IJMS 2019 vol. 6 (2): 17 - 25

International Journal of Multidisciplinary Studies (IJMS)

Volume 6, Issue 11, 2019

Toxicity evaluation and volatile component analysis of tropical marine sponge *Clathria* sp. (Schmidt, 1862)

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ABSTRACT

Marine sponge Clathria sp. holds a significant position in marine natural product research due to its wide range of secondary metabolites. Extensive distribution of genus Clathria in the world causes variances in chemical profile in relation to their environment. The purpose of this study was to evaluate toxicity and profile secondary metabolites of sponge Clathria sp. collected from coastal waters off Mannar, Sri Lanka. Methanolic crude extract of the sponge was subjected to preliminary phytochemical screening and GC-MS analysis. Alkaloids, steroids, terpenoids, and saponins were subjected to phytochemical screening. Seven steroidal derivatives; Cholesta-3,5-diene, stigmastan-3,5-diene, cholesta-7,14-diene, (5. alpha.), ergosta-4,6,22-triene, cholesta-2,4-diene and cholest-2-ene were identified through GC-MS analysis. Brine shrimp lethality assay was carried out to test the toxicity of the extract. LC50 value estimated at 624 ppm. Results of the present study suggest that ethanolic extract of marine sponge Clathria sp. contains steroidal derivatives and certain toxicity, which will be important in future studies to understand the antifouling property of Clathria sp.

KEYWORDS*: Clathria sp., phytochemical, GC-MS, secondary metabolites, cytotoxicity*

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1. INTRODUCTION

Marine sponges are an abundant source of secondary metabolites presenting a diverse collection of biological activities and one of the most researched marine organisms. Due to their structural diversity and geographical variation, they have a collection of novel compounds with high antimicrobial, anticancer activity as well as antifouling activity (Newman and Cragg, 2004). Additionally, these secondary metabolites act as chemical cues for larval settlement and as well as to prevent fouling (Zimmer and Butman, 2000). Kobayashi et al., (1991) mentioned alkaloids, in particular, are one of the main chemical groups exhibiting these properties. According to Mol et al., (2009) alkaloids from marine sponge *Haliclona exigua* showed positive response against fouling. Steroids and terpenoids extracted from marine sponges also showed a range of biological activity including antimicrobial, anticancer, antifouling activity as well as antiplasmodial activity (Qiu et al., 2008; Krishnan and Keerthi, 2016; Orhan et al., 2010) Furthermore, a number of microorganisms associated with sponges produce diverse classes of chemical compounds in relation to the changes in their habitats (Zaro, 1982).

Genus Clathria is a widely distributed marine sponge. An assembly of secondary metabolites such as alkaloids, steroids, terpenoids, and their derivatives have been extracted from *Clathria* sp. through the years. Multiple research has been conducted on *Clathria* sp. to determine the role of these compounds for their biological and chemical importance (Sun et al., 2015). Clathsterol, clathrin A-C, pseuduchynazaine A-C, clathriol, clathrinamide A-C are few of the major compounds that have resulted during past studies. These compounds were extracted from *Clathira* sp. through various methods (Davis et al., 2004; Rudi et al., 2001; Capon et al., 2001, 2000; Ohta et al., 1993). Rudi et al., (2001) mentioned the extraction of a clathsterol from *Clathria* sp. The sponge sterol, clathsterol has shown significant medicinal potential as it showed anti-HIV-1 reverse transcriptase activity during the study. Furthermore, a nortriterpene compound isolated from *Clathria gombawuiensis* mentioned in Woo et al., 2015 exhibited antibacterial activity. Moreover, Sun et al., 2015 described the isolation of extremely active antibacterial crambescidin 800, from *Clathria cervicornis*. It is vital for the future of marine natural products to understand the chemical, biological and structural changes of marine organisms that can occur with environmental changes. The present study was conducted on marine sponge *Clathria* sp., which is one of the most common marine organisms in Sri Lankan waters. Sponge *Clathria* sp. was screened for its phytochemical constituents and further analyzed for volatile organic compounds as an attempt to discover secondary metabolites and toxicity. Bio-assay-guided toxicity evaluation was carried out parallel to the chemical screening and the brine shrimp lethality assay (BSLA) was used in the primary screening of the crude extract in this study.

2. MATERIALS AND METHODS

Sponge *Clathria* sp. was collected at 4m - 5m depth from coastal waters off Mannar in March 2017. Collected sponge samples were chilled and transported to the laboratory for further analysis.

2.1 Extract preparation

Extraction in ethanol was carried out according to the procedure described by Yusuf et al., (2014) with slight modifications following Harborne (1984), Ebada (2008) and Evans (2009).

Sponge samples were rinsed with fresh water to remove associated debris and lyophilized at the laboratory. A weight of 20 g of the dried sponge was macerated with ethanol at room temperature and solvent level was always maintained higher than the sample level. The extract was filtered out after 24 h and the cycle was repeated twice for the residue. Combined extracts were evaporated in a pre-weighed flask and dry weight was determined. A ration of the dry product was re-dissolved in ethanol to make 200 mL of 1 mg/ mL concentrated solution for phytochemical screening and GC-MS analysis. Another 0.5 g of dry product was extracted for cytotoxicity determination.

2.2 Preliminary phytochemical screening

Phytochemical screening was carried out in the laboratory using 1 mg/ mL ethanolic extract. The presence of alkaloids, sterols, triterpenes, saponins, phenols and flavonoids were qualitatively determined.

Aalkaloids

3 mL of warm 1% HCl was stirred with 3 mL of extract. Three test tubes were filled with 1 mL of the extract separately. One test-tube was considered as the control.

Mayer's test: Mayer's reagent was added dropwise to one test tube. Formation of cream the precipitate or suspension indicates the presence of alkaloids (Evans, 2009; Yusuf et al., 2014; Bargah, 2015).

Wagner's test: Reagent was added dropwise to 1 mL of the solution. The appearance of a reddish-brown precipitate is taken as an indication for alkaloid (Evans, 2009; Yusuf et al., 2014; Bargah, 2015).

Sterols and triterpenes

The presence of sterols and triterpenes were identified following the methods mentioned in Yusuf et al., (2014) with slight modification.

Salkowski's test: Equal volumes of extract and chloroform in a test tube was treated with concentrated H_2SO_4. The appearance of a reddish-brown colour indicates the presence of sterol.

Liebermann Burchard test: The appearance of a brownish-red ring at the interface when 1 mL of H_2SO_4 was added from the side to 2 mL of extract and treated with 2 mL of chloroform with an equal amount of acetic anhydride indicates the presence of sterols and triterpenes.

Saponins

Frothing test: 5 mL of distilled water was added to 5 mL of extract and was shaken strongly for 15-30 s and observed for 30 min. The presence of honeycomb froth indicates the entity of saponins (Rhandour et al., 2016).

Phenols and tannins

Ferric chloride test mentioned in Yusuf et al., (2014) and Jaradat et al., (2015) with slight modifications was carried out to identify the presence of phenols and tannins.

Ferric chloride test: 3 mL of solution was stirred with three drops of 5% $FeCl_3$ solution. Blue or greenish-black colour turns in to olive green with excess $FeCl_3$ indicating the presence of tannins and phenols.

Flavonoid

Shinoda's test: Few drops of concentrated HCl following 0.5 g of magnesium ribbon was added to 1 mL of methanolic extract and development of pink or magenta-red colouration within 3 min was observed for flavonoids.

GC-MS

The GC-MS analysis was conducted on an Agilent 7890A GC coupled with Agilent 5975C inert MS with Triple-Axis Detector. In order to remove residue water from ethanolic extraction anhydrous Na_2SO_4 was added and filtered through Whatman No.1 filter paper. The filtrate was subjected to centrifugation at 10000 rpm for 30 minutes after the optimization of the method. The supernatant was filtered through 0.45 µ filter paper. Helium (99.999%) was used as carrier gas at a constant flow rate of ± 1 mL mn-1. Injector volume of 2 µm with a split ration 10:1 and the injector temperature and source temperature were set at 250 ^{0}C and 280 ^{0}C respectively. The oven temperature was programmed from 110 ^{0}C for 2 min to 200 ^{0}C at 10 ^{0}C min-1 rate, then increased to 280 0C at 5 ^{0}Cmin-1 and maintained for 9 min (Meenakshi et al., 2013).

Cytotoxicity determination

Cytotoxicity was determined in terms of mortality of Artemia larvae after 48 h of hatching against crude extractions of *Clathria* sp. using 6 well plates. Dry product (0.5 g) was dissolved in 5% dimethyl sulfoxide (DMSO) and diluted with filtered seawater, final volume was adjusted to 5 mL with aerated seawater to get different concentrations (0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 mg/ mL). The solution from each concentration was introduced to a well plate containing 10 individuals per well. Mortality was recorded after 24 h of incubation in each well. The experiment was conducted with positive and negative controls and different concentrations of the test extract in a set of three wells per dose. Lethal concentration (LC50) was determined using Finney's probit analysis (Finney, 1952).

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

Table 1. Phytochemical screening of ethanolic extract marine sponge *Clathria* sp.

Compound	Presence
Alkaloid	+
Sterols and triterpenes	+
Saponins	+
Phenols	-
Tannins	-
Flavonoid	-

"+" Presence, "-" Absence

Phytochemical screening of crude ethanolic extract of marine sponge *Clathria* sp. indicates the presence of alkaloids, sterols, triterpenes as well as saponins while resulting in the absence of phenols, tannins, and flavonoid (Table 1). The quality and quantity of secondary metabolites of sponge *Clathria* sp. may differ from one geographical region to another and from one environment to another. Wulf (2012) has discussed extensively with examples, the patterns of sponge distribution and growth due to the difference in abiotic factors and ecological interactions thus, resulting in the alteration in secondary metabolites qualitatively and quantitatively.

3.2 GC-MS analysis of secondary metabolites

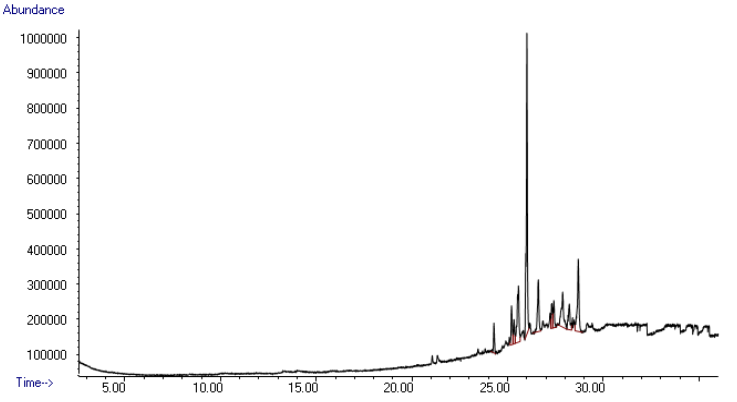

Figure 1. Volatile organic component profile of *Clathria* sp.

Table 2. Volatile organic components of crude ethanolic extract of marine sponge *Clathria* sp**.**

Retention Time (min)	Name of the compound	Peak area %
24.281	Cholesta-3,5-diene	3.122
25.202	Cholesta-2,4-diene	4.952
25.331	Cholest-2-ene	2.989
25.561	Cholesta-7,14-diene,(5.alpha.)	11.850
26.001	Cholesta-3,5-diene	34.761
26.598	Ergosta-4,6,22-triene	8.283
28.687	Stigmastan-3,5-diene	12.815

After the preliminary phytochemical screening, the ethanolic extract was further analyzed using GC-MS. The chromatogram of *Clathria* sp. only shows the presence of steroids (Figure 1). Degrading the compounds due to the high temperature during the ionization process might be one of the reasons for the absence of alkaloids in the resulted chromatogram. According to Table 2, cholesta-3,5-diene also known as cholesterylene shows the most noteworthy occurrence in GC-MS analysis with the significant presence of stigmastan-3,5-diene, cholesta-7,14-diene, (5. alpha.) and ergosta-4,6,22-triene. Cholesta-2,4-diene and cholest-2-ene also result in volatile component analysis as well. Stigmastan-3,5-diene is commonly found in vegetable oil as a result of thermal alteration, and the compound is also reported to have antibacterial and antifungal activities which as described in Boligon et al., 2012. Except for the seven compounds identified from GC-MS analysis, none of the other compounds were successfully annotated. This suggests the possibility of these compounds having very low concentrations or has not previously been identified or cataloged. Future studies will be conducted to investigate the presence of other volatile components. Studies of Rudi et al., (2001), Ravichandran et al., (2011) and Woo et al., (2015) mentioned the potential antiviral and antibacterial activity of steroids extracted from *Clathria* sp. The presence of terpenoids and derivatives in *Clathria* sp. were also discussed in Capon et al., (2000) and Gupta et al. (2012). Comprehensive studies should be conducted to confirm the presence of these compounds in *Clathria* sp. found in the coastal waters off Mannar. LC_{50} of 624 ppm (497-784 ppm 95% fiducial confidence interval) and lethargy of Artemia at all concentrations were observed in the cytotoxicity bioassay. The lethality against brine shrimp can be considered as a primary indicator for bioactivity of

Clathria sp. when responding to chemical cues for invasion and fouling of other organisms. Their abundant presence in marine waters with minimum fouling is an attestation for their utilization practically (Wulff, 2012).

However further biological testing should be pursued in order to explore the potential of antimicrobial and antifouling activities of *Clathria* sp.

4. CONCLUSION

Preliminary phytochemical screening of crude ethanolic extract from marine sponge *Clathria* sp. shows the presence of steroids, triterpenes, saponins, and alkaloids. The GC-MS analysis shows the presence of seven known steroids. Chemical compounds result from phytochemical screening and GC-MS analysis followed by significant lethality against Artemia for cytotoxic bioassay concluded that ethanolic extract of marine sponge *Clathria* sp. contains steroidal derivatives and certain bioactivity. Further studies should be conducted in the future to explore the bioactivity of *Clathria* sp. A comprehensive microbial analysis and a settlement biological assay should be conducted to the extracts isolated from Clathria sp. according to the polarity in order to identify the potential of the antibacterial and antifouling activities.

ACKNOWLEDGMENT

The authors express gratitude to the University of Sri Jayewardenepura, Sri Lanka (Research grant no: ASP/01/RE/SCI/2015/28), Central Instrument Facility, University of Sri Jayewardenepura and Sri Lanka Ports Authority for their support during the study.

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