INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 14, No. 3, p. 30-34, 2019

RESEARCH PAPER OPEN ACCESS

The brine shrimp toxicity of the philippine marine sponges *Axinyssa* sp., *Plocamionida* sp., *Forcepia sp.*, *Pachymatisma sp.*, and *Placospongia sp.*

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Key words: Marine sponges, Bioactivity, Toxicity, Brine shrimp lethality test.

http://dx.doi.org/10.12692/ijb/14.3.30-34 Article published on March 15, 20199

Abstract

Marine sponges have been considered as a gold mine with respect to the diversity of their secondary metabolites and biological activities. Five marine sponges were collected off Agusan del Norte, Philippines and taxonomically identified as *Axinyssa* sp., *Plocamionida* sp., *Forcepia* sp., *Pachymatisma* sp., and *Placospongia* sp. Nonpolar and polar extracts of the marine sponges were prepared by sequential partitioning of their freeze-dried samples with 1:1 ethyl acetate-methanol and 1:1 ethanol-water, respectively. The toxicity of the sponge extracts was evaluated against the brine shrimp *Artemia salina* after six (6) and twenty-four (24) hours exposure to indicate acute and chronic toxicity, respectively. The results indicated significant brine shrimp toxicity for most of the marine sponge extracts. The highest potencies were exhibited by the nonpolar and polar extracts of *Forcepia* sp. (acute LC_{50} = 22.24 □g/mL; chronic LC_{50} = 3.98 □g/mL), the polar extract of *Axinyssa* sp. (chronic LC_{50} = 17.90 □g/mL), and the polar extract of *Placospongia* sp. (chronic LC_{50} = 4.48 □g/mL). Accordingly, these active sponge extracts are worthy of further investigation for the isolation and determination of potential anti-tumor and pesticidal compounds.

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Introduction

The marine biology existing in the oceans and seas which blanket nearly three-quarters of the Earth comprise a continuous resource of immeasurable biological activities and immense chemical entities (Ebada *et al.*, 2010). The marine ecosystem rapidly competes for potential therapeutics in drug discovery researches due to their inherent chemically bioactive properties that could lead to new drug advancement from natural sources (Haefner, 2003; Davis, 2006).

One of the rationale of searching drugs from marine environment stem from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition, predation, surface fouling, and reproduction (Jirge and Chaudhari, 2010).

Many invertebrates produce secondary metabolites of diverse and novel types, but marine sponges elaborate by far the greatest number. Marine sponges have been considered as a gold mine during the past 50 years, with respect to the diversity of their secondary metabolites. The biological effects of new metabolites from sponges have been reported in hundreds of scientific papers (Sipkema *et al.*, 2005).

The brine shrimp lethality test (BSLT) is a simple bioassay for natural product research utilizing bring shrimp (*A. salina*, Leach). The method is developed whereby compounds and extracts are tested at different concentrations.

The procedure determines the lethal concentration required to kill 50% of the population (LC_{50}) values in μg/mL of active compounds in the brine medium. This simple bioassay system is readily utilized by natural product chemists in the detection and isolation of higher plant constituents with a variety of pharmacologic activities (Meyer *et al.*, 1982).

The main objective of this study was to determine the potential cytotoxicity of the extracts obtained from the five marine sponges collected off the coasts of Agusan del Norte, Philippines.

Materials and methods

Collection and Taxonomic Identification of Marine Sponge Samples

Fresh samples of the five marine sponges were collected off the coasts of Brgy. Vinapor, Carmen, Agusan del Norte, Philippines (9°05'13.6"N, 125°13'12.4"E) on November 2015. The collected samples were properly labelled, stored in containers filled with ice and transferred to the Natural Products and Bioorganic Research Laboratory of the Department of Chemistry of Mindanao State University – Iligan Institute of Technology, Iligan City, Philippines. The collected sponges were then immediately cut and freeze-dried (Eyela FDU-2200 Freeze-dryer). Voucher specimens of the five marine sponges collected were prepared in accordance with a standard protocol (Hooper and Soest, 2002) and taxonomical identification was done one of the authors (EB Metillo). The marine sponges were identified as *Axinyssa* sp., *Plocamionida* sp., *Forcepia* sp., *Pachymatisma* sp., and *Placospongia* sp.

Solvent Extraction of Marine Sponge Samples

Each of the five marine sponge samples was soaked in adequate amount of 1:1 ethyl acetate-methanol mixture for 72 hours. The resulting mixture was then filtered, concentrated *in vacuo* using rotary evaporator, and weighed to give the nonpolar extract. The sponge residue from the first extraction was then soaked in 1:1 ethanol-water mixture for 72 hours. The resulting mixture was then filtered, concentrated *in vacuo*, freeze-dried, and weighed to give the polar extract.

Brine Shrimp Lethality Test

Brine shrimp lethality assay was carried out to investigate the potential cytotoxicity of the marine sponge extracts and the method for the cytotoxicity test was based from the study conducted by Meyer *et al.* (1982). For each sponge extract, different concentrations were prepared which are 1000-, 500-, 100-, and 10-□g/mL at three replicates. The resulting test solutions were then subjected to toxicity test against brine shrimp *A. salina*. The number of

survivors and deaths were monitored, counted, and recorded after 6 and 24 hours at each dose. The results obtained were analysed by Reed-Muench method of the mortality data at 6- and 24-hour periods to determine the acute and chronic concentrations (LC_{50}), respectively (Reed and Muench, 1938).

Results and discussion

Brine shrimp lethality assay is considered as a simple bioassay for natural product research that has the advantages of being rapid and inexpensive and was carried out to investigate the potential cytotoxicity of the marine sponge extracts (Meyer *et al.*, 1982).

Table 1. Brine Shrimp Lethality Test Results of the Marine Sponge Extracts.

Marine Sponge	Extract	Acute toxicity	Chronic toxicity
		LC_{50}, 6h (µg/mL)	LC_{50}, 24h (µg/mL)
Axinyssa sp.	Nonpolar	630.96	239.88
	Polar	263.03	17.90
Plocamionida sp.	Nonpolar	>1000.00	630.96
	Polar	>1000.00	1000.00
Forcepia sp.	Nonpolar	22.24	<10.00 (3.98)[b]
	Polar	41.69	<10.00 (2.56)[b]
Pachymatisma sp.	Nonpolar	>1000.00	501.19
	Polar	>1000.00	>1000.00
Placospongia sp.	Nonpolar	1000.00	354.81
	Polar	707.94	<10.00 (4.48)[b]

[b]extrapolated value from the Reed-Muench plot.

The quantal data analysis by Reed-Muench method of the mortality data at 6- and 24-hour periods allowed the estimation of the acute and chronic LC_{50} values, respectively, and are presented in Table 1. Majority of the crude extracts exhibited remarkable cytotoxic potency. Evaluated brine shrimp mortality data have revealed both acute and chronic LC_{50} values well below 100 µg/mL for both the polar (41.69 and <10.00(2.56) µg/mL, respectively) and nonpolar (22.24 and <10.00(3.98) µg/mL, respectively) extracts of *Forcepia* sp., which is considered significantly toxic according to the National Cancer Institute (NCI) standards and is the one which has the highest cytotoxic activity among all of the tested crude extracts of the marine sponge samples (Cordell *et al.*, 1993; Krishnaraju *et al.*, 2005). This result can be associated to the study previously done by Wright *et al.* in 2004, where five marine-derived macrolide compounds have been isolated from the marine sponge *Forcepia* sp. collected off a site in the U.S. Gulf of Mexico. These compounds isolated were found to inhibit the *in vitro* proliferation of A-549 human lung adenocarcinoma cells, PANC-1 human pancreatic carcinoma cells, and NCI-ADR-RES cell line (Wright *et al.*, 2004).

Other marine sponge extracts which also exhibited remarkable cytotoxic potency include the polar and nonpolar extracts of *Axinyssa* sp., and the polar extract of *Placospongia* sp. A prior study revealed that twelve bicyclic sesquiterpenes axinisothiocyanates isolated from a marine sponge of the genus *Axinyssa* exhibited biological activities such as antihelmintic, antimicrobial, and cytotoxic properties which have been attributed to these nitrogen-containing sesquiterpenes (Zubia *et al.*, 2008). In 2013, two phosphorus-containing iodinated polyacetylenes were isolated from a Korean marine sponge *Placospongia* sp. Polyacetylenic natural products have been isolated from a number of different marine sponges and they exhibited diverse bioactivities such as cytotoxic, antiviral, antifouling,

RNA-cleaving, and enzyme-inhibitory activities (Faulkner, 1997; Youssef *et al.*, 2003; Minto and Blacklock, 2008; Kim *et al.*, 2013). The overall high toxicity implies abundance of toxic principles distributed within the crude extracts of the marine sponge samples.

Conclusion

The results indicated most of the marine sponge extracts exhibited high cytotoxicity potential against the brine shrimp *A. salina* with some extracts. Notable among them are the nonpolar and polar extracts of *Forcepia* sp., and polar extracts of *Axinyssa* sp. and *Placospongia* sp., exhibiting LC_{50} values well below 100 µg/mL. The remarkable cytotoxic potential exhibited by the marine sponge extracts can be related to a variety of pharmacological properties that may be present in the secondary metabolites in the marine sponge species.

Acknowledgment

SRS Murcia would like to express her earnest appreciation to the Department of Science and Technology of the Philippine Government for the scholarship grant through its Accelerated Science and Technology Human Resource Development Program. MM Uy is grateful to the Philippine Council for Health Research and Development of the Department of Science and Technology for the research grant.

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