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Screening of Antibacterial Activity of Marine Sponges Extract Against Clinically Significant MDR Pathogens.

KEYWORDS

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ABSTRACT *The large amounts of antibiotics used for human therapy, as well as for farm animals and even for fish in aquaculture resulted in the selection of pathogenic bacteria resistant to multiple drugs. Most bioactive compounds from sponges include anti-inflammatory, antitumor, antiviral, anti-malarial, and antibiotic or antifouling, immunosuppressive or neurosupressive. Hence, our present investigation of marine sponges extracted and fraction was tested against MDR (Multi Drug Resistant) pathogens isolated from clinical samples. Ethanol extract of marine sponges was effective in controlling the pathogen. Ethanol extract of marine sponges was most effective against E.coli (16.3mm) observed in extract at 50 µg concentration followed by salmonella and P.aeuroginosa (16.0mm). Qualitative and quantitative analysis of the Ethanol extract of marine sponges revealed the presence of flavones, alkaloids, tannins and sponins. Thin layer chromatography and GC-MS, FT-IR analysis confirmed the presence of secondary metabolites.*

INTRODUCTION:

The ocean covers 70% of the surface of the earth, and is still to a great extent unexplored. Accelerated technological development in sub-sea level sampling and harvesting equipment has increased the availability to areas below sea level (Synnes, 2007). The most promising organisms are microorganisms, sponges and cnidarians, and microorganisms and sponges have also had a significant increase in number of discovered compounds per year from 1965 to 2007. The marine environment resulted as early as1951 in three reports on nucleosides from marine sponges. This lead to the development of the chemical derivatives ara-A and ara-C, nucleosides that have been in clinical use in anticancer treatment for decades (Molinski et al., 2009). The habitats of marine organisms incline them to make adaptive adjustments, and to develop unique systems, which make them differ from terrestrial organisms. Sponges were first described from the Precambrian era over 700 million years ago(Finks,1970) and were the major-reef building organisms during the Palaezoic and Mesozoic eras(Hooper and Van Soest,2002).Sponges occupy a diverse array of marine and fresh water habitats, from the polar waters of Antarctica to tropical coral reefs. Despite being classified as the most primitive and simple metazoans sponges are demonstrated sophisticated cellular systems(Muller and Muller,2003) complex developmental and reproductive processes(Kaye and Reiswig,1991),versatility in feeding behavior (Vacelet and BouryEsnault,1995),production of unique natural products. Marine organisms living in the Arctic are exposed to extreme conditions with regard to temperatures and light. Because of this, organisms are developing unique qualities and biochemical processes in order to survive under these extreme circumstances. Such extremophile organisms are interesting in the search for unique, bioactive compounds (McMinn et al., 2005). A mineral skeleton composed of silica or calcium carbonate ,is found in most sponge groups and, along with collagen and spongin fibres provide the supporting structure. Sponges can filter large quantities of sea water(Bell et al.,1998),hence a large proportion of microbes residing within the tissue may be transient components of Pharmaceutical interest in sponges was aroused in the early 1950s by the discovery of a nucleosides spongothymidine and spongouridine in the marine sponge Cryptotethia crypta (Bergmann and Feeny,1950,1951).These nucleosides were the basis for the synthesis of Ara-C, the first marine derived anti-cancer agent, and the Anti-viral drug Ara-A(Proksh et al.,2002) Ara-C is currently used in the routine treatment of patients with leukemia and lymphoma. More than 15,000 marine products have been described so far from sponges.(Marinlit,1999.,Faulkner,2000,2001,2002). Sponges in particular, are responsible for more than 5300 different product, and every year Hundreds of new compounds are being discovered.(Faulkner.,1999,2001,2002). Most bioactive compounds from sponges include anti-inflammatory, antitumor, antiviral, anti-malarial, antibiotic or antifouling, immunosuppressive or neurosupressive. In the present investigation of marine sponges extracted and

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fraction was tested against MDR (Multi Drug Resistant) pathogens isolated from clinical samples.

Materials and Methods:

Isolation of Microorganisms

The collected samples were transferred into aseptic conditions. The sample was cultured in nutrient agar medium plates incubated at 37 degree C for 24 hours. After incubation periods characteristic colonies were subjected to standard tests.

Identification of the Isolates:

The isolated colonies were purified using pure culture technique, and stock culture was maintained in Nutrient agar slants for further studies. Pure cultures were further differentiated and characterized by the following standard morphological, physiological and biochemical tests (Bergys manual John Holt,1994).

Sponge Collection:

The marine sponges Monanchora arbuscula, Arenosclera brasiliensis and Caminussphaero chonia were collected at depths of shallow region of Gulf of Mannar and Thondi coastal region of South east coast of India in Nov2009 (GPS:9DEGREE14"N:79DEGREE 14).Sponge material was frozen and transported to the laboratory and subsequently stored . Organic extract preparation 30g of biomass of sponge Monanchora arbuscula , Arenosclera brasiliensis and Caminussphaero conia was lyophilized with a freeze dryer.10g of the dried mass from each sponge were extracted successfully with hexane and methanol(1L of each solvent) to obtain milligram amounts of the three different crude extracts per sponge. Obtained crude extracts were submitted to bioactivity testing.

Extraction Procedure

Organic fractions of the selected marine sponge obtained by standard cold extraction method.Hot extraction method was followed for aqueous extraction Harbone(1984)

Antibacterial Assay

Based on disc diffusion method the antibacterial assay was performed.Sterile Petriplates containing 20ml of Puller Hinton Agar medium were seeded with 0.01ml of 18 hours old culture with calibrated loop(Himedia)and lawned evenly using sterile cotton swabs.Wells were made using well cutter and added with one drop of sterile agar at the bottom of the well to seal it(Honda reference).All the extracts and fractions were added at different concentrations viz., 40microlitre,60microlitre,80microlitre,100 microlitre.Incubation was made at 37C for 24 hours.The assessment activity was based on the measurement of the inhibition zone formed around the disc,using Hi-media scale.Gentamycin, Ampicillin,Ceftoxamine,Ciproflaxime and various other antibiotics of 100microlitre were used as controls.Triplicates were made for all experiments.

Separation and Characterization of Active Principles:

Crude extracts which showed maximum MDR pathogen activity was subjected to further separation and characterization procedures.

Qualitative analysis of secondary metabolites by chromatography techniques:

Column chromatography

Column chromatography is frequently used by organic chemists to purify liquids or solids. An impure sample is loaded into a column of absorbent, such as silica gel or alumina. An inorganic solvent or a mixture of solvents flows down through the column. Components of the sample separate from each other by partitioning between the stationery packing material (silica or alumina)and the mobile eluent. Molecules with different extents move through the column at different rates. The eluent is collected as fractions. Fractions are typically analyzed by thin layer chromatography to see if seperation of compounds.

Thin Layer Chromatography:

TLC (Thin Layer Chromatography)plate was prepared by mixing silica gel-G in distilled water.Mixture was prepared in a colloidal form, poured and spread to the glass slide as a thin layer.

Result and Discussion

In the present investigation antibacterial activity of marine sponges extracts was assayed against the MDR pathogens. The clinical samples (sputum and urine sample) were collected from Govt-Hospital in Trichy, to isolate MDR pathogens. MDR pathogens were isolated using plating techniques (Blood Agar plates, Macconkey agar plates), identified based on standard morphological and biochemical screening which includes, staining, motility test, IMVIC test etc (Table 1).

Ciprofloxacin, Norfloxacin, Ofloxacin, Novobiocin, Gentamycin, and Erythromycin are the routine antibiotics prescribed for UTI. But in our study that higher rate of resistance in these antibiotics against the clinical isolates (Table-2). These may be due to frequent use of antibiotics, incomplete course of antibiotic, incomplete therapy and low quality antibiotics. Noyce et al., 2006 Ragunath et al., 2008 and Elizabath Ponciae 2008 reported that Enterbacteriaceae have changed their resistant patterns extensively. According to them the resistance is wide spreaded among enterobacteriacae due to vertical us usual as horizontal acquired resistance patterns.

A grained material of marine sponges was successively extracted with aqueous and organic solvents and the extraction values were depicted. Extraction was high in water (4.5g) followed by hexane and ethyl acetate (3.5g). Least quantity of secondary metabolites were extracted in alcohol (2.5g) (Table: 3).

The extractive value in water (4.5) and ethyl acetate (4.1) indicated the presence of polar constituents like alkaloids, flavones and sugars. Hexane and cholorofrom extractive values (3&3.1) suggested the presence of non -polar chemical constituents (Rajapandiyan et al., 2011).

Antibacterial activity of hexane fraction of marine sponges was against MDR pathogens were depicted in table-3. Pseudomonas aeruginosa was highly sensitive to hexane fraction at 800µg/well concentration and produced (13.6 mm) of zone of inhibition. E.coli, Klebsiella pnemoniae, Salmonella were also controlled by these fractions at 800µg concentration. S,aureus was highly resistant to all the fractions of marine sponges (Table -4).

Singh et al., (1999) reported that aqueous, ethanolic and hexane extracts of marine sponges have insecticidal and bactericidal activity. The hexane extract, which exhibited much higher activity than the two extracts.

Antibacterial activity of chlorofrom fraction of marine spongeswastested against MDR pathogen. E.coli (12.6mm) and Pseudomonas aeruginosa (12.6mm) was highly sensitive to chlorofrom fraction of marine spongesfollowed by

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Klebsiella pnemoniae (11.6mm), Salmonella sp.,(11.6mm), S.aureus was highly resistent to all the fractions of marine sponges.(Table -5).

Antibacterial activity of ethyl acetate fraction of marine sponges was tested against MDR pathogens. E.coli was highly sensitive to ethyl acetate fraction at 800µg/well concentration and produce (13.6mm) of zone of inhibition. , Klebsiella pnemoniae, Salmonella sp., pseudomonas aeruginosa were also controlled by these fraction at 800µg concentration. The zone of inhibition ranged between (13.6-12mm). Absence of zone of inhibition was observed in S.aureus.(Table-6).

Antibacterial activity of aqueous, ethanol and acetone extracts of marine spongeswas determined against E. coli. Acetone and ethanol extracts exhibited significant activity (Anjanaet al., 2009).

Antibacterial activity of alcohol fraction of marine spongeswas assayed against MDR pathogens. Klebsiella pnemoniae was highly sensitive to alcohol extract only at highest concentration (800µg/well) concentration and produce (14.6mm) of zone of inhibition. E.coli.,Pseudomonas aeruginosa were also controlled by these fraction at 800µg concentration. The zone of inhibition ranged between (14.6-11mm).Salmonella and Staphylococcus aureus were completely resistent to alcohol fraction. (Table -7).

Ethanol extract of marine spongeswhich showed maximum antibacterial activity (9th day) was purified by column chromatography. The eluents with different colours were subjected to Thin Layer Chromatography (TLC). Spots with Rf value 0.66, 0.51 and 0.79 indicated the presence of marine sponges compound. Purified compounds were tested for their antibacterialactivity. (Table: 17). Singh et al., (1999).

The GC-MS analysis of ethanol extract of marine sponges indicated the presence of 55% of oxirane hepatadecyl (M.wt. 282) with highest retention time 60.22. 1, 2- benzenedicarboxylic acid mono(2- ethylhexyl) ester (M.wt. 278) was present around 16%, Eicosane, 12% (M.wt. 282) , dibutylphthalate, 1.93% (M.wt. 278) and phenol 2,4 – bis (1,1- dimethyl ethyl) 1.8% (M. wt. 206), are some of the fragments and groups of significance. (Table-18 ,fig-1).

FT-IR analysis of ethanol extract of marine spongesshowed nearly 15 peaks. Peaks at 1359 cm^{-1}, 1219 cm^{-1}, and 1024 cm^{-1} frequencies may be considered as new. Frequencies having 1465 may be having C-O stretching, frequency 1219 cm^{-1} may have C-O stretching 1025 may have C-N stretching which may be confirmed by further studies.(fig-2).

In the present investigation, GC-MS analysis of ethanol extract of marine spongesindicated the presence of oxirane hepatadecyl- 1, 2- benzenedicarboxylic acid mono(2- ethylhexyl) ester, Eicosane, dibutylphthalate, and phenol- 2,4 – bis (1,1- dimethyl ethyl). The GC-MS result indicated high percentage of oxirane derivates while the quantitative analysis indicated flavanoid in higher proportion. Dodecane- 2,6,11- tri methyl tridecane, Dodecane- 2,7, 10 trimethyl eicosane, Octadecane- 2- methyl hexadecane- 2,6,11,15- tetramethyl were reported in cow urine extract . These are all the sub groups of Decosane. As such decosane has antibacterial activity (Exarchou et al., 2002; Christoforidou et al., 2005)

Table No: 1 Identification of Pathogen Based On Biochemical Tests.

S.No	Test	E.coli	Salmonella	S.aureus	Pseudomonas	Klebsiella
1	Simple staining	Rod	Rod	Cocci	Rod	Rod
2	Gram staining	-	-	+	-	-
3	Motility	M	M	NM	M	NM
4	Indole test	+	-	-	-	-
5	Methyl red	+	+	+	+	-
6	Voges-proskaur	-	-	-	-	+
7	Citrate utilization	-	-	+	-	+
8	Urease	-	-	+	-	+
9	TSI test	A/A	A/K	K/K	A/K	A/A
9a	H_2s Production	-	+	-	-	-
9b	Gas Production	+	+	-	+	+
10	Nitrate reduction	+	+	-	+	+
11	Catalase test	+	+	+	+	+
12	Oxidase test	+	+	-	+	+
13	Carbohydrate fermentation test					
13a	Glucose	+	+	+	+	+
13b	Maltose	+	+	+	-	+
13c	Sucrose	+	+	-	-	+

Table No -2: Antibiotic Profile of clinical isolates

Name of the organisms	Bacitracin		Carbencillin		Cefixime		Ciprofloxacin		Erythromycin		Novobiocin	
	R%	S%	R%	S%	R%	S%	R%	S%	R%	S%	R%	S%
E.coli	26	74	35	65	32	68	21	79	26	74	21	79
K.pneumoniae	31	69	38	62	38	62	8	92	8	92	15	85
p.aeuroginosa	82	18	82	18	36	64	9	91	27	73	36	64
S.aureus	23	77	23	77	23	77	13	87	20	80	13	87
S.typhi	100	-	50	50	50	50	-	100	100	-	100	-

Table No : 3 Extraction values ofmarine sponges in different solvents

S.No	Solvents	Volume of Solvent Added (ml)	Powder taken in grams	Incubation Period	Volume of solvent collected (ml)	Wet weight in grams	Final dry weight in grams
1	Hexane	300	100	3 days	250	95.5	3.5
2	Chloroform	300	100	3 days	220	84.6	3.0

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S.No	Solvents	Volume of Solvent Added (ml)	Powder taken in grams	Incubation Period	Volume of solvent collected (ml)	Wet weight in grams	Final dry weight in grams
3	Ethyl acetate	300	100	3 days	190	79.2	3.5
4	Alcohol	300	100	3 days	150	71.9	2.5
5	Water	300	100	Boiled at 100°C	150	82.6	4.5

Table No: 4 Antibacterial Activity of Hexane Fraction ofmarine spongesAgainst MDR Pathogens

Name of the organisms	Concentration of Extract in µg/ well zone of inhibition in mm					
	P	N	200µg	400µg	600µg	800µg
Klebsiella pnemoniae	12.6± 0.47	-	11±0.00	11.3 ± 0.47	12 ± 0.81	13.3 ± 1.24
E.coli	13.6 ±0.47	-	10.0±0.00	10.0 ± 0.00	10.3 ±0.47	11.6 ± 0.94
Salmonella sp	14.3 ±0.47	-	10.0 ±0.00	10.6 ± 0.94	12.6± 0.47	13.3 ± 0.47
Pseudomonas aeruginosa	16.3 ±0.47	-	12 ±0.81	12.6 ± 0.47	12.6 ± 0.47	13.6 ± 0.47
Staphylococcus aureus	12.0 ±0.00	-	-	-	-	-

Positive control - Ciprofloxacin for Gram^{+ve} organisms and Erythromycin for Gram^{-ve} organisms
Negative control - DMSO

Table No: 5 Antibacterial Activity of Chlorofrom Fraction of marine sponges against MDR Pathogens:

Name of the organisms	Concentration of Extract in mg/well zone of inhibition in mm					
	P	N	200µg	400µg	600µg	800µg
Klebsiella pnemoniae	24.3± 0.47	-	10.0±0.00	10.6± 0.47	11.0 ± 0.00	11.6 ± 0.47
E.coli	14.7 ±0.47	-	10.3±0.47	11.3 ± 0.47	12.0 ±0.81	12.6 ± 0.47
Salmonella sp	20.6 ±0.94	-	10.0 ±0.00	10.3 ± 0.47	11.3± 0.47	11.6 ± 0.47
Pseudomonas aeruginosa	20.6 ±0.94	-	10.0 ±0.00	11.0 ± 0.00	11.3 ± 0.47	12.6 ± 0.47
Staphylococcus aureus	15.3 ±0.47	-	-	-	-	-

Positive control - Ciprofloxacin for Gram^{+ve} organisms and Erythromycin for Gram^{-ve} organisms
Negative control - DMSO

Table No: 6: Antibacterial Activity of Ethylacetate Fraction ofmarine spongesAgainst MDR Pathogens

Name of the organisms	Concentration of Extract in µg/ well zone of incubation in mm					
	P	N	200µg	400µg	600µg	800µg
Klebsiella pnemoniae	20.3± 0.47	-	10.0±0.00	10.6± 0.47	11.3 ± 0.47	12.3 ± 0.47
E.coli	14.3 ±0.47	-	10.3±0.47	11.6 ± 0.47	12.6 ±0.47	13.6 ± 0.47
Salmonella sp	20.6 ±0.94	-	11.3 ±0.47	11.6 ± 0.94	12.3± 0.47	13.3 ± 0.47
Pseudomonas aeruginosa	17.3 ±0.47	-	-	-	-	12.0 ± 0.00
Staphylococcus aureus	15.3 ±0.47	-	-	-	-	-

Positive control - Ciprofloxacin for Gram $^{+ve}$ organisms and Erythromycin for Gram^{-ve} organisms. Negative control - DMSO

Table No: 7 Antibacterial Activity of Alcohol Fraction ofmarine sponges MDR Pathogens

Name of the organisms	Concentration of Extract in µg/well zone of incubition in mm					
	P	N	200µg	400µg	600µg	800µg
Klebsiella pnemoniae	15.3± 0.47	-	-	-	-	14.6 ± 0.47
E.coli	16.0 ±0.00	-	10.0±0.00	10.5 ± 0.5	12.0 ±0.00	12.0 ± 0.00
Salmonella sp	15.0 ±0.00	-	-	-	-	-
Pseudomonas aeruginosa	15.0 ±0.00	-	-	-	10.0 ± 00	11.0 ± 0.00
Staphylococcus aureus	14.6 ±0.47	-	-	-	-	-

Positive control - Ciprofloxacin for Gram^{+ve} organisms and Erythromycin for Gram^{-ve} organisms
Negative control - DMSO

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Table No: 8 Antibacterial Activity of Aqueous Fraction ofMarine SpongesAgainst MDR Pathogens

Name of the organisms	Concentration of Extract in µg/well zone of incubition in mm					
	P	N	200µg	400µg	600µg	800µg
Klebsiella pnemoniae	17.3± 0.47	-	10.0±0.00	11.0±00	11.3±0.47	12.6± 0.47
E.coli	15.6 ±0.47	-	10.0±0.00	10.3 ± 0.47	1.0 ±0.00	11.6 ± 0.47
Salmonella sp	16.3 ±0.47	-	-	10.00±0.00	10.6±0.47	11.3±0.47
Pseudomonas aeruginosa	14.3 ±0.47	-	-	-	12.0±0.00	13.6 ± 0.47
Staphylococcus aureus	15 ±0.00	-	-	-	-	-

Positive control - Ciprofloxacin for Gram^{+ve} organisms and Erythromycin for Gram^{-ve} organisms
Negative control - DMSO

SUMMARY AND CONCLUSION

The sputum and urine sample were collected from Govt. Hospital Trichy. The samples were aseptically processed. Microorganisms isolated were identified using microscopic and macroscopic examinations as E.coli, K.pneumoniae, P.aeuroginosa, S.aureus, Salmonella sp. They are the predominant flora of respiratory tract and urinary tract. Sensitivity profile of all the isolates against several antibiotics were tested. Most of the isolates were resistant to ciprofloxacin, Erythromycin, Gentamycin, Cefixime, Carbencillin, Novobiocin, bacitracin. Antibacterial activity of organic fractions, aqueous extract marine sponges screened against MDR pathogen. The zone of inhibition for ethanol fraction ranged (10-14.6mm). It was most effective against K.pneumoniae followed by hexane and aqueous fraction (13.6mm). For chloroform fractions they exhibited 12.6mm zone of inhibition. Ethanol extract of marine sponges was effective in controlling the pathogen. Ethanol extract of marine sponges was most effective against E.coli (16.3mm) observed in extract at 50 µg concentration followed by salmonella and P.aeuroginosa (16.0mm). Qualitative and quantitative analysis of the Ethanol extract of marine spongesrevealed the presence of flavones, alkaloids, tannins and sponins. Thin layer chromatography and GC-MS, FT-IR analysis confirmed the presence of secondary metabolites. The Ethanol extract of marine sponges could be used as a natural, viable, cost effective, alternate medicine to control RTI and UTI.

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