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Bioactive potential with antimicrobial activity of selected gastropod *Euchelus asper*

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ABSTRACT

The present study aims to identify the bioactive potential of the extracts of *Euchleus asper* collected from the west coast of India Mumbai (Coast). The technique used was Disc diffusion technique to test the bioactive potential of gastropods against microbes. GC-MS and NMR spectroscopy was carried out to characterize and structurally analyze the bioactive compounds. The crude extract of *E. asper* in methanolic extract showed a maximum zone of inhibition against fish pathogens and human pathogens. The minimum zone of inhibition in ethyl acetate extract was recorded against human pathogens. The present study reveals that intertidal gastropod *E. asper* has bio potential activity in terms of antibacterial which needs to be explored.

Keywords: Bioactive potential, *Euchleus asper*, Mumbai coast, antimicrobial activity, Gastropod.

INTRODUCTION

The ocean, which covers more than 70% of earth's surface, is the largest and the most stable ecosystem of all biomes. It is well known that the ocean of the world is truly the last frontier for human exploration and exploitation of the planet. Further, these vast salty liquid expanses have inexhaustible resources of food and minerals. Therefore, today the major focus of marine activity tends to lie for food and energy exploration in the benefit of human beings. Nevertheless, the sea and coast worldwide are being used more and more to provide the basics of life. Among many uses of the oceans, discovery of novel products from marine organisms has inspired scientists all over the world to search for bioactive molecules of medicinal applications. Molluscs are extremely important members of many ecological communities of marine ecosystems. These organisms have been important to humans throughout history as a source of food, jewelry, tools, and even pets. Among Mollusca, Gastropods inhabit every niche in the ocean from the

intertidal zone to the deepest ocean trenches. Macrobenthic diversity is a nutrient resource of shell fisheries which can also be looked upon as a source of marine drugs.

Chemically these novel compounds are amino-acids, peptides, nucleosides, alkaloids, terpenoids, sterols and polycyclic esters. In marine ecosystems seasonal, tidal and diurnal changes influence organisms to produce a variety of different, often unique molecules that serve as protection against the enemies or that are of vital importance for feeding and reproduction (Lanora *et al*., 2006). These chemicals are referred to as secondary metabolites or natural products. These natural products from marine organisms are useful sources of developing novel therapeutics for human diseases. These marine natural products have been investigated primarily for their antimicrobial, anti-viral, cytotoxic, Anti-tumor, anti-inflammatory properties (Jha and Zi-rong, 2004). Till now sponges of marine environment are found to be a potent species for many bioactive compounds. Similarly other groups like tunicates, bryozoans and horseshoe crabs have also shown potential of active biomolecules (Jha and Zi-rong, 2004). The majority of the research on the natural products from marine mollusc has been concentrated on the gastropods. Extraction of ziconotide from *conus magus* has motivated the scientist to screen marine mollusc for bioactive compounds and more than 2600 scientific studies over the last 20 years have added an important contribution of toxins extracted from cone snails to medicine and cellular biology (Pickrell, 2003).

However, despite deteriorating coastal zones, plentiful diversity of molluscs including gastropods and bivalves in and around Mumbai has been reported recently (Datta *et al.,* 2010). Since gastropods inhabit polluted environments, they might be adapting to such polluted conditions with the help of their intrinsic physico-chemical mechanisms. Production of bioactive compounds may be one of the mechanisms of defense used by these marine macrobenthos. But insufficient information is available on bioactive compounds in intertidal macrobenthos of Mumbai, therefore it is rational to search for novel antimicrobial agents in these gastropods, as this benthos survive amidst high density of microorganisms. During present investigation the extracts of *E. asper* were processed to study and identify the potential antimicrobial activity of tissue treated with different solvents against four fish pathogens and eleven clinical isolates of human pathogens. The extract was also analyzed by GC-MS and NMR Spectrometry.

MATERIALS AND METHODS:

All the samples were collected manually from the Marine Drive rocky shore (Mumbai Coast) at low tide. The samples were brought to the laboratory and immediately frozen at -20^{0}C. The whole animal was removed from the shell and subsequently washed with autoclaved distilled water to remove any attached debris. The opercula of all the gastropods were detached from their respective bodies and discarded.

Extract Preparation:

The whole-body tissue was cut into small pieces & organic extracts were prepared by soaking the homogenized tissue in solvents such as Hexane, Petroleum ether, Ethyl acetate, Methanol and Acetonitrile.

Antibacterial Assay:

Antibacterial effect of extract of *Euchelus asper* was determined against 4 different fish pathogens viz., *Vibrio harveyi, V. alginolyticus, V. parahaemolyticus* and *Aeromonas hydrophila* and 8 human pathogens viz., *Edwardsiella tarda, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus, Klebsiella pneumoniae, Enterobacter gergoviae* and *Salmonella typhimurium* and antimicrobial activity was carried out using disc diffusion method (Bauer *et al.* 1966). Filter paper (Whatman No.1) discs with 4mm diameter were impregnated with known amount test samples of the crude extract obtained from *Euchelus asper* and *Trochus radiatus* and positive control contained a standard (Tetracycline) antibiotic disc. The discs were loaded each with 20 µl of crude extract, first applying 10 µl with the pipette, allowed to evaporate, then applying another 10 µl, then drying again. The positive control contained a standard antibiotic disc. The respective solvents are used as negative control. The impregnated discs along with control (tetracycline) were kept at the center of Nutrient agar plates seeded with test bacterial cultures. The discs were then placed individually using sterile forceps in appropriate grids which were marked on the under surface of the plated petri plates and kept for incubation at room temperature (28 ± 2°C) for 24 hr. After incubation, plates were observed for zones of inhibition which were recorded in millimetres.

Minimum Inhibitory Concentration (MIC):
The crude extract from *Euchelus asper* showing broad spectrum activities were examined for Minimum Inhibitory Concentration (MIC) by serial dilution technique method (Noble and Sykes, 1977) in the presence of a standard tetracycline. The different concentrations viz., 100, 90, 80, 70, 60, 50, 40, 30, 20 and 10 µg/ml each active sample was tested thrice for confirmation of activity.

GC-MS (Gas Chromatography - Mass Spectroscopy):
GC-MS analysis was used to identify compounds present in active Fractions. An Agilent 6890 Series GC System equipped with a BPX5 capillary column (50 m x 0.22 mm I.D., film thickness 1 µm, SGE) was used. The oven temperature was held at 50° C for 1 minute following injection and programmed to increase to 330°C at a rate of 5°C/min. The temperature of the injection port and interface was set at 250°C. Helium was used as the carrier gas, at a flow rate of 1.9 mL/min. The mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70 eV. One microliter of each sample was injected (10 mg/mL in Methanol) in the split less mode (Jeol, AccuTOF GCV)

NMR (Nuclear Magnetic Resonance):
NMR (^{1}H, ^{13}C) data were obtained on Bruker Avance 300 spectrometer with TMS as internal standard. EIMS and MS were recorded on QSTARXL MS/MS, applied Biosystems, Switzerland.

RESULTS

Antimicrobial Assay:
In fish pathogens maximum activity was observed on *Aeromonas hydruphila* and minimum activity was observed against *Vibrio harveyi.* The zone inhibition varied from 2 to 13 mm. Maximum 13±0.01mm diameter was noted against *V. parahaemolyticus* in methanol extract of *E. asper* and minimum 2±0.02mm diameter was recorded against *A. hydrophila* in acetonitrile extract. Compared to methanol and acetonitrile crude extract, other respective extract of ethyl acetate, petroleum ether and hexane did not show any activities. In the case of human pathogens, maximum zone of inhibition was observed in *S. aureus* and minimum was recorded in *E. coli.* The extract of methanol and acetonitrile, showing potential activities compared to other solvent extracts. Methanol solvent extract showed maximum activities for five different pathogens as; *E. gergoviae* (12.5±0.02mm), *P. aeruginosa* (11.5±0.01mm), *E. tarda* (10±0.03mm) and fungus *A. niger* (10±0.01mm). In the case of acetonitrile solvent extract, potential activity was observed from only one pathogen (*P. aeruginosa)*. However very negligible results were observed for human pathogens treated with ethyl acetate solvent extract. The *E. asper* crude extract MIC ranges varied between 10±0.02µg/ml - 40±0.03µg/ml at different pathogens.

GC-MS:
The fractionation and isolation of compounds on a C18 column using methanol/isopropanol gradient was carried out. The GC-MS was used to identify compounds present in the active fractions and to compare the profiles with standards. The GC-MS total ion chromatogram of the methanol extract peaks was carefully matched with commercial MS libraries of National Institute of standard and Technology (NIST). The results revealed that methanolic extract consists of various fatty acids and fatty acid methyl esters. The prevailing compounds in the methanol extract of *Euchelus asper* are Decanoic acid (RT. 12.4), Hexadecanoic acid (RT. 14.1), Hexadecanoic acid, methyl ester (RT. 15.2), Hexadecanoic acid, ethyl ester (RT. 15.3), Octadecanoic acid (RT. 15.6) and Octadecanoic acid, methyl ester (RT. 17.3). However, the present study shows potential fatty acid bioactive compounds from methanol extract of *E. asper.*

Identification of fatty acids and their derivatives compounds from purified extract of *E. asper* by using ^{1}H-NMR:
The results revealed that methanolic extract consists of various fatty acids and fatty acid derivative compounds like methyl esters, ethyl esters etc. The ^{1}H-NMR spectrum of fatty acid compounds is virtually identical to that of the strong peak caused by the methyl ester protons at about 3.34 ppm. The ^{1}H-NMR spectrum of other derivatives shows the following peaks: 2.17 ppm: CH_2 α to COOH (C2 methylene; triplet); 1.89 ppm: CH_2 of C3; multiplet and 1.3-1.4 ppm: CH_2 of C3-C16 and 0.899 ppm: CH_3 (C18 terminal methyl; triplet). In earlier NMR studies of a full series of *cis*-octadecenoic and some acetylenic fatty acids are studied by Gunstone and Ismail, 1967. The ^{1}H-NMR spectrum of the C18 fatty acid methyl ester in which the *cis* double bond is located at C6, however, shows minor changes in the chemical shifts compared to methyl ester, Double bond configuration affects the

NMR signals of the unsaturated fatty compounds. On the basis of foregoing evidence, it is concluded that the antimicrobial activity observed in the extracts is due to the presence of lipids particularly, fatty acids, fatty esters as the major components and Octadecanoic acid as the minor components of the purified extract. This observation is further supported by the GC-MS profile of the methanolic extract of purified active fraction of *E. asper.* However, the present study has potential bioactive compounds from methanol extract of *E. asper* in the presence of fatty acids and their derivative compounds.

Table -1: Antimicrobial activity of *E. asper* crude extracts against fish and human pathogens

Test organisms	Zone of Inhibition (mm)					Standard (Tetracycline)
	Methanol	Acetonitrile	Ethyl acetate	Petroleum ether	Hexane	
Fish pathogens						
Vibrio harveyi	10±0.02	10±0.02	-	-	-	12±0.03
V. alginolyticus	-	-	-	-	-	10±0.02
V. parahaemolyticus	13±0.01	4±0.03	-	-	-	10±0.03
Aeromonas hydrophila	11±0.02	2±0.02	-	-	-	9±0.01
Human pathogens						
Edwardsiella tarda	10±0.03	-	-	-	-	12±0.01
E. coli	-	-	1±0.03	-	-	8±0.03
Pseudomonas aeruginosa	11.5±0.01	12±0.02	-	-	-	10±0.02
Staphylococcus aureus	13±0.01	-	1±0.01	-	-	11±0.02
Bacillus cereus	-	-	-	-	-	6±0.03
Klebsiella pneumoniae	-	-	-	-	-	7±0.03
Enterobacter gergoviae	12.5±0.02	-		-	-	13±0.01
Salmonella typhimurium	-	-	2±0.02	-	-	6±0.02
Aspergillus niger	10±0.01	-	-	-	-	8±0.03
Penicillium citrinum	9±0.03	-	-	-	-	9±0.03
Candida albicans	-	-	-	-	-	10±0.02

The data presented as the mean ± value standard deviation of three replicates

Table -2: MIC (Minimum Inhibitory Concentration) value of E. asper crude extracts against fish and human pathogens

Test organisms	Zone of Inhibition (mm)					Standard (Tetracycline)
	Methanol	Acetonitrile	Ethyl acetate	Petroleum ether	Hexane	
Fish pathogens	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
Vibrio harveyi	10±0.02	30±0.02	-	-	-	20±0.02
V. alginolyticus	-	-	-	-	-	35±0.02
V. parahaemolyticus	10±0.02	-	-	-	-	35±0.03
Aeromonas hydrophila	10±0.03	20±0.01	-	-	-	20±0.01
Human pathogens						
Edwardsiella tarda	20±0.01	-	40±0.03	-	-	15±0.01
E. coli	-	-	-	-	-	20±0.02
Pseudomonas aeruginosa	10±0.02	20±0.02	-	-	-	15±0.02
Staphylococcus aureus	10±0.03	-	30±0.03	-	-	15±0.03
Bacillus cereus	-	-	-	-	-	20±0.02
Klebsiella pneumoniae	-	-	-	-	-	20±0.01
Enterobacter gergoviae	10±0.03			-	-	30±0.03
Salmonella typhimurium	-	-	30±0.02	-	-	20±0.02
Aspergillus niger	20±0.01	-	-	-	-	15±0.01
Penicillium citrinum	20±0.02	-	-	-	-	20±0.02
Candida albicans	-	-	-	-	-	25±0.03

The data presented as the mean ± value standard deviation of three replicates.

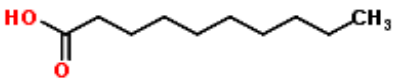
Table-3: Components detected in the purified methanolic extract of *E. asper*

No. of Peaks	Retention time (RT)	Peak width	Peak Area	Compound Name	Molecular Formula	Activities*
1	12.4	0.0348	2097239.21	Decanoic acid	$C_{10}H_{20}O_2$	Antifungal agent, Antibacterial agent.
2	14.1	0.0290	105499.54	Hexadecanoic acid	$C_{16}H_{32}O_2$	Antioxidant, Hypocholesterolemic Nematicide, Pesticide, Lubricant, Antiandrogenic, Flavor, Hemolytic 5-Alpha reductase inhibitor
3	15.2	0.0288	106631.31	Hexadecanoic acid, methy ester	$C_{17}H_{34}O_2$	Antioxidant, Hypocholesterolemic Nematicide, Pesticide, Lubricant, Antiandrogenic, Flavor, Hemolytic 5-Alpha reductase inhibitor
4	15.3	0.0290	669694.62	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	Antibacterial, Antioxidant, Antitumor, Cancer preventive, Immunostimulant, Chemo preventive
5	15.6	0.0291	384687.64	Octadecanoic acid	$C_{18}H_{36}O_2$	Antimicrobial Anti-inflammatory Anti cancer Diuretic
6	17.3	0.0241	131837.30	Octadecanoic acid, methyl ester	$C_{19}H_{36}O_2$	Antimicrobial and Antifouling

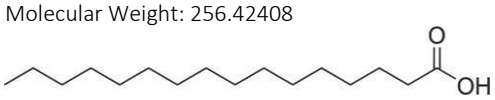
***Dr. Duke's :** Phytochemical and Ethnobotanical Databases

Fig. 1: Identified Compounds from purified methanolic extract of *E. asper*

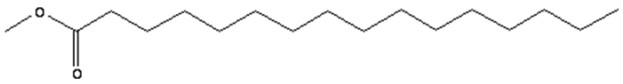
Decanoic acid:
Molecular Formula: $C_{10}H_{20}O_2$
Molecular Weight: 172.2646



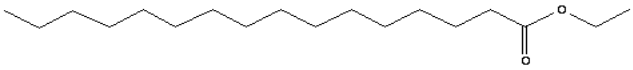
Hexadecanoic Acid:
Molecular Formula: $C_{16}H_{32}O_2$
Molecular Weight: 256.42408



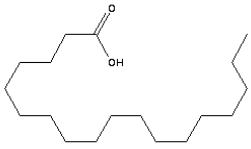
Hexadecanoic acid, methyl ester:
Molecular Formula: $C_{17}H_{34}O_2$
Molecular Weight: 294.4721



Hexadecanoic acid, ethyl ester:
Molecular Formula: $C_{18}H_{36}O_2$
Molecular Weight: 284.4772



Octadecanoic acid:
Molecular Formula: $C_{18}H_{36}O_2$
Molecular Weight: 284.4772



Octadecanoic acid, methyl ester:
Molecular Formula: $C_{19}H_{34}O_2$
Molecular Weight: 294.4721

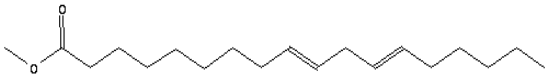


Fig.2: Antibacterial activity of methanolic extract of *E. asper* against some fish and human pathogens

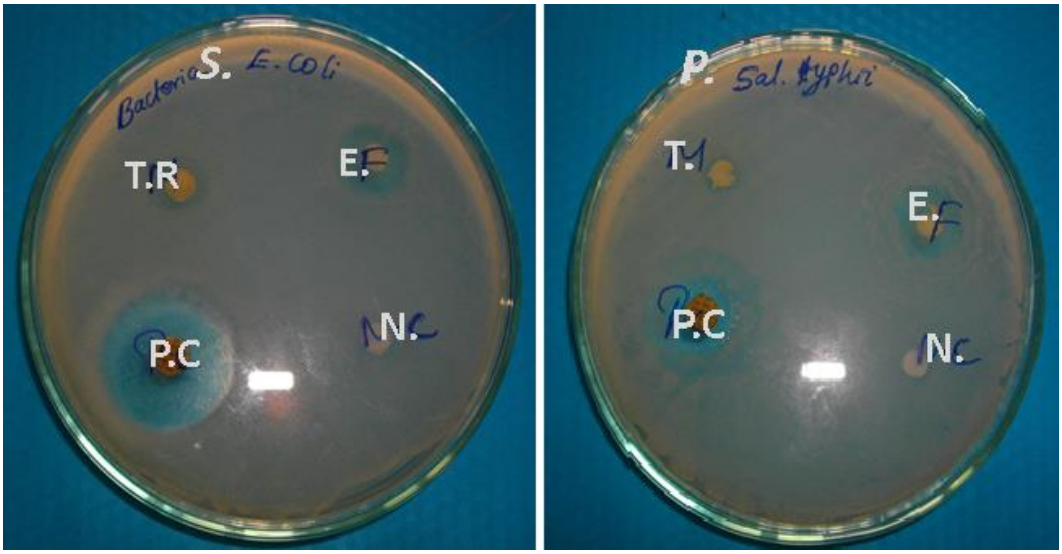


Fig.3: Antibacterial activity of methanolic extract of *E. asper* against some fish and human pathogens

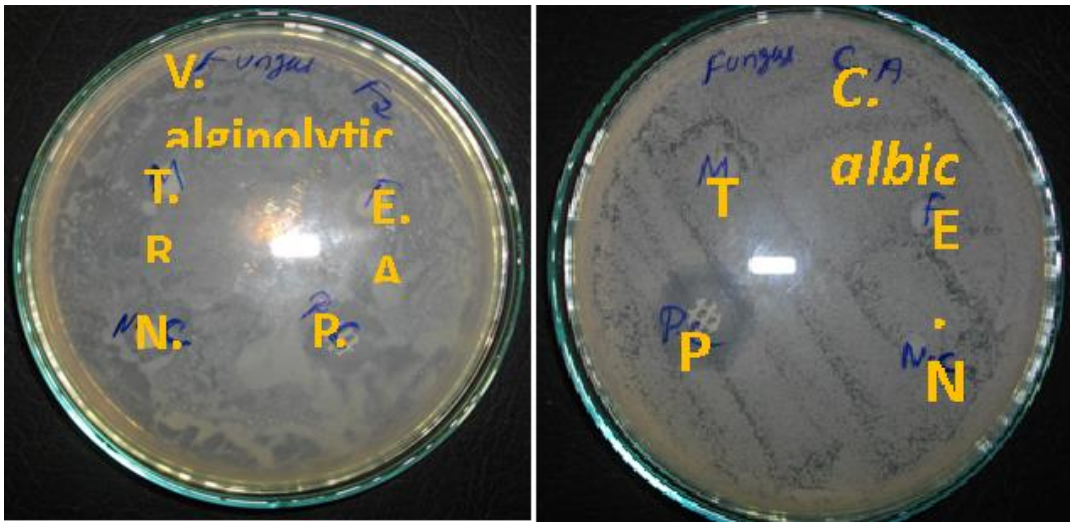


Fig.4: No activity of methanolic extract of *E. asper* against some fish and human pathogens
***E. asper* (E.A) extracts (P.C positive control; N.C negative control)**

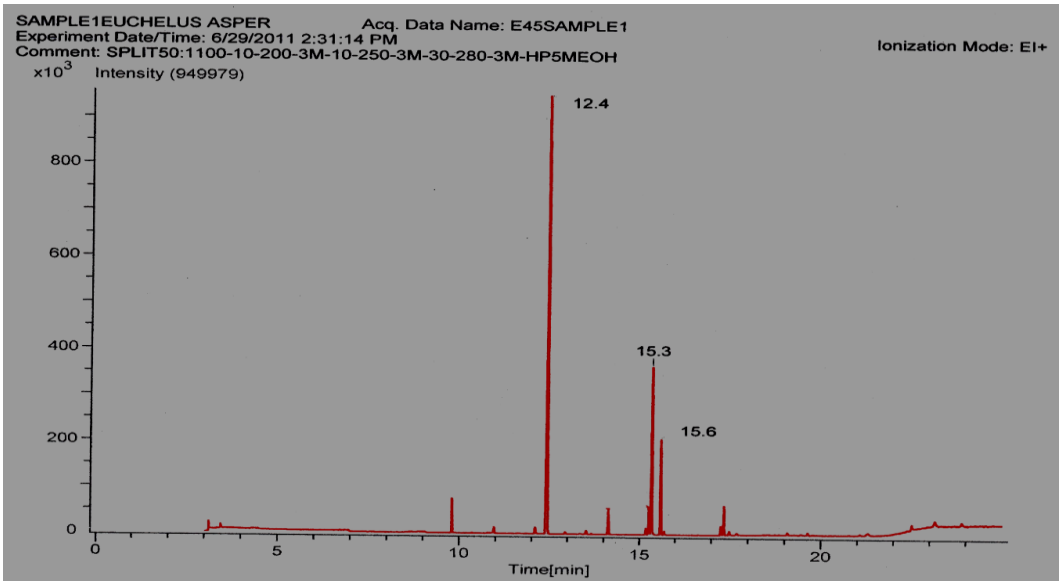


Figure 5: GC-MS chromatogram of bioactive compounds of purified methanolic extract of *E. asper*

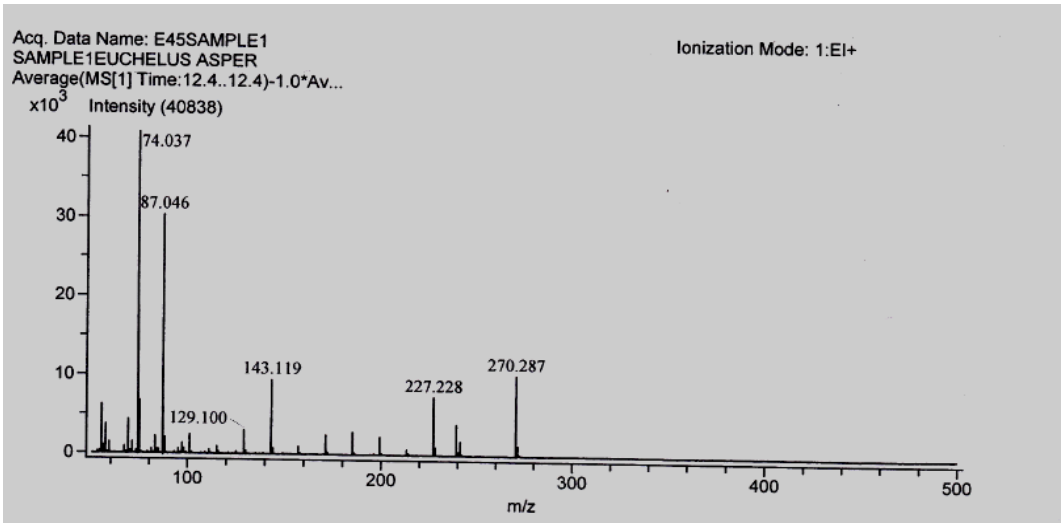


Figure 6A. Positive mode GC-MS of the molecular species of purified *E. asper* extract with intensity at 40638 (100-500 m/z)

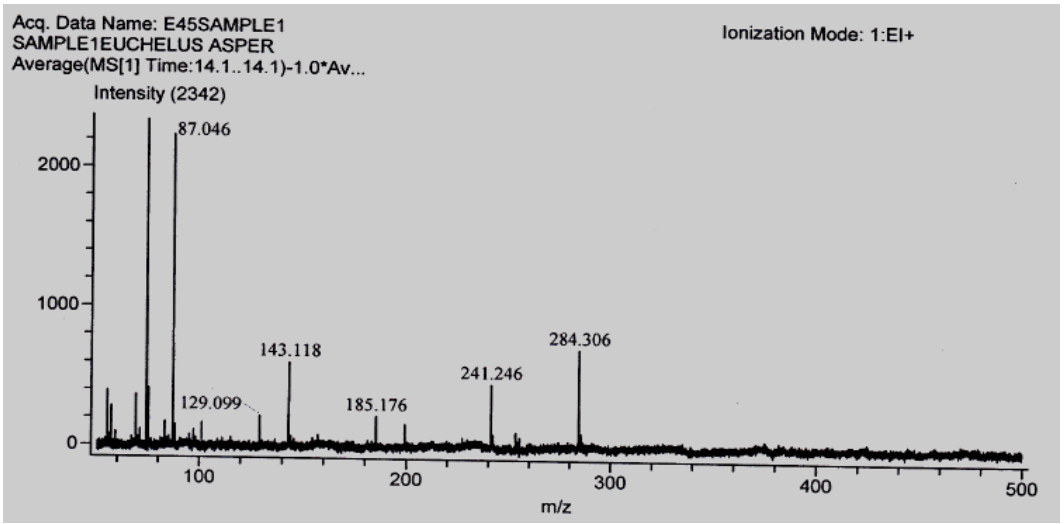


Figure 6B. Positive mode GC-MS of the molecular species of purified *E. asper* extract with intensity at 2342 (100-500 m/z)

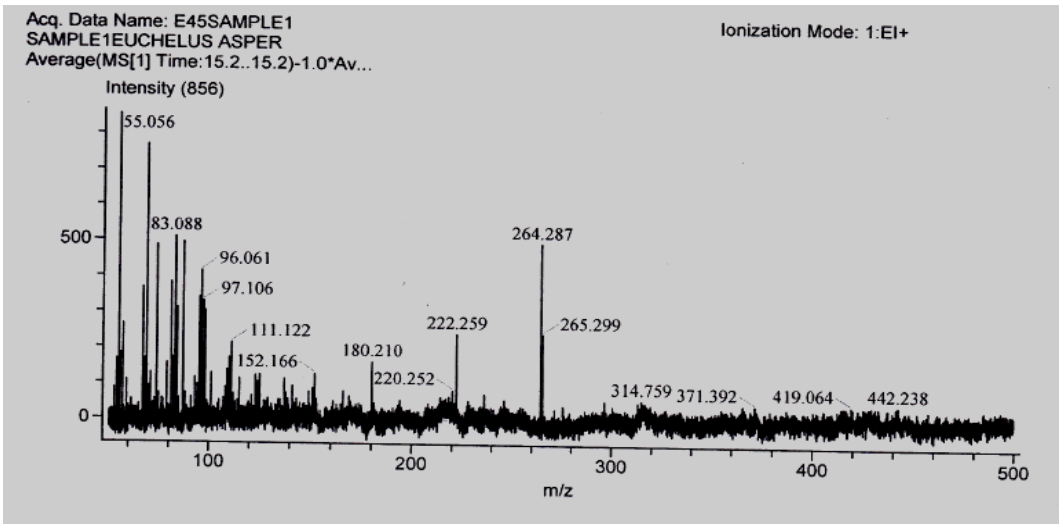


Figure 6C. Positive mode GC-MS of the molecular species of purified *E. asper* extract with intensity at 856 (100-500 m/z)

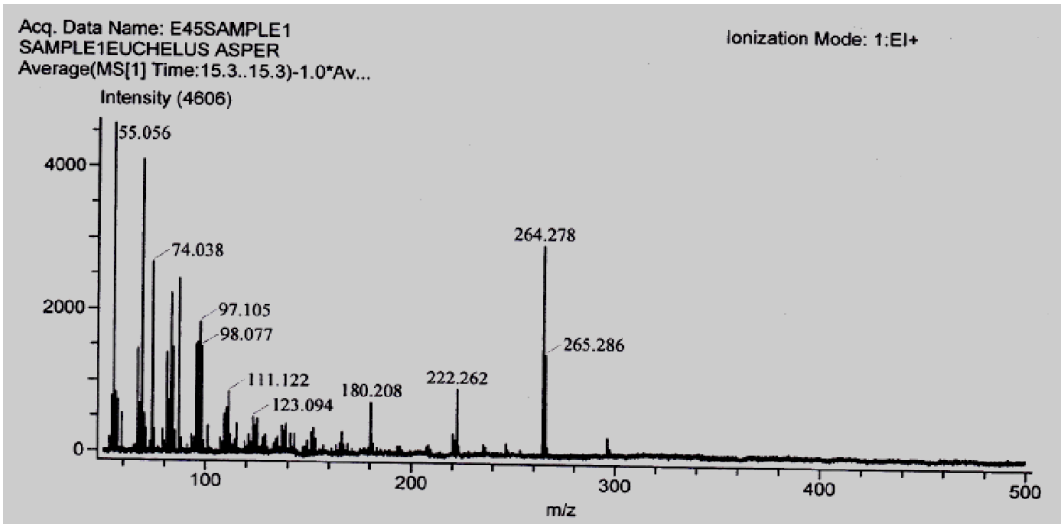


Figure 6D. Positive mode GC-MS of the molecular species of purified *E. asper* extract with intensity at 4606 (100-500 m/z)

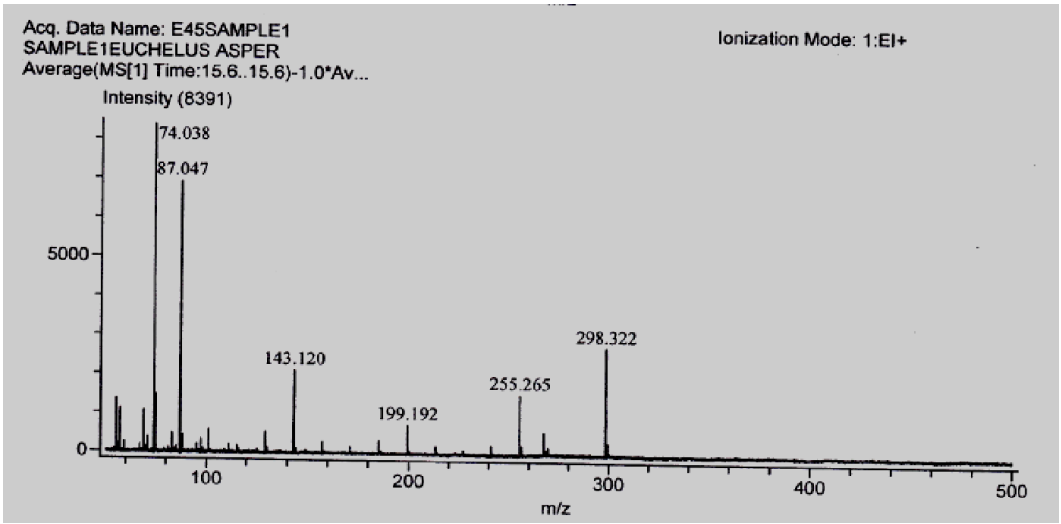


Figure 6E, Positive mode GC-MS of the molecular species of purified *E. asper* extract with intensity at 8391 (100-500 m/z)

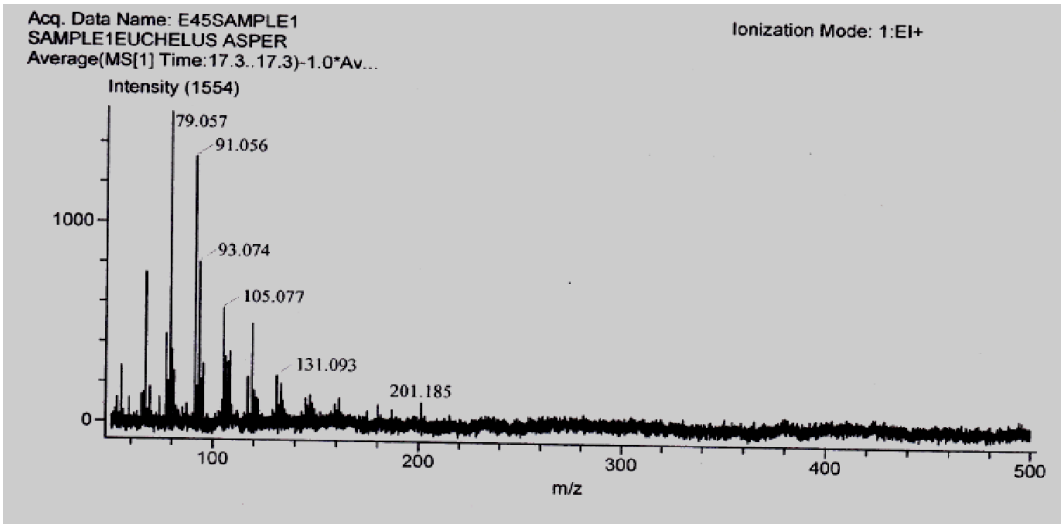


Figure 6F. Positive mode GC-MS of the molecular species of purified *E. asper* extract with intensity at 1554 (100-500 m/z)

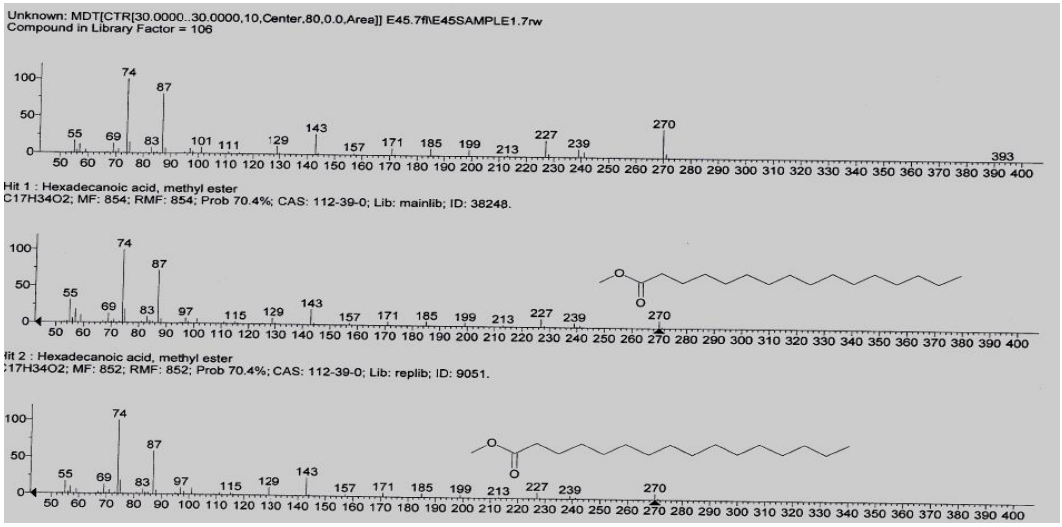


Figure 6G. Presence of Hexadecanoic acid, methyl ester in purified methanol extract of *E. asper* R.T at 12.4 min

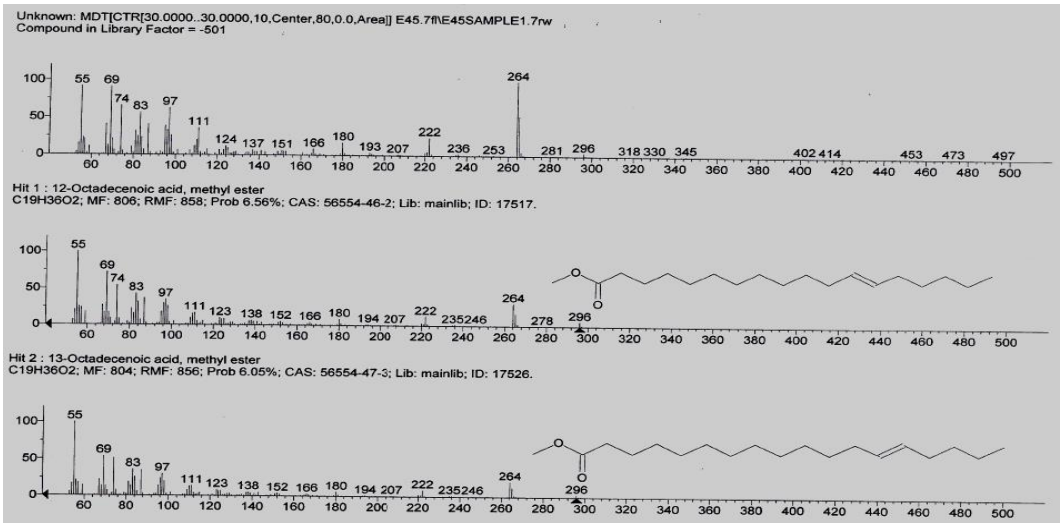
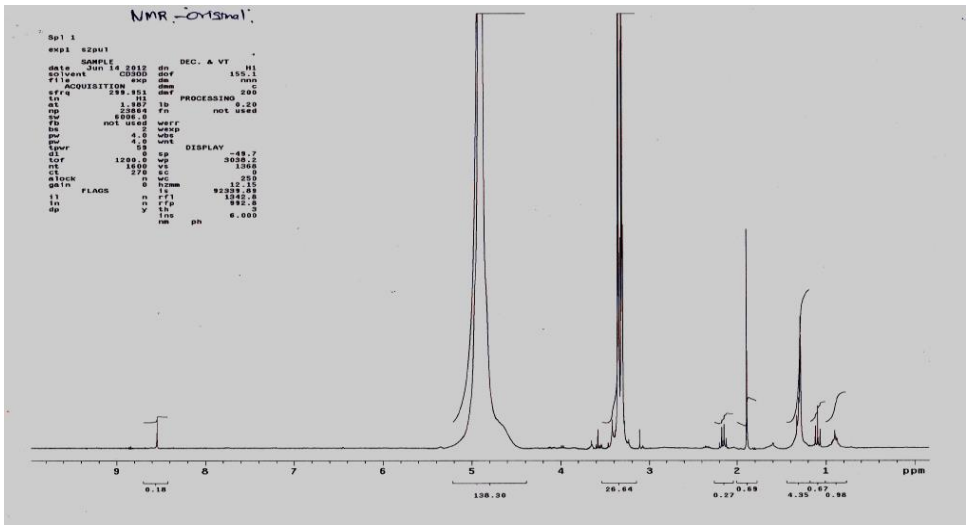
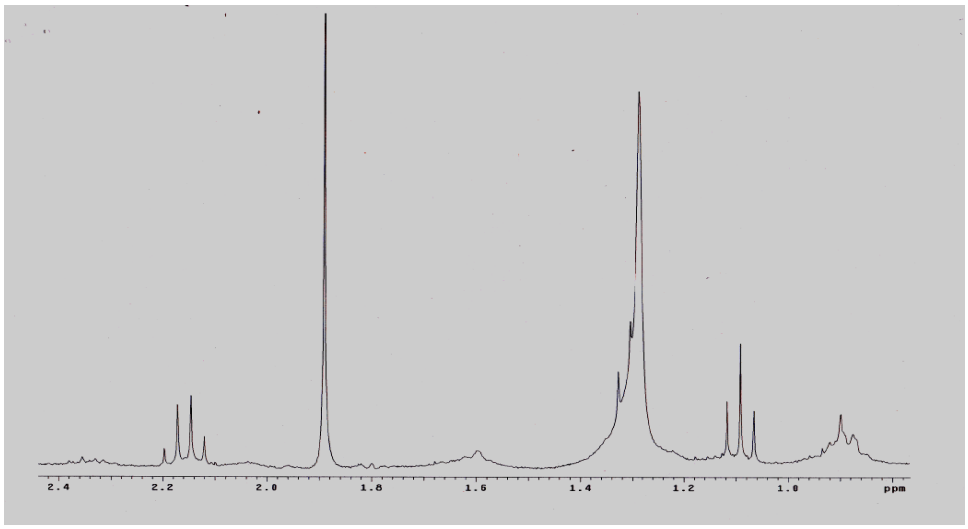


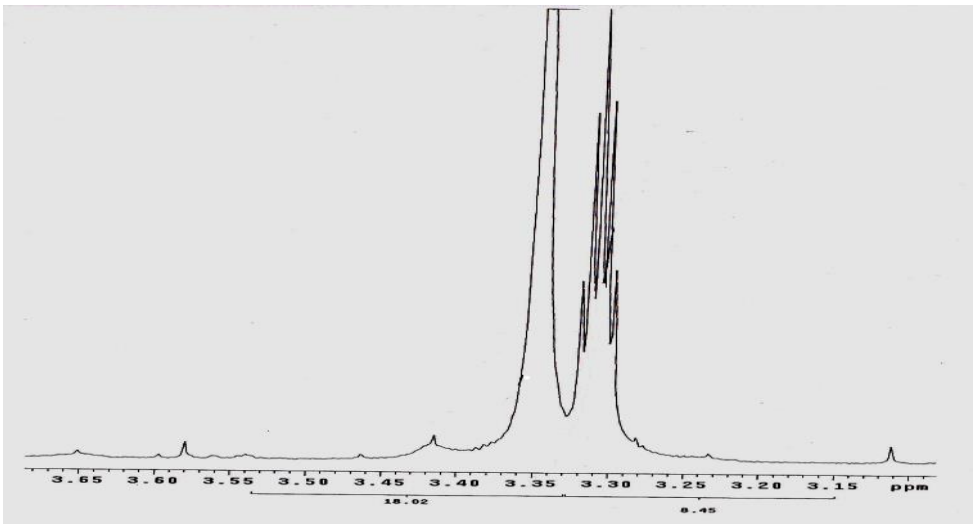
Figure 6H. Presence of Octadecenoic acid, Methyl ester in purified methanol extract of *E. asper* (R.T at 15.3 min)



(A): ^{1}H-NMR spectrum of purified methanol extract of *E. asper* (1 – 10ppm)



(B): ^{1}H -NMR spectrum of purified methanol extract of *E. asper* (0 – 2.2ppm)



(C): ^{1}H-NMR spectrum of purified methanol extract of *E. asper* (3 – 3.7 ppm)

Fig. 7 (A, B & C): ^{1}H-NMR spectroscopy of fatty acids and their Derivatives Compounds from purified methanolic extract of *E. asper*

(D): ^{1}HNMR spectrum Frequency with Height on different ppm

FREQUENCY	PPM	HEIGHT	FREQUENCY	PPM	HEIGHT
1469.014	4.898	1368.2	975.968	3.254	2.4
1419.526	4.733	12	974.501	3.249	2.4
1197.38	3.992	1.1	973.035	3.244	2.3
1190.782	3.97	1.1	969.736	3.233	3.3
1095.105	3.651	2.7	933.455	3.112	7.2
1078.976	3.597	1.1	922.081	3.074	1.1
1073.844	3.85	7	659.245	2.198	2
1061.747	3.54	1.4	651.546	2.172	8
1039.019	3.464	1.9	643.848	2.147	9.2
1024.356	3.415	10.8	636.15	2.121	3.7
1019.957	3.4	4.6	567.234	1.891	84.6
1017.391	3.392	4.5	478.888	1.597	1.8
1015.925	3.387	5.5	398.241	1.328	12.5
1014.458	3.382	6.8	391.276	1.304	19.3
1012.992	3.377	8	386.144	1.287	50.5
1004.194	3.348	1192.8	353.519	1.179	1
996.129	3.321	81.2	335.19	1.117	8.5
994.296	3.315	157.6	327.492	1.092	16.3
992.83	3.31	210.1	319.794	1.066	7.3
990.997	3.304	162.9	280.203	0.934	2.2
989.531	3.299	86.3	275.805	0.92	3
984.399	3.282	10.5	269.573	0.899	6.8
982.933	3.277	6.9	262.608	0.876	4.1

DISCUSSION

More than 1,100 antibiotic substances have been isolated from invertebrates. Among these, 50 have found widespread use in the prevention and treatment of bacterial diseases in animals and man (Gale and Kiser, 1967). Many of these organisms have antimicrobial properties, although most of the antibacterial agents that have been isolated from marine sources have not been active enough to compete with classical antibiotics obtained from microorganisms (Rinehart *et al.,* 1981). More than thousand new compounds such as peptides, terpenes, polypropionates, nitrogenous compounds, polypeptides, macrolides, prostaglandins as fatty acid derivatives, sterols and miscellaneous compounds have been characterized from marine invertebrates **(**Maktoob and Ronald, 1997**)**. The bioactive compounds isolated from gastropods are considered to have a role in the chemical defense against their predators (Avila, 1995). Bioactive compounds isolated from marine molluscs are also used for treatment of rheumatoid arthritis and osteoarthritism (Chellaram and Edward, 2009). In the present study antimicrobial activities were recorded from different kinds of fishes and human pathogens. Defer *et al.* (2009) reported that the extracts from molluscs have antimicrobial and antibacterial activities against human pathogens and potential activity against some fish pathogens. Periyasami *et al.* (2012) reported antimicrobial activity of the tissue extract of *Conus betulinus* and *Conus insccriptus* at low molecular weight. In this present study, MIC value confirm that *E. asper* methanolic extract has potential antimicrobial effect against the strains (*Vibrio harveyi, V. parahaemolyticus, Aeromonas hydrophila, Pseudomonas aeruginosa, Staphylococcus aureus*) at 10µg/ml and *Edwardsiella tarda* (20µg/ml). Petroleum ether and hexane extract were not active against any of the organisms it has been observed, generally the methanolic extract was more active than the other extracts. This may be attributed to the presence of soluble compounds

(Kowalski and Kedzia, 2007) present in the extract. The methanolic extract of *Hemifusus pugilinus* showed maximum antibacterial activity against human pathogens (Sugesh *et al*., 2013) and similar observation has been made by Anand *et al.* (1997) who reported antibacterial activities in marine gastropods.

Likewise, Jayaseeli *et al*. (2001) studied the antibacterial activity of four bivalves against few pathogens and found that the extracts showed significant activity against *K. oxytoca* and *Bacillus subtilis*. Anand and Edward (2001) studied the antibacterial activities in ethanol extracts of gastropod species *Babylonia spirata* and *Turbo brunneus* and observed highest activity against *E. coli, K. pneumoniae, P. vulgaris* and *S. typhi.* Diane *et al.* (2009) reported the antibacterial and antiviral activities on three bivalves and two gastropods' species.

The present finding agrees well with the result of broad-spectrum activity for methanolic extract of *E. asper* against three fish pathogens and six human pathogens (Anand *et al*., 1997). In traditional Indian medicine, especially siddha medical preparation, the opercula of gastropods are used as ingredient to combat different disease (Anbuselvi *et al*., 2009). In the present investigation, the tissue extracts of *E. asper* showed a broad-spectrum antimicrobial activity similar to *Lambish lambish* (Vimala and Thilaga, 2012) and *Chicoreus virgineus* (Rajaganapathy *et al*., 2000). There is a possibility that, the presence of derivative compounds in the tissue extracts of *E. asper* which could be responsible for antimicrobial activity.

In the present investigation, as compared to all crude extracts of *E. asper*, the methanol extracts of *E. asper* showed maximum antimicrobial activities against human pathogens as well as fish pathogens. It is revealed that some of the bioactive compounds are responsible for these activities.

CONCLUSION

The present study reviews that intertidal gastropod *E. asper* has bio potential activity in terms of antibacterial activities which needs to be explored. The GC-MS analysis shows the presence of a bioactive compound. The ^{1}H-NMR analysis also confirmed fatty acid functional groups in methanolic extract of *E. asper*.

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