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In vitro Antibiotic Bustle of Coral Reef Associated Gastropod, *Drupa margariticola* (Broderip, 1832) of Tuticorin Coastal Waters, Southeastern India

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Abstract: To test the antibacterial effect of the extracts of *Drupa margariticola* obtained using low to high polar solvents, like ethyl acetate, dichloromethane, acetone and methanol. Partial purification of the active crude extract was carried out using column chromatography employing a step gradient solvent system. A maximum inhibition of 7 mm against *E. coli* was shown by the 100% acetone column purified fractions of *D. margariticola* at a concentration of 0.125 mg. Minimum Inhibitory Concentration values were found to be lower for the 100% acetone fraction for pathogens, *E. coli* (0.05 mg), *Klebsiella pnuemoniae* (0.05 mg), *Pseudomonas aerogenosa* (0.07 mg) and *Streptococcus pnuemoniae* (0.07 mg). Thus 100% acetonated fraction of the extract of *D. margariticola* was considered as potent antibacterial compounds against some human pathogens. The antibacterial potential of the mollusc, *Drupa maragariticola* becomes a corner stone for the future development of novel biologically active compounds.

Key words: Reef associated mollusc, *Drupa margariticoala*, antibacterial activity, MIC

INTRODUCTION

The oceans are the source of a large group of structurally unique natural products that are mainly accumulated in bacteria, invertebrates such as sponges, tunicates and bryozoans and also in molluscs. Research on bioactive compounds from marine organisms has provided the broad and better support of marine natural products research throughout the past quarter century. Serious attempts to tap the vast potential of marine organisms as sources of bioactive metabolites that may be directly utilized as drugs or serve as lead structures for drug development started in late 1960s. Marine natural products chemistry is essentially a child of the 1970's that developed rapidly during the 1980's and matured in the last decade (Faulkner, 2005).

As a consequence of an increasing demand for the biodiversity in the screening programmes seeking therapeutic drugs from natural products, there is now a greater interest in marine organisms. There is a copious number of works pertaining to the discovery of antibacterial agents from marine bacteria (Fenical and Jensen, 1993; Kobayashi and Ishibashi, 1993), seaweeds (Fuller, 1994; Gerwick, 1993), sponges (Ireland *et al*., 1993; Fusetani *et al*., 1987; De Silva *et al*., 1992; Longley *et al*., 1991), molluscs (Zhang *et al*., 1994; Schmitz *et al*., 1993; Wright, 1998; Fenical, 1997; Chellaram and Edward, 2009) and ascidians (Davidson, 1993; Rinehart *et al*., 1993; Sakai *et al*., 1992). The cone shaped gastropod *Drupa margariticola* is commonly occurring long the Tuticorin costal waters were chosen in an attempt to test the antibacterial activity with the crude and column purified extracts. Also, the Minimal Inhibitory Concentration (MIC) of the column-purified fractions was tested.

MATERIALS AND METHODS

Extraction of crude extracts: The samples were collected in the intertidal region (using SCUBA diving) of the Tuticorin coastal waters (Lat 8°45 and Long 78°13'E) (Fig. 1) and immediately brought to the laboratory. The animals were thoroughly washed with fresh water to remove the salt and debris and air-dried. Approximately 20 g of the air dried sample was taken and immersed separately in different solvents such as ethyl acetate, acetone, dichloromethane and methanol and cold steeped at 18°C. The extract from each solvent was filtered

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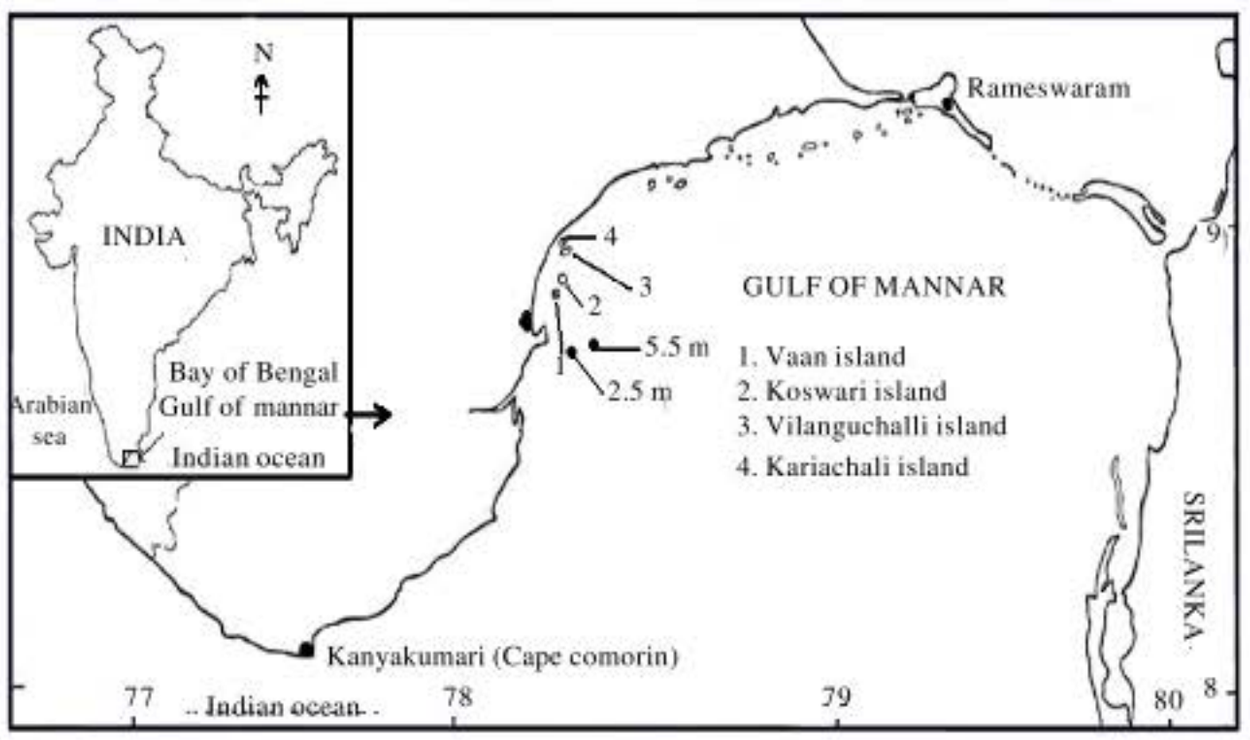

Fig. 1: Study area

separately using Whatmann No.1 filter paper. The filtrate was poured in previously weighed petri plates and evaporated to dryness (Chellaram and Edward, 2009b; Becerro *et al*., 1994; Riguera, 1997; Wright, 1998) and the dried extract was used for the antibacterial assay.

Antibacterial assay: To test the antibacterial effect of the extracts obtained using different solvents, *E. coli*, *Shigella dysentriae*, *Stphylococcus epidermidis*, *S. areus*, *Klebsiella pneumoniae*, *Pseudomonas aerogenosa*, *Salmonella typhimurium*, *S. paratyphi*, *Vibrio cholerae*, *Streptococcus pneumoniae*, *S. faecalis*, *Bacillus subtilis*, *B. cereus*, *Enterococcus aerogenosa* and *Citrobacter* sp. were used as a test strains. All the test strains were cultured in Nutrient Broth (NB) and the 12-18 h old cultures were used for the tests. The antibacterial assay was performed by using of the standard Nathan's Agar Well Diffusion (NAWD) technique (Nathan *et al*., 1978) against the test strains on 50% Nutrients Agar (NA) in petridishes with drilled wells of 6 mm diameter. The 0.2 and 0.4 mg of the dried extract in 50 µL Dimethyl Sulfoxide (DMSO) was loaded onto each well. The well at the center served as the control (without the extract). After 22-24 h of incubation at room temperature, the susceptibility of the test organisms was determined by measuring the radius of the zone of inhibition around each well which is the distance between the border of the well and the edge to where the test strains are completely inhibited.

Partial purification of the active crude extract: Partial purification of the active crude extract was carried out following the method of Wright (1998). After primary screening, the extract showing activity obtained from acetone was fractionated using normal phase silica gel (200-400 mesh, LOBA CHEMIE, Mumbai) column chromatography employing a step gradient solvent system from low to high polarity. The step gradient protocol used was: 100% hexane; 80% hexane: 20% acetone; 60% hexane: 40% acetone; 40% hexane: 60% acetone; 20% hexane: 80% acetone; 100% acetone; 80% acetone: 20% methanol; 60% acetone: 40% methanol; 40% acetone: 60% methanol; 20% acetone: 80% methanol and finally 100% methanol. Each of the dried fractions was dissolved in 5 µL DMSO and were again tested for antibacterial activity. After 24 h of incubation, the susceptibility of the test organisms was determined by measuring the radius of the zone of inhibition around each well.

Determination of Minimal Inhibitory Concentration (MIC): The Minimal Inhibitory Concentration (MIC) of the active column-purified fractions were determined by serially diluting the active column purified fractions so that concentrations of 250, 200, 150, 100 and 50 µg in 50 µL DMSO were loaded in to each well for the individual pathogenic strains that were found to be highly susceptible. The work was carried out at Suganthi Devadason Marine Research Institute, Tuticorin and Veltech Multitech Dr. RR Dr. SR Engineering College, Chennai. India, during April 2008 to April' 2009 and one of the author is professionally trained international certified advanced level SCUBA diver.

RESULTS

Antibacterial Activity of crude extracts: Out of the 4 solvents used for the extraction of the gastropod, *Drupa margariticola* the extract with acetone was found to

produce a distinct zone of inhibition 8 and 7 mm against *Salmonella typhimurium*, *Vibrio cholerae*, *Bacillus subtilis* and *Staphylococcus epidermidis*, *Enterobacter earogenes* and *Citrobacter* sp., respectively at a concentration of 0.2 mg. Similarly the methanol extract was also able to produce zone of 8 mm against *Vibrio cholerae* at the concentration of 0.2 mg. However, the ethyl acetate was able to produce zone of 7 mm against *V. cholerae*. On the other hand, methanol crude extract was able to produce a zone of 2 mm against Pseudomonas *aerogenosa* and *Salmonella paratyphi* at the same concentration (Table 1).

Antibacterial activity of the column purified extracts of *Drupa margariticola* against human pathogens: Table 2 shows the effect of the column purified extracts of *D. margariticol* against human pathogens. A maximum inhibition of 7 mm against *E. coli* was shown by 100% acetone column purified fractions at concentration of 0.125 mg. Similarly, the 100% acetone fraction of column purified fraction of *D. mrgariticola* produced a 6 mm against *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Pseudomonas aerogenes* and *Vibrio cholerae* at the same concentration. However, somewhat lesser inhibition of zone was shown by combination of 80% acetone and 20% hexane followed by 60% acetone and 50% hexane fractions. On the other hand, 100% hexane; 80:20 hexane: acetone; 20:80 acetone: methanol and 100% methanol fractions showed only trace inhibition.

Minimal Inhibitory Concentration (MIC): The Minimal Inhibitory Concentration (MIC) values were found to be lower for the 100% acetone column purified fractions for the pathogens, *E. coli* (0.05 mg), *Klebsiella pneumoniae* (0.05 mg), *Pseudomonas aerogenes* (0.07 mg), *Streptococcus pneumoniae* (0.07 mg) (Table 3).

Table 1: Antibacterial activity of gastropod *Drupa margariticola* against human pathogens

	Radius of the zone of inhibition (mm)			
Pathogens	EA	A	DCM	ME
Escherichia coli	5	6	5	4
Shigella dysentriae	4	5	4	3
Staphylococcus epidermidis	5	7	5	4
S. aureus	5	6	4	3
Klebsiella pneumoniae	3	5	4	4
Pseudomonas aerogenosa	4	6	4	2
Salmonella typhimurium	4	8	5	3
S. paratyphi	4	4	3	2
Vibrio cholerae	7	8	5	8
Streptococcus pneumoniae	5	6	5	4
S. faecalis	4	5	4	3
Bacillus subtilis	4	8	6	3
B. cereus	6	6	5	6
Enterobacter aerogenes	6	7	6	6
Citrobacter sp.	5	7	6	5

Note: A: Acetone, EA: Ethyl acetate, DCM: Dichloromethane and ME: Methanol

Table 2: Antibacterial activity of the column purified fractions of *D. margariticola* against pathogens

	Radius of the zone of inhibition (mm)										
Pathogens	H	80: 20	60: 40	40: 60	20: 80	A	80: 20	60: 40	40: 60	20: 80	ME
E. coli	-	T	T	2	4	7	6	5	3	2	T
S. dysentriae	-	-	2	2.5	3.5	5	3	2	T	-	-
S. epidermidis	-	T	2	3	4	6	5.5	3	2.5	T	-
S. aureus	-	T	T	3	3.5	5.5	5	3.5	2	1.5	T
K. pneumoniae	-	-	2	3	4	6	5	4	3	T	-
P. aerogenosa	-	T	2.5	4	5	6	4.5	3	2	T	T
S. typhimurium	-	T	2	2.5	3.5	5.5	3.5	2	T	T	T
S. paratyphi	-	T	T	2.5	4	5	4	3.5	2	T	T
V. cholerae	-	-	2.5	4	5	6	5	3	2.5	2	T
S. pneumoniae	-	-	T	2.5	3	5.5	3	2	2	T	T
S. faecalis	-	T	3	3	4.5	5.5	4.5	3	2.5	2	2
Bacillus subtilis	-	T	2.5	3.5	4	5	4	3	2	T	T
B. cereus	-	T	T	2	2.5	5	3.5	2.5	2	2	T
E. aerogenes	-	T	2.5	3	3.5	4.5	3	2.5	2	2	T
Citrobacter sp.	-	T	T	2.5	3	4	3	2	T	T	T

Note: A: Acetone, H: Hexane, ME: Methanol and T: Trace

Table 3: MIC of the column purified fractions of *Drupa margariticola* against human pathogens

	Minimal inhibitory concentration (mg)										
Pathogens	H	80:20	60:40	40:60	20:80	A	80:20	60:40	40:60	20:80	ME
E. coli	-	0.43	0.43	0.40	0.18	0.05	0.13	0.15	0.35	0.43	0.43
S. dysentriae	-	-	0.38	0.38	0.20	0.07	0.20	0.35	0.43	-	-
S. epidermidis	-	0.43	0.38	0.35	0.18	0.10	0.15	0.33	0.38	0.43	-
S. aureus	-	0.43	0.43	0.35	0.20	0.08	1.50	0.30	0.38	0.40	0.43
K. pneumoniae	-	-	0.38	0.35	0.18	0.05	0.18	0.30	0.38	0.43	-
P. aerogenosa	-	0.43	0.35	0.28	0.15	0.07	0.20	0.33	0.38	0.43	0.43
S. typhimurium	-	0.43	0.38	0.35	0.20	0.12	0.20	0.38	0.43	0.43	0.43
S. paratyphi	-	0.43	0.43	0.35	0.18	0.08	0.15	0.30	0.38	0.43	0.43
Vibrio cholerae	-	-	0.35	0.28	0.18	0.10	0.23	0.30	0.35	0.38	0.43
S. pneumoniae	-	-	0.43	0.38	0.23	0.07	0.18	0.35	0.38	0.43	0.43
S. faecalis	-	0.43	0.33	0.35	0.15	0.80	0.18	0.33	0.35	0.38	0.43
Bacillus subtilis	-	0.43	0.35	0.30	0.18	0.12	0.20	0.33	0.38	0.43	0.43
B. cereus	-	0.43	0.43	0.40	0.28	0.08	0.25	0.35	0.38	0.38	0.43
Ent. aerogenes	-	0.43	0.38	0.35	0.20	0.10	0.26	0.35	0.38	0.38	0.43
Citrobacter sp.	-	0.43	0.43	0.38	0.23	0.12	2.25	0.38	0.43	0.43	0.43

A: Acetone, H: Hexane and ME: Methanol

DISCUSSION

The sea has immense biomedical potential which can be exploited not only as a source of drugs for treatment of disease but also of new and novel structures with useful biological activities. In the past 25 years, marine organisms- mollusks, algae, plants and microbes have provided key structures and compounds that proved their potential in several fields, particularly as new therapeutic agents for a variety of diseases. The interest in the field is reflected by the number of scientific publications, the variety of new structures and wide scope of organisms investigated (Faulkner, 1996).

In the present study, pronounced inhibition was conferred by the acetone extracts of *D. margariticola* against the 15 dreadful human bacterial pathogens. Earlier work performed on the antibacterial activity of the winged mollusc, *Pteria chinensis* reported that out of the 6 solvents used, the extract obtained from acetone and chloroform exhibited higher antibacterial activity against human pathogens which stands by the present work (Chellaram *et al*., 2004). Also, there is finding to report that the acetone extracts of different seaweeds showed antibacterial properties against human pathogens (Sureshkumar *et al*., 2002). On the contrary, Anand and Edward (2002) reported that the crude methanol extracts of *Cypraea errones* exhibited higher antibacterial and antifungal activity. In this study, extracts from other solvents tested showed only moderate type of inhibition suggesting that these extracts may not possess potent antibacterial compounds. But a work executed on the in vitro antimicrobial susceptibility test of the red, green and brown macroalgae showed that the methanolic extracts were efficient in their action (Gonzalez del Val *et al*., 2001). Similarly, Anand *et al*. (1997) have also reported a broad spectral activity for the methanolic extract of *Rapana rapiformis* egg capsules against nine pathogenic bacteria.

The acetone extract of the *D. margariticola* which was found to possess higher antibacterial activity was hence chosen to localize the active component through column purification. Yet again the 100% acetone column purified fractions were found to possess utmost antibacterial activity. The inhibition zone of 7 mm was shown by the 100% acetone column purified fractions against *E. coli*, at a concentration of 0.125 mg which is much lesser than the concentration of the crude extract (0.2 mg). Chellaram *et al*. (2004) have reported that the acetone fractions of mollusk, *Pteria chinensis* exhibited broad spectral antibacterial activity that substantiates the present finding. But on the contrast, the crude extract of *Chicoreus virgineus*, after antibacterial assay-guided elution, showed activity only in 100% methanol fraction (Ramasamy and Murugan, 2003). The Minimal Inhibitory Concentration of *D. margariticola* was found to be lower for the 100% acetone phases (0.05 mg) for *E. coli*. However, MIC values of 20 µg mL^{-1} were recorded for the metabolites of soft corals, *Caldiella* sp. and *Sinularia* sp. for human pathogenic bacteria (Radhika *et al*., 2003). The study by Kelman *et al*. (2001) has focused in determining the MIC values of the pure compounds obtained from the sponge, *Amphimedon viridis* for selected marine bacteria and the authors have estimated that the values are greater than 250 µg. This finding suggests that since antibacterial activity was more pronounced at the 100% acetone phase with very minimal concentration against dead full human pathogens.

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