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Short Communication

Lethal concentration of methanol extract of sponges to the brine shrimp, *Artemia salina*

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Abstract

Methanol extracts of five marine sponges *viz*., *Acanthella elongata, Echinodictyum gorgonoides, Axinella donnani, Callyspongia subarmigera* and *Callyspongia diffusa* collected as fresh bycatch from fishing nets off Kanyakumari were screened for estimating the lethal concentration to the brine shrimp (*Artemia salina*). The extract of C*allyspongia subarmigera* had the highest toxicity with LC_{50} at 0.46% in 24 h whereas the extract of *Eichinodictyum gorgonoides* was least toxic with LC_{50} at 8.58% in 24 h.

Keywords: Sponges, brine shrimp assay, methanol extract, lethal concentration

Introduction

Sessile, soft-bodied marine invertebrates, lacking obvious physical defenses are considered as prime candidates for bioactive metabolites. They have a very long evolutionary history with ample opportunity to perfect their chemical defenses. Among them, the sponges (Phylum: Porifera) continue to be a rich source of novel secondary metabolites, with a diversity of biological activities that continue to inspire the efforts of synthetic organic chemicals (Blunt *et al*., 2005). Sponges produce bioactive molecules to defend themselves from predators or spatial competitors (Pawlik *et al*., 2002). These molecules exhibit species–specific toxicity against a wide range of organisms, including microorganisms, invertebrates and vertebrates. Using different modes of screening, many authors have found out the biological activity of sponge extracts (Duckworth and Battershill, 2001). It has been demonstrated that some of these metabolites have biomedical potential and in particular, Ara-A and Ara-C are clinically used as antineoplastic drugs in the routine treatment of patients with leukemia and lymphoma (Thakur and Muller, 2004). Moreover, *in vitro* studies have shown that the sponge metabolites exhibit several biological activities such as antimicrobial, antifungal, antiviral, neurotoxic and cytotoxic properties (Rangel *et al*., 2001). Considering the pharmaceutical potential of many bioactive molecules, a large number of studies have been carried out to examine the effects of marine sponge crude extracts and isolated molecules on several kinds of cytotoxicity bioassays.

It is possible to detect the toxicity of marine natural products using the brine shrimp lethality bioassay rather than more tedious and expensive *in vitro* and *in vivo* antitumour assays. The brine shrimp, *Artemia* species, live in extreme saline environments and play an important role in the energy flow of the food chain (Sanchez - Fortun *et al*., 1995). They can be used in laboratory bioassay to determine cytotoxicity through the estimation of the medium lethal concentration (LC_{50} values) (Parra *et al*., 2001). We screened *in vitro* cytotoxicity in extracts of chosen marine sponges collected from the coastal waters of Kanyakumari using brine shrimp lethality bioassay.

Material and methods

Collected the sponges which were entangled in fishing nets operating off Kanyakumari (08º 04' N

lat., 77º 36' E long.) at depths ranging from 10 to 15 m. Five species of sponges segregated and identified were *Acanthella elongata* (Dendy), *Echinodictyum gorgonoides* (Dendy), *Axinella donnani* (Bowerbank), *Callyspongia subarmigera* (Ridley) and *Callyspongia diffusa* (Ridley). All these sponges belong to the Class Demospongiae Sollas. For the extraction of secondary metabolites, sponges were cut into small pieces and extracted thrice with distilled methanol and the pooled organic solution was filtered through Whatman No.1 filter paper fitted in a Buchner funnel using suction. Solvents were removed by rotary evaporator (Buchi-type) under reduced pressure so as to get the crude methanol extract.

The experiments were conducted at Vizhinjam Research Center of CMFRI. The brine shrimp (*Artemia salina*) lethality assay was performed as described by Meyer *et al*. (1982). For the bioassay, the cysts were allowed to hatch in a beaker filled with filtered seawater (32 ppt) under constant aeration. After 48 h, the phototropic brown coloured nauplii were siphoned out using a glass pipette. The nauplii were counted against an illuminated background and ten nauplii were transferred to each cavity cup containing 2 ml of filtered seawater dissolved with varying dilutions of sponge extract ranging from 0.2% to 10%. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not due to starvation, control was maintained without adding the sponge extract. The larvae were not fed during the experiment, as the newly hatched brine shrimp nauplii can survive for up to 48 h without food due to the presence of yolk-sac (Lewis, 1995). The cavity cups were maintained under constant illumination. Experiments were carried out in triplicates to get statistically significant results and the mean value was recorded as mortality after 24 h. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. Based on the per cent mortality, LC_{50} value of the extract was determined using the probit scale (Miller and Tainter, 1944).

Results

The results showed the presence of bioactive compounds in all the tested sponge extracts. The secondary metabolites of the sponge *Callyspongia subarmigera* exhibited high toxicity against *Artemia* nauplii followed by *Acanthella elongata*, *Axinella donnani*, *Callyspongia diffusa* and *Eichinodictyum gorgonoides* (Table 1). Mortality increased linearly with increase of concentration of every extract.

Table 1. Mortality of brine shrimp exposed for 24 hours to different concentrations of methanol extract from sponges (± represents standard deviation)

Sponges	Concentration(%)	Mortality(%)
	Control	0
	0.2	20.0±0.0
Acanthella	0.4	36.0±1.26
elongata	0.6	52.0±0.57
	0.8	76.6± 1.52
	1.0	100±0.0
	Control	0
	2	0
Echinodictyum	4	10.2±5.4
gorgonoides	6	32.8±1.3
	8	44.5±7.2
	10	60.0±2.3
	Control	0
	2	10.3±4.1
Axinella	4	32.4±5.7
donnani	6	61.0±0.57
	8	80.4±1.8
	10	100±0.0
	Control	0
	0.2	25.0±0.17
Callyspongia	0.4	40.0±0.2
subarmigera	0.6	65.0±1.52
	0.8	80.5±3.16
	1.0	100±0.0
	Control	0
	2	10.0±0.57
Callyspongia	4	35.0±0.50
diffusa	6	55.3±0.29
	8	74.0±0.10
	10	98.2±4.3

The results of LC_{50} evaluation are presented in Table 2. The median lethal dose indicated that *E. gorgonides* was the least toxic as it produced 50% mortality at 8.58% concentration. The mortality was nil at 2% level whereas 10% mortality was noted against *A. donnani* and *C. diffusa*. The LC_{50} values of *C. subarmigera* and *A. elongata* were 0.46% and 0.51%, respectively whereas *Axinella donnani* and *C. diffusa* extract produced 50% mortality at 5.3% and 5.4%.

Table 2. LC_{50} (% extract) value of sponge metabolites to *Artemia salina*

Sponges	LC_{50} (in % extract)
Acanthella elongata	0.51
Echinodictyum gorgonoides	8.58
Axinella donnani	5.30
Callyspongia subarmigera	0.46
Callyspongia diffusa	5.40

Discussion

The brine shrimp larval mortality assay is widely accepted as a convenient probe for potential *in vitro* cytotoxicity and pharmacological activity in marine natural products (Carballo *et al*., 2002). The assay showed a good correlation with cytotoxicity in cell lines such as 9KB, P388, L5178Y and L1210 (De Rosa *et al*., 1994; McLaughlin *et al*., 1998). The brine shrimp assay has a number of advantages such as experimental simplicity, sensitivity, reproducibility, rapid (24 hours), inexpensive, simple and reliable way of determining the intensity of cytotoxicity (Lieberman, 1999).

Sponges of the class Demospongiae are known to produce the largest number and diversity of secondary metabolites isolated from marine invertebrates, most of them with medically relevant biological activities and important ecological roles (Faulkner, 2002). The toxicity of sponges has been well-documented, which could be ascribed to the diverse and potent cytotoxic compounds (Lee and Qian, 2003). Studies made by Zhang *et al.* (2003) revealed that more than 10% of the investigated marine sponge species exhibited cytotoxic activity suggesting production of potential medicines. In the present study, the results of brine shrimp lethality indicated that the extract of *C. subarmigera* was highly active with LC_{50} value of 0.46% followed by other extracts. *E. gorgonoides* was found to contain the least toxicity (LC_{50} = 8.58%). Selvin and Lipton (2004) studied brine shrimp toxicity of sponges like *Clathria gorgonoides, Dendrilla nigra* and A*xinella donnani* and reported that the LC_{50} was 0.20%, 0.28% and 5.5%, respectively. Variations in toxicity could be attributed due to the changes in the ecology and metabolism of the respective species (Becerro *et al*., 2003).

Cytotoxicity of marine sponges against brine shrimp has been studied by a few authors. The bioactive acetylenic compound isolated from the Caribbean sponge *Cribrochalina vasculum* was found to be highly toxic to *Artemia salina* (Aiello *et al*., 1992). The crude extracts of the marine sponges *Pachastrella* sp. and *Jaspis* sp. collected from the South Sea of Korea exhibited significant brine shrimp cytotoxicity; further purification gave Pectenotoxin and Psammaplin A, which were cytotoxic to human cancer cell lines (Jung *et al*., 1995). Qureshi *et al*. (1998) found that peroxides found in the Philippine marine sponge, *Plakinastrella* sp. was toxic to *Artemia salina*. Earlier studies conducted using the methanol extract of the Korean sponge, *Petrosia* sp. showed potent brine shrimp cytotoxicity. Guided by this assay, further fractionation and purification gave potent cytotoxic polyacetylenes (Kim *et al*., 1999). The Indonesian sponge, *Callyspongia pseudoreticulata* yielded diyne which was found to be toxic in the brine shrimp assay (Braekmam *et al*., 2003). The present study indicates that the extract of *Callyspongia subarmigera* could be further fractionated to yield potent cytotoxic drugs.

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