acetone extract maximum zone of inhibition (19.11±0.005mm) was observed against *P. aeruginosa* at concentration of

100mg/ml and minimum in *P. mirabilis* (9.97±0.035mm) at a concentration of 25mg/ml. In the present investigation, high concentration of methanol extract showed strong antimicrobial activity against gram negative and gram positive pathogens. From the tested bacteria tested *P. aeruginosa*, *E. coli* and *P. mirabilis* showed high sensitivity against methanolic extract at concentration of 100mg/ml. Antibacterial activity of ascidian *A. multiplicatum* extract was found to increase with respect to increasing concentration. In the present study the methanol extract showed higher activity than the ethanol and acetone extracts.

Table 1: Antibacterial activity of *A. multiplicatum* against human pathogens

Pathogens	Methanol	Ethanol	Acetone
Staphylococcus aureus	3.98±0.03	3.27±0.24	2.96±0.03
Salmonella typhi	2.14±0.13	2.14±0.13	1.97±0.03
Klebsiella pneumonia	0	1.06±0.12	0
Pseudomonas aeruginosa	12.05±0.10	10.35±0.72	10.14±0.12
Escherichia coli	5.15±0.14	7±0.1	5.95±0.05
Proteus mirabilis	4.07±0.22	8.06±0.11	4.09±0.17

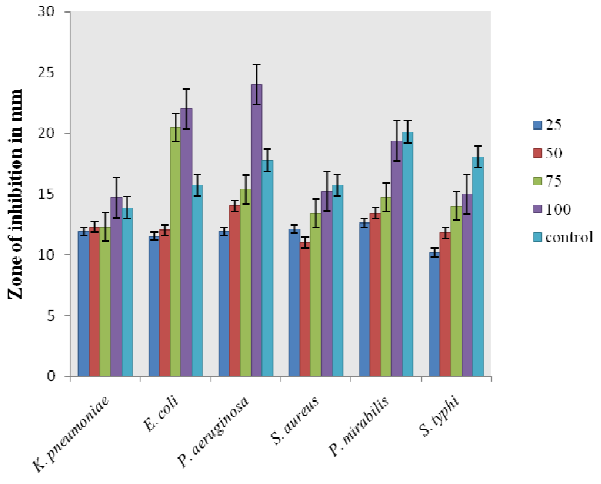

Figure 1: Antibacterial activity of methanol extract of *A. multiplicatum* against bacteria

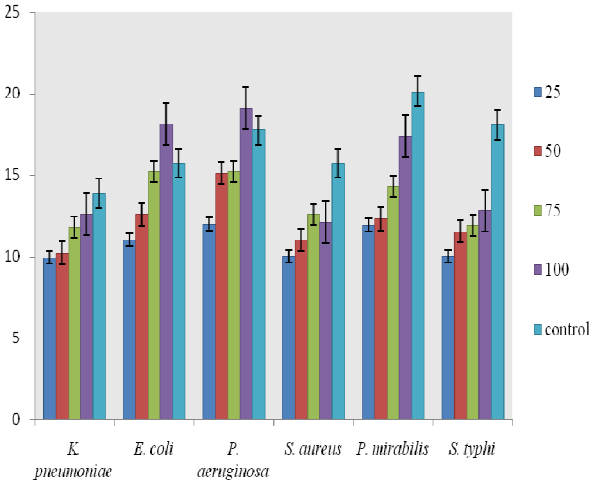

Figure 2: Antibacterial activity of ethanol extract of *A. multiplicatum* against bacteria

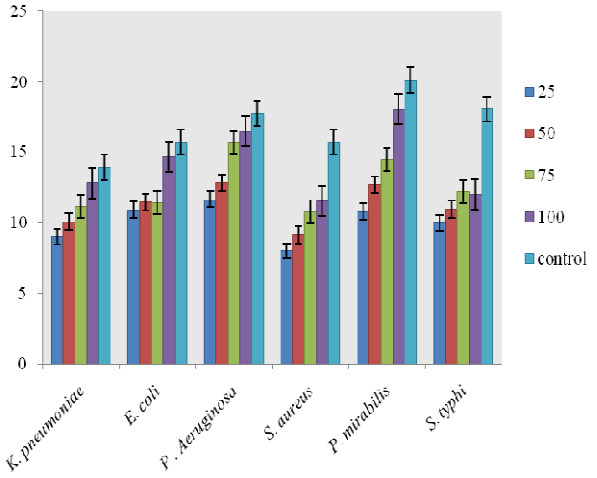

Figure 3: Antibacterial activity of acetone extract of *A. multiplicatum* against bacteria

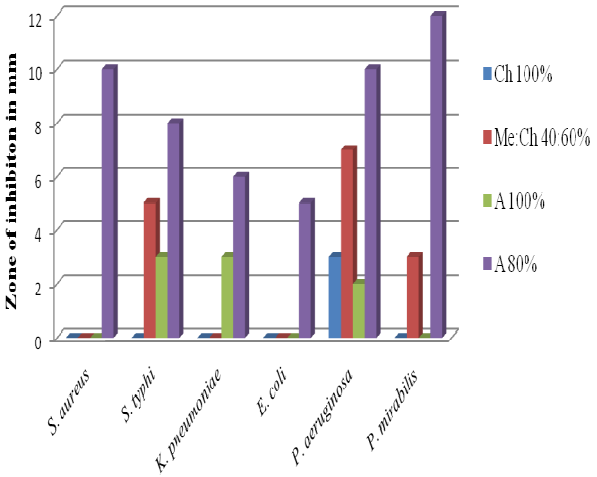

Figure 4: Antibacterial activity of column purified fractions of *A. multiplicatum* in methanol extract

Purified Column Chromatography

The methanol extracts of *A. multiplicatum* were further fractionated to examine their inhibitory effects. The results of antimicrobial activity of different fractions of the colonial ascidian *A. multiplicatum* were shown in the Fig. 4 . 80% of acetone fraction of the *A. multiplicatum* revealed a higher antibacterial activity indicated by zone of inhibition ranging from 5 to 2mm in diameter against all bacteria tested and highest activity was observed in *P. mirabilis* (12mm). The lowest activity was found in *E. coli* (5mm). In chloroform 100% fraction the antibacterial activity was found only in *Pseudomonas aeruginosa* and no activity found on other bacteria. In this study extracts of *A. multiplicatum* 40:60% methanol and chloroform fraction showed highest activity against *P. aeruginosa* (7mm) followed by *S. typhi* (5mm). The lowest activity was found on *P. mirabilis* and no activity to other bacteria. The 100% acetone fraction showed the maximum activity in *S. typhi* (3mm) and *K. pneumoniae* (3mm). And no activity was found on 100% acetone fraction against *S. aureus*, *E. coli*, and *P. mirabilis.*

DISCUSSION

Marine organisms have been found to produce a great diversity of novel bioactive secondary metabolites and are potential source of drug discovery. Extensive investigations of ascidians in chemical and pharmacological studies have been already reported. Several drug discovery projects have screened ascidians for antibiotic activities. Overall, ascidian extracts caused growth inhibition in gram positive and gram negative bacteria, indicating that these extracts do not selectively inhibit one group of microorganism (Thompson *et al.,* 1985). Here we have examined antibacterial activity of the crude methanol and ethanol extracts of *A. multiplicatum* against gram positive and gram negative bacteria. The present study is contrary with the findings of Ali *et al*. (2008) who have reported the maximum antibacterial activity of the crude methanol extract of the test and mantle bodies of *P. nigra* against gram positive strains with inhibitory zones of 12.3±0.8mm and 8.2±0.8mm in diameter respectively.

In the present study *A. multiplicatum* extract has showed promising source of antibacterial activity. It showed high and moderate antibacterial activity against six pathogens assayed. From the tested bacteria, *P. aureus* was most sensitive against methanol extract (12.05±0.10mm). Minimum zone of inhibition (2.16±0.13 mm) was observed in *S. typhi* against methanol extract. The crude ethanol extract showed maximum activity against *P. mirabilis* (10.35±0.72mm) followed by *P. aureus,* (8.06±0.11mm), *E. coli* (7±0.1mm), *S. aureus* (3.27±0.24 mm) and *S. typhi* (2.14±0.13mm) respectively and minimum activity was noticed against (1.06±0.12 mm) *K. pneumoniae*. Acetone extract showed maximum activity against *P. aereuas* (10.14±0.12 mm) followed by zone of inhibition of 5.95±0.05 mm in *P. mirabilis,* 4.09±0.17mm against *E. coli,* 2.96±0.03 mm in *S. aureus* and minimum zone of inhibition (1.97± 0.03 mm) in *S. typhi.* No activity was observed in both methanol and acetone extracts against *K. pneumoniae*. Anand and Patterson (2002) reported that the ascidians *D. psammathodes* seems to be the promising source of antibacterial compound.

In the present study antimicrobial activity of the crude methanol of the *A. multiplicatum* against gram negative strains revealed that the gram negative bacteria were more resistant than gram positive bacteria. Sivaperumal *et al*. (2010) reported that the crude ethyl acetate extract of *A. multiplicatum* was more effective against gram negative bacteria than gram positive bacteria. Methanol extract at 100mg /ml concentration produced a maximum zone of inhibition against *P. aeruginosa* (24±0.035) followed by *E. coli* (22.03±0.035), *P. mirabilis*, (19.38±0.020) *S. typhi* (15.23±0.001) and *S. aureus* (13.106±0.060) and minimum zone of inhibition was exhibited by *S. typhi* (11.91±0.037) in 25mg/ml. Similar results were reported by Ravi Kumar *et al*. (2002). They observed that the crude diethyl ether extract of seaweed showed good antibacterial activity against both gram positive and gram negative bacteria. Regarding ethanol extract, maximum antimicrobial activity of 18.0±0.08 mm was found against *P. aeruginosa*.

Acetone extract of *A. multiplicatum* produced maximum zone of inhibition against *P. aeruginosa* (19.11±.005mm) at a concentration of 100mg/ml and minimum in *P. mirabilis* (9.97±0.035mm) concentration of 25mg/ml. Selva Prabhu *et al*. (2011) reported that the crude methanol extract of 1mg/ml concentration produced a maximum zone of 12mm against *P. aeruginosa* and minimum in *E. coli* (3mm). The corresponding ethanol extract produced 10mm and 3mm against *P. aeruginosa* and *E. coli*. Both acetone and ethanol extracts showed a broad spectrum antibacterial activity against *P. aeruginosa* followed by *K. pneumoniae*. This observation is consistent with the findings of Ananthan *et al*. (2011b) who reported that both methanol and ethyl acetate extract of *P. nigra* showed a broad spectrum antibacterial activity against

tested gram negative pathogen. The 80% acetone column purified fraction was found to possess highest antibacterial 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 antibacterial activity only in (100%) methanol fraction (Bragadeeswaran *et al*., 2011). Mariappan *et al*. (2010) reported that the minimum inhibitory concentration (MIC) was found to be lesser for the 100% acetone phase of *T. tentorium* (0.8mg) 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 bacterium after treatment with all fractions. This study corroborates with the previous report of Amutha *et al*. (2010a). They reported that *P. aeruginosa* was most susceptible bacterium after treatment with all fractions tested. As 80% acetone fraction showed more potent antibacterial activity than the rest of the fractions and further studies are needed to elucidate structure and mechanism of action of these marine ascidians extracts. From the present results the ascidian extracts showed hopeful source of antimicrobial compounds towards isolated pathogens. The observed results strongly suggest that the *A. multiplicatum* extracts can be used as antimicrobial agent.

Meenakshi (2006) in the preliminary screening of nine species of ascidian indicate, the presence of antibacterial activity of the three different solvents tested and methylene extract showed maximum activity followed by methanol and hexane. Methanol and methylene chloride extracts of *A. indicum* were active against all pathogens. The continuing and overwhelming contribution of ascidian metabolites to the development of new pharmaceuticals are clearly evident and need to be explored. Thus the current study revealed the presence of antibacterial activity from ascidians of Vizhinjam bay.

Further studies are needed to the isolation, purification and structural determination of the chemical compounds responsible for the biological activities which may be lead to the discovery of drug molecules as chemotherapeutic agents in combating various diseases of mankind.

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