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Short Communication

Antimicrobial activity in the tissue extracts of five species of cowries *Cypraea* spp. (Mollusca: Gastropoda) and an ascidian *Didemnum psammathodes* (Tunicata: Didemnidae)

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Five species of Cypraea (*Cypraea errones, C. arabica, C. onyx, C. tigris* and *C. vitellus*) were assayed for their antibacterial and antifungal activity against 15 bacterial pathogens and 3 fungal pathogens. Antibacterial and antifungal activities were exhibited only by *C. errones* extracts. An ascidian, *Didemnum psammathodes*, the principal diet of *C. errones* was also assayed for antimicrobial activity to evaluate whether the activity exhibited by *C. errones* was derived from its diet. The ascidian extract exhibited broad-spectrum antibacterial activity. Chromatographic fractionation of the extracts of *C. errones* and *Didemnum psammathodes* confirmed that the activity exhibited by *C. errones* was not diet derived.

[**Key words** : Marine natural products, antibacterial , molluscs, ascidians]

The world's oceans, covering more than 70% of the earth surface represent an enormous resource for the discovery of potential chemotherapeutic agents. Because of the diversity of marine organisms and habitats, marine natural products encompass a wide variety of chemical class, including terpenes, shikimates, ployketides, acetogenins, peptides, alkaloids of varying structures and multitude of compounds of mixed synthesis. In the past decade alone structures of over 5000 marine natural products have been published[1]. During past 20 years, pharmaceutical industry has been relatively successful in containing problems due to single resistant determinants; however the advent of multiple resistance mechanism has severely limited the effective use of many major classes of drugs[2]. The need for the discovery of new and novel antibiotics is imperative because evidence suggests that development and spread of resistance to any new antimicrobial agents is inevitable. However new drug classes with novel mechanisms of action will create effective therapy, at least for a period of time[3]. Molluscs, which are widely distributed throughout the world, have many representatives in the marine and estuarine ecosystem. Many studies on bioactive compounds from molluscs exhibiting antitimour, antileukemic, antibacterial and antiviral activities have been reported worldwide[4-8].

Present study was carried out to investigate the antibacterial and antifungal properties of the whole body of five species of cowries from Gulf of Mannar, southeast coast of India. In the field, *Cypraea errones* species were observed to graze exclusively on a compound ascidian *Didemnum psammathodes*. The antibacterial activity of this ascidian was screened to find out whether the antibacterial activity of *C. errones* was derived from its diet.

Five species of *Cypraea* namely, *C. errones, C. arabica, C. onyx, C. tigris, C. vitellas* (Linne, 1758) and the ascidian *Didemnum psammathodes* (Renganathan, 1996) were collected from Tuticorin coastal waters. The shells were broken and the soft body was removed, cut into small pieces and air-dried; the ascidian was also air-dried (24-48 h) to remove the water content. The air-dried *Cypraea* flesh and ascidian were extracted with methanol and cold steeped overnight at –18ºC. The crude methanol extract was assayed for antibacterial and antifungal activity using the standard disc diffusion method in Antibiotic assay medium (Himedia, Bombay) using 6 mm discs. The following bacterial and fungal pathogens were used for the assay: *Escherichia coli (ATCC 25922), Bacillus subtilis (ATCC 6633), Pseudomonas aeruginosa (ATCC 29336), Shigella flexneri (ATCC 12022), Staphylococcus aureus (ATCC 29737), Proteus vulgaris (ATCC 13315), Proteus mirabilis (ATCC 25922), Klebsiella pneumoniae (ATCC 10031), Vibrio cholerae (ATCC 15748), Salmonella typhi (ATCC 6539), Enterobacter*

Table 1 — Antibacterial and antifungal activities of crude extracts in methanol of *Cypraea* spp and the ascidian *Didemnum psammathodes*

Species	Pathogens (inhibition zone in mm **)				
	Staphylococcus aureus	*Enterobacter aerogenes*	Streptococcus pyogenes	*Aspergillus niger*	Candida *albicans*
Cypraea errones	3	–	3	5	1
Cypraea vitellus	–	–	–	–	–
Cypraea tigris	–	–	–	–	–
Cypraea onyx	–	–	–	–	–
Cypraea arabica	–	–	–	–	–
Didemnum psammathodes	1	5	7	–	–

**- Zone in mm indicates the distance from the border of the disc to the edge of the clear zone.

aerogenes (ATCC 13048), Streptococcus pyogenes (ATCC 19615), Aspergillus niger (ATCC 16404), and *Candida albicans (ATCC 10231).*

Partial purification of the active compounds was carried out following the method out lined by Wright[1]. After initial screening of all the extracts, the ones with a potentially promising activity were partitioned first between ethyl acetate and water and the water phase subsequently partitioned against n-butanol. The three phases thus obtained were evaporated and concentrated. Once again, antibacterial and antifungal assay was carried out. The active compounds were found to be in the ethyl acetate phase. The ethyl acetate phase was then fractionated using normal phase silica gel column chromatography employing step gradient solvent system from low to high polarity. The step gradient protocol used was: heptane 100% → ethyl acetate 20%: heptane 80% → ethyl acetate 40%: heptane 60% → ethyl acetate 60%: heptane 40% → ethyl acetate 80%: heptane 20% → ethyl acetate 100% → methanol 25%: ethyl acetate 75% → methanol 50%: ethyl acetate 50% → methanol 100%. The fractions thus obtained were once again evaporated and concentrated. They were then assayed for antibacterial and antifungal activity.

In the crude methanol extracts of *Cypraea* only *C. errones* exhibited moderate antibacterial and antifungal activity against *Staphylococcus aureus, Streptococcus pyogenes, Aspergillus niger,* and *C. albicans* (Table 1). The crude methanol extract of *Didemnum psammathodes* exhibited wide spectral antibacterial activity but no antifungal activity was noticed. Extracts from *C. errones* and *Didemnum psammathodes* were chosen for further partitioning and chromatographic separation. After partitioning, *C. errones* extracts exhibited moderate activity in the ethyl acetate phase (Table 2). Fractions obtained by column chromatography of ethyl acetate phase of *C. errones* extracts exhibited wide spectral activity against 6 pathogens but highest activity was observed only against *Shigella flexneri* (heptane fraction –9 mm) (Table 2).

Table 2 — Antibacterial activity (inhibition zone in mm) of *Cypraea errones* extracts of ethyl acetate phase (EA) and 100 % heptane (HP) fraction

Pathogens	Ethyl acetate phase	Heptane phase
Escherichia coli	–	–
Pseudomonas aeruginosa	2.5	1
Shigella flexneri	5	9
Bacillus subtilis	–	–
Proteus vulgaris	–	–
Klebsilla pneumoniae	–	–
Salmonella typhi	–	–
Enterobacter aerogenes	–	–
Vibrio cholerae	1	–
Proteus mirabilis	–	–
Staphylococcus aureus	1	–
Streptococcus pyogenes	–	–

**- Zone in mm indicates the distance from the border of the disc to the edge of the clear zone.

In the partitioning of the ascidian *Didemnum psammathodes* methanol extract, the ethyl acetate phase exhibited higher antibacterial activity against 11 out of 15 pathogens assayed (Table 3). The highest activity was seen against *P. mirabilis* (7 mm), *Shigella flexneri* (8 mm) and *Salmonella typhi* (6 mm). The chromatographic fraction of the ascidian also exhibited broad spectral activity against 13 out of 15 pathogens, assayed with highest activity against

Table 3—Antibacterial activity (as indicated by inhibition zones** in mm) of various extracts from the ascidian, *Didemnum psammathodes*

Pathogens	Solvent phases			
	Ethyl acetate	Butanol	Water	Methanol
Pseudomonas aeruginosa	2	–	–	3
Shigella flexneri	8	–	–	7
Bacillus subtilis	2	2	–	4
Proteus vulgaris	–	–	–	2
Klebsilla pneumoniae	5	–	–	10
Salmonella typhi	6	1	–	6
Enterobacter aerogenes	5	–	2	3
Vibrio cholerae	5	2	1	2
Proteus mirabilis	7	–	–	1
Staphylococcus aureus	5.5	1	–	1
Streptococcus pyogenes	1	–	–	–
Escherichia coli	6	–	–	2

**- Zone in mm indicates the distance from the border of the disc to the edge of the clear zone.

K. pneumoniae (10 mm) and *Shigella flexneri* (7 mm). The activity was confined only to the methanol fraction indicating the activity to be due to a polar compound. No antifungal activity was observed in the ascidian extract column fractions (Table 3)

In the present investigation only *C. errones* exhibited higher antibacterial activity and all other species gave negative results. Activity of the 100% heptane fraction against *Shigella flexneri* (9 mm) can be considered a drug lead against this specific pathogen. Antifungal activity of *C. errones* was totally lost, at the second step of isolation (partitioning). The active compound may have degraded or modified during this separation process, this possibility has been discussed in detail earlier[9].

In this study, the ascidian *Didemnum psammathodes* was screened to evaluate if *C. errones*, which feeds exclusively on it, derives its activity from its diet, because ascidians have been already established as a group with higher percentage of bioactivity[10]. This phenomenon has been well studied in nudibranchs and it has been argued that the nudibranchs owe their evolutionary success to the development of a chemical defence obtained from their invertebrate diets like sponges, bryozoans, ascidians and coelenterates[11]. Results of this study do not indicate that *C. errones* active compound is derived from its diet and this may be due to the breakdown of the ascidian antimicrobial molecules by the digestive enzymes of *C. errones*. The ascidian exhibited broad spectral antibacterial activity at the polar end of the step gradient in column chromatography but *C. errones* exhibited such activity at the non-polar end of the step gradient, also the activity exhibited by the ascidian was broad spectral but *C. errones* activity was more specific.

Comparatively the ascidian *Didemnum psammathodes* seems to be a promising source of antibacterial compound. Ascidians are already reported to be rich source of nitrogen compounds with a wide range of biological activities[12]. The most potent metabolite from ascidian discovered is Didemin-B from a Caribbean tunicate *Tridemnum solidum*, which became the first marine compound to enter human cancer clinical trails as a purified natural product[13]. Hence further purification of the *Didemnum psammathodes* extracts may lead to the discovery of novel antibacterial compounds.

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