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Full Length Research Paper

Bioactivity potential of extracts from ascidian *Lissoclinum fragile*

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Ascidians are marine biofouling animals with a proven ability to synthesis bioactive substances. This study investigated the bioactive potential of ascidian *Lissoclinum fragile,* found in the coastal waters of Tuticorin Southeast coast of India. Freshly collected ascidian soaked in methanol for five days and filtered through filter paper. The solvent was concentrated by rotary evaporator which reduced the pressure to give a dark brown gummy mass. The collected gummy mass was separately extracted successively with acetone, n-butanol, chloroform, ethyl acetate and dichloromethane. These extracts were used for antimicrobial, heamolytic and cytotoxic assays. In antibacterial assay bacterial pathogen, *S. typhi* exhibit high zone of inhibition (14.1 ± 0.01 mm) against dichloromethane extract. In antifungal assay, fungal pathogen *Penicillium* species showed high zone of inhibition (12.3 ± 0.07 mm) against n-butanol extract. In haemolytic assay, n-butanol extract showed high heamolytic activity in chicken erythrocytes (64 HU), goat erythrocytes (16 HU) and cow erythrocytes (16 HU). In cytotoxic activity, n-butanol extract exhibited high LC_{50} value (97 µg/ml) against brine shrimps. These results indicate that the ascidian *L. fragile* has remarkable antimicrobial, haemolytic, and cytotoxic activities.

Key words: Ascidian, *Lissoclinum fragile,* haemolytic and cytotoxic activities, antimicrobial.

INTRODUCTION

The biodiversity of the marine environment constitute a practically unlimited resource of active substances for the development of natural bioactive products. Of the natural products isolated from marine organisms, less than 1% have been examined for pharmacological activities (Fusetani, 2002). Biological activities that have been frequently observed in marine invertebrate crude extracts include antibiosis against human microbial pathogens, marine microorganisms, and cytotoxicity (Mayer et al., 2007). Many marine invertebrate secondary metabolites have presented both antibiotic and cytotoxic activities as a result of increased research into these bioassays for new drugs (Newman et al., 2003). A large proportion of natural compounds that have been extracted from marine invertebrates, especially sponges, ascidians, bryozoans, of biologically active compounds, especially amino acid derived nitrogenous secondary metabolites (Rinehart, 2000) and antimicrobial, antiviral, and anti-inflammatory compounds (Davidson, 1993). Although, research on bioactive compounds from ascidians was recently initiated, the first marine natural product entering human clinical trials, Didemnin B, is an ascidian metabolite. Several ascidian compounds in anticancer preclinical or clinical trials include the tetrahydroisoquinolone alkaloid, 'Ecteinascidin 743' from *Ecteinascidia turbinata*; cyclic depsipeptides, 'Dehydrodidemnin B' and 'Didemnin B' from *Tridemnum solidum*; cyclic peptide, 'Vitilevuamide' from *Didemnin cuculiferum*; and 'Diazonamide', from *Diazona angulata* (Jain et al., 2008). Potential ascidians need to be explored for pharmaceutical use, and a broad based screening of ascidians for bioactive compounds is necessary. This study evaluates the biological properties from the biofouling ascidian *Lissoclinum fragile,* collected

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Figure 1. Map of collection site.

from the Tuticorin coast of India.

MATERIALS AND METHODS

Sampling site

Ascidians were collected from rocks near Hare Island, Tuticorin coast (Lat. 8° 46' 20.72" and Long. 78° 11' 57. 91"E), India via SCUBA diving at depths ranging from 1 to 2 m in September, 2010 (Figure 1).

Sample collection and extraction

The samples were thoroughly washed with sea water to remove sand, mud and overgrowing organisms at the collection site. The species was identified by the standard literature of Cole and Lambert (2009) and Rocha and Bonnet (2009). A voucher specimen No. AS 2234 has been deposited in the National Collections of Ascidians in the Museum of the Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin - 628002.

Extraction procedure

The ascidians were soaked in methanol for 5 days. The extract was filtered through Whatman®No.1 filter paper and was concentrated by rotary evaporator (VC100A Lark Rotavapor® at 30°C) with reduced pressure, resulting in a dark brown gummy mass. The methanolic crude was separately extracted successively with acetone, n-butanol, chloroform, ethyl acetate, and dichloromethane. These extracts were used for antimicrobial, haemolytic, and cytotoxic assays.

Test microorganisms and microbial culture

Human bacterial and fungal pathogen assay

Antimicrobial assays were performed using bacterial pathogens such as, *Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Klebsiella aerogenes*, *Proteus mirabilis, Salmonella paratyphi*, *Salmonella typhi*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus;* and fungal pathogens such as *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Candida tropicalis, Mucor* species, *Penicillium* species, *Rhizopus* species, *Trichophyton mentagarophytes*, and *Trichophyton rubrum*. Pathogens were obtained from Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar. The bacterial and fungal strains were maintained on nutrient agar and fungal agar slants at 4°C, respectively.

Antibacterial activity

Antibacterial activity was carried out using the standard disc diffusion method by Laouer et al. (2009). The tetracycline discs (30 mg/disc) were used as a control. All extracts were tested with

Table 1. Antimicrobial activity of *L. fragile* against human pathogens.

Human bacterial pathogen	Methanol	Acetone	Dichloromethane	n-Butanol	Chloroform	E. acetate	Control
E. coli	5.02 ± 0.1	4.01 ± 0.18	8.05 ± 0.26	7.05 ± 0.15	8.05 ± 0.1	5.02 ± 0.1	13.01 ± 0.15
K. oxytoca	9.01 ± 0.11	4.11 ± 0.16	10.2 ± 0.33	4.15 ± 0.11	6.15 ± 0.15	9.01 ± 0.11	14.02 ± 0.17
K. pneumoniae	9.11 ± 0.12	3.02 ± 0.12	7.3 ± 0.12	6.01 ± 0.5	7.35 ± 0.18	9.11 ± 0.12	15.03 ± 0.15
P. aeruginosa	7.01 ± 0.1	3.01 ± 0.15	11.02 ± 0.15	6.04 ± 0.22	6.01 ± 0.25	7.01 ± 0.1	14.02 ± 0.5
P. mirabilis	7.02 ± 0.14	3.11 ± 0.2	13.5 ± 0.18	6.01 ± 0.5	7.02 ± 0.11	7.02 ± 0.14	13.05 ± 0.1
S. paratyphi	5.1 ± 0.1	3.01 ± 0.23	11.11 ± 0.15	4.07 ± 0.21	4.02 ± 0.5	5.1 ± 0.1	14.06 ± 0.33
S. typhi	6.02 ± 0.2	2.03 ± 0.11	14.1 ± 0.01	5.11 ± 0.11	7.02 ± 0.66	6.02 ± 0.2	16.07 ± 0.23
S. aureus	5.05 ± 011	3.01 ± 0.1	9.08 ± 0.11	5.08 ± 0.8	9.04 ± 0.2	5.05 ± 011	14.06 ± 0.1
V. cholerae	6.01 ± 0.1	2.04 ± 0.1	7.06 ± 0.13	4.08 ± 0.15	6.05 ± 0.11	6.01 ± 0.1	13.04 ± 011
V. parahaemolyticus	6.2 ± 0.2	3.05 ± 0.2	8.02 ± 0.2	4.04 ± 0.1	7.01 ± 0.2	6.2 ± 0.2	14.05 ± 0.14
Fungal pathogen							
A. alternata	3.01 ± 0.1	4.06 ± 0.1	7.05 ± 0.4	12.02 ± 0.17	6.05 ± 0.15	3.01 ± 0.1	13.02 ± 0.22
A. flavus	4.05 ± 0.33	4.08 ± 0.17	5.08 ± 0.1	11.06 ± 0.15	5.06 ± 0.16	4.05 ± 0.33	14.05 ± 0.25
A. niger	4.06 ± 0.3	6.1 ± 0.18	9.04 ± 0.8	10.01 ± 0.16	4.04 ± 0.15	4.06 ± 0.3	13.02 ± 0.12
C. albicans	3.07 ± 0.11	5.05 ± 0.14	7.07 ± 0.01	11.02 ± 0.14	5.01 ± 0.14	3.07 ± 0.11	14.02 ± 0.16
C. tropicalis	3.06 ± 0.1	3.01 ± 0.05	6.06 ± 0.06	9.01 ± 0.47	5.05 ± 0.1	3.06 ± 0.1	12.03 ± 0.17
Mucor sp.	3.07 ± 0.8	3.2 ± 0.4	5.08 ± 0.01	11.09 ± 0.6	5.04 ± 0.16	3.07 ± 0.8	12.01 ± 0.12
Penicillium sp.	3.1 ± 0.16	4.1 ± 0.3	5.04 ± 0.22	12.3 ± 0.07	4.02 ± 0.15	3.1 ± 0.16	15.02 ± 0.14
Rhizopus sp.	2.11 ± 0.14	3.01 ± 0.15	3.05 ± 0.04	10.04 ± 0.1	3.04 ± 0.1	2.11 ± 0.14	14.01 ± 0.11
T. mentagarophytes	3.06 ± 0.04	4.03 ± 0.14	5.01 ± 0.11	9.03 ± 0.14	4.06 ± 0.24	3.06 ± 0.04	14.02 ± 0.14
T. rubrum	3.04 ± 0.15	3.01 ± 0.16	4.05 ± 0.1	7.05 ± 0.11	6.02 ± 0.18	3.04 ± 0.15	14.03 ± 0.36

3 Replicates; Mean ± SD (Standard error); Inhibition zones (Diameter in mm).

triplicate at a concentration of 30 mg/disc.

Antifungal activity

Antifungal activity was carried out using the standard disc diffusion method described by National Committee for Clinical Laboratory Standards (2006).

Haemolytic assay

Solvent crude extracts were assayed on chicken, goat, and cow erythrocytes following the method of Bragadeeswaran et al. (2010).

Cytotoxicity using brine shrimp (*Artemia salina*)

The toxic effects of the extracts were determined by the method of Meyer et al. (1982). The LC_{50} values of brine shrimp were obtained from counts using the probit analysis method described by Litchfield and Wilcoxon (1949).

RESULTS

The ascidians *L. fragile* (455 g in wet weight) was collected from Hare Island Tuticorin coast of India. Methanol extract of *L. fragile* was concentrated under reduced pressure to give a dark brownish gummy mass of 18.73 g (in wet weight).

In the present investigation, different extracts of *L. fragile* showed promising antimicrobial activity against human bacterial and fungal pathogens. Antimicrobial activities of *L. fragile* represent the radius of the zone of inhibition around the disc (Table 1). The human bacteria *S. typi* exhibit high inhibition zone (14.1 ± 0.01 mm) against dichloromethane extract and fungal *Penicillium* sp.

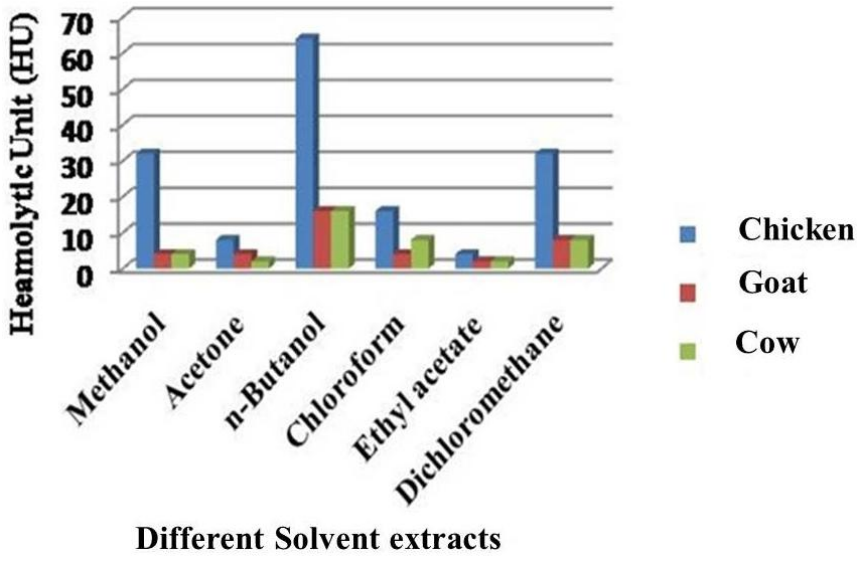

Figure 2. Haemolytic activity of *L. Fragile* extracts.

shows high inhibition zone (12.3 ± 0.07 mm) against n-butanol extract. In heamolytic assay, n-butanol extract showed high heamolytic activity against chicken erythrocytes (64 HU), goat erythrocytes (16 HU), and cow erythrocytes (16 HU). The different extracts of *L. fragile* also show cytotoxic properties against *A. salina* larva. The brine shrimp assay is considered as a reliable indicator for the preliminary assessment of toxicity. This assay is widely employed in the screening process for the isolation of bioactive metabolites. In this cytotoxic study, n-butanol extract showed high LC_{50} value 97 µg/ml. These results indicate that the ascidian *L. fragile* was found to have remarkable cytotoxic activities.

DISCUSSION

In the present investigation, different extract of *L. fragile* showed promising antimicrobial activity against human bacterial and fungal pathogens. The antibacterial activities of *L. fragile* extracts were measured as radius of zone of inhibition around the disc (Table 1). From the human pathogenic bacteria and fungal tested, *S. typhi* exhibit high inhibition zone (14.1 ± 0.01 mm) against dichloromethane extract, *Penicillium* sp. shows high inhibition zone (12.3 ± 0.07 mm) against n-butanol extract. From the result, the ascidian extracts showed hopeful source of antimicrobial compounds towards isolated pathogens. The observed result strongly suggests that the ascidian extracts can be used as antimicrobial agents. These findings are consistent with previous studies on ascidians. Antibacterial activity has been previously reported from the extracts of some ascidians (Santhana and Murugan, 2003). Overall, ascidian extracts caused growth inhibition in Gram positive and Gram negative bacteria, indicating that these extracts do not selectively inhibit one group of microorganisms. This view is contrary with the findings of Ali et al. (2008) who reported the maximum antibacterial activity exhibited by the Gram positive bacteria than in Gram negative bacteria of crude methanol extracts of the test and mantle bodies of *Phallusia nigra*. Meenakshi (2002) revealed that the preliminary screening of nine species of ascidian indicated the presence of antibacterial activity of the three different solvent that were tested (methanol, methylene chloride, and hexane); methylene chloride extracts showed maximum activity followed by methanol and hexane. Abourriche et al. (2003) evaluated the antibacterial activity against *Agrobacterium tumifaciens, E. coli, Pseudomonas aeruginosa* and *S. aureus* from the extracts of Morocco Atlantic sea ascidian, *Cynthia savignyi*. In the shown activity, all extracts were active against bacteria, except the dichloromethane extract. It is clearly evident that the antibacterial activity has been previously reported from extracts of some ascidian extracts that caused growth inhibition in Gram positive and negative bacteria. Several drug discovery investigations have screened ascidians for antibiotic activities. Chemical antibacterial defense has been suggested as one of the arrays of defenses potentially available to sessile invertebrates.

In the present study, different extracts of *L. fragile* were assayed on chicken goat and cow erythrocytes (Figure 2). In this assay, n-butanol extract showed high heamolytic activity against chicken blood (64 HU), goat blood (16 HU) and cow blood (16 HU). The present study supports the previous reports, that is, haemolysis of human and sheep red blood cells has been studied by Al

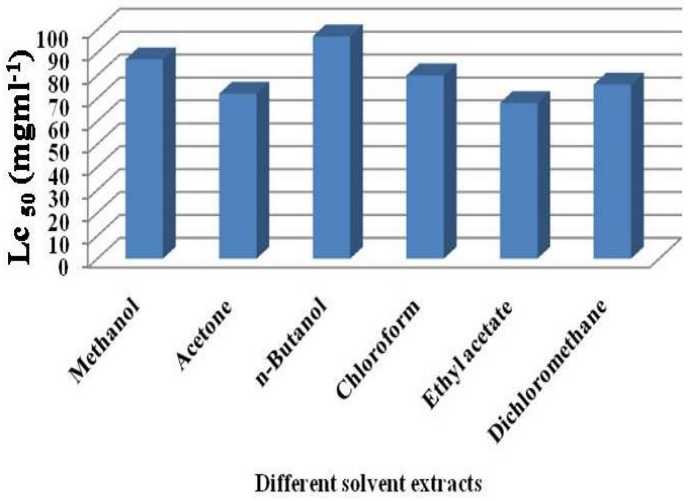

Figure 3. Cytotoxic activity of *L. Fragile* extracts.

Hassan et al. (1982). The haemolytic activity of tunicate, *Halocynthia aurantium* disrupted 8 and 16% of human erythrocytes, respectively (Jang et al., 2002). Lee et al. (2001) revealed that the haemolytic activity of the extracts from the ascidian, *H. aurantium* showed 21% lysis against human red blood cells. From the results, lytic protein substances are present in these animals.

Some compounds exhibited antibacterial activity as well as toxicity towards to brine shrimps. Coproverdine is a cytotoxic alkaloid isolated by bioassay direct fractionation of a unidentified ascidians collected at the three Kings Islands, New Zealand (Urban et al., 2002). Cytotoxicity towards a variety of murine and human tumor cells lines was observed. Rubrolide-M, recently isolated from a Spanish collection of the ascidians *Synoicum blochanni* (Ortega et al., 2000) was synthesized using palladium catalyzed coupling methodology (Bellina et al., 2002). The epidioysterol is toxic against *A. salina* larva, derived from morocco ascidian, *C. savignyi*; LC_{50} value showed 71 µg/ml. In the present study, n-butanol extract showed high LC_{50} 97 µg/ml (Figure 3). These results indicate that the ascidian *L. fragile* was found to have remarkable cytotoxic activities. These findings are consistent with previous studies on ascidians. Gouiffes et al. (1988) demonstrated that the Bistramide A compound derived from the ascidian, *Lissoclinum bistratum* from UA islet in New Caledonia, France showed cytotoxicity against *A. salina* larva in less than 1 µg/ml. Methanolic and crude extract of *Polyclinum madrasensis* are more toxic and they showed LC_{50} values of 97 and 95, respectively (Bragadeeswaran et al., 2010) like many species of ascidians that showed cytotoxicity. The ascidian, *L. fragile* seems to be a promising source of antimicrobial, haemolytic, and cytotoxic activities. These results indicate that ascidians exhibits remarkable activity against microbes. Therefore, this study exposed the presence of potent antimicrobial and cytotoxic compounds from ascidians of Tuticorin coast. Hence, further purification may lead to the discovery of novel antimicrobial and cytotoxic compounds.

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