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Research Article

BIOPROSPECTING OF MARINE SPONGE (*CALLYSPONGIA DIFFUSA*) FOR ANTIBACTERIAL COMPOUND

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ABSTRACT

Objective: Marine sponges are a rich source of new antimicrobial drugs. The present study was aimed to evaluate the antibacterial activity of the marine sponge (*Callyspongia diffusa*) against human pathogenic bacteria and to analyze the presence of bioactive compounds in the sponge.

Methods: Antibacterial activity of the marine sponge *C. diffuser* was examined using petroleum ether, chloroform, n-butanol, methanol, ethanol, and water as solvents and tested against human pathogenic bacteria such as *Esherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* by agar well diffusion method. Zoochemical analysis was performed to screen for the presence of secondary metabolites. Bioactive compounds were purified by thin layer chromatography (TLC) and were identified by gas chromatography-mass spectrometry (GC-MS) analysis.

Results: The results obtained show that the sponge extracts had significant antibacterial activity against the tested strains. The methanol extract was found to be the most effective and exhibited the highest potency against all pathogens tested. Zoochemical analysis revealed the presence of alkaloids, terpenoids, and sterols. In TLC, spots corresponding to a Rf value of 0.67 were found to possess antibacterial activity against the test bacteria. GC-MS chromatogram showed seven major peaks at retention time of 12.69, 13.81, 24.21, 24.65, 28.01, 28.93, 30.87 minutes. The mass of the compounds and fragments were matched with the National Institute of Standard and Technology (NIST) database for identification of probable compounds present in the sample. GC-MS analysis revealed the presence of bioactive compounds in the sponge.

Conclusion: This study confirms the marine natural species provides an excellent source of bioactive metabolites that can exploit to develop novel and potential therapeutic agents.

Keywords: *Callyspongia diffusa*, Solvent extraction, Antibacterial activity, Thin layer chromatography, Gas chromatography-mass spectrometry.

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INTRODUCTION

Sponges belong to the phylum *Porifera,* and they are ancient metazoans that have existed since 700-800 million years. They are abundantly present not only in the tropical oceans but also occur in temperate waters and even in fresh water [1]. They are simple multicellular invertebrates, and they are attached to solid substrates in benthic habitats [2]. The sponges are of three classes, namely, the Calcarea (5 orders and 24 families), Demospongiae (15 orders and 92 families), and Hexactinellida (6 orders and 20 families). About 15,000 species of sponges have been described so far [3].

Marine sponges are rich sources of pharmacological compounds [4]. Studies show that secondary metabolites of sponges play an important role in their survival in the marine water [5,6]. These secondary metabolites have interesting biomedical, pharmaceutical, and biotechnological applications [7]. These bioactive compounds are attributed to their antimicrobial, antiviral, antitumor, and general cytotoxic properties [8]. *Porifera* is the most important phylum, as it provides a number of natural products, especially novel pharmacologically active compounds [9].

Bioactive compounds from marine sponges have extensive application for the treatment of many diseases. Several molecules isolated from various sponges are involved in the advanced stage of clinical trials. In this study, we tested the organic extracts of marine sponge (*Callyspongia* sp.) for antibacterial activity against three strains of pathogenic bacteria followed by gas chromatography-mass spectrometry (GC-MS) analysis to separate and identify the major chemical compounds present in the sample.

MATERIALS AND METHODS

Collection of marine sponge

The sponge was collected from the Krusadai Island, Gulf of Mannar, Ramanathapuram District, Tamil Nadu, India. It is 3 km from Pamban and 500 m from Kundugal point (Lat 9°15'00" N, Long 79°13'25"E). Sponges were collected by scuba diving at a depth of 5-10 m. A part of the specimen has been deposited in the Microbiology Research laboratory of Dr. N. G. P. Arts and Science College for records.

Identification of sponge

The sponge was identified as *Callyspongia diffusa* by Dr. R. Saravanan, Scientist, Central Marine Fisheries Research Institute, Mandapam Coast based on the skeletal characteristics (size and shape of the spicules), and external morphology. The color of its exterior was yellowish to purplish. Its body has small protrusions, cavities, and branches.

- Kingdom: Animalia
- Phylum: *Porifera*
- Class: Demospongiae
- Order: Haplosclerida
- Family: Callyspongidae
- Genus: *Callyspongia*
- Species: *C. diffusa*
- Generic name: Sponges
- Common name: Callyspongid sponge

Extraction of bioactive compounds from sponge

The sponge was rinsed thoroughly with sterile seawater to remove algae or if any bacteria present on the surface of the sponge. The sponge was then cut into small pieces of about 5 cm and then homogenized

by crushing in a mortar and pestle [10]. The homogenized sponge was subjected to sequential solvent extraction employing solvents of varying polarity, namely, petroleum ether, chloroform, n-butanol, methanol, ethanol, and water. The solvents employed for extraction were of analytical grade purchased from HiMedia Laboratory, Mumbai, India. The extraction was carried out using a Soxhlet apparatus for 48 hrs [11]. After extraction, the extracts were concentrated in a Rotavapor (Equitron) and the concentrated extracts were collected in airtight glass screw cap tubes and preserved at 4°C for further use [12].

Collection of bacterial culture
The pathogenic pure cultures of *Escherichia coli*, *Pseudomonas aeruginosa,* and S*taphylococcus aureus* were obtained from Jansons MRI Diagnostic Pvt. Ltd., Erode, Tamil Nadu. The pure cultures were then subcultured on nutrient agar slants and preserved at −4°C until further use.

Determination of antibacterial activity of the extracts
The antibacterial activity of the extracts of *C. diffusa* was determined by the standard agar well diffusion assay on Mueller-Hinton agar (HiMedia) [13,14]. The concentrated extracts were dissolved in dimethyl sulfoxide (DMSO) [15]. Wells of 8 mm diameter were punched using sterilized cork borer. 1 ml exponential phase culture of the test bacteria corresponding to 0.5 ml McFarland opacity was swabbed with a sterile cotton on the surface of the medium. 50 µl of each extract in DMSO (500 µg/ml) was dispensed into different wells. DMSO was maintained as a control for each test organism. The plates were incubated at 37°C. Areas of inhibited microbial growth were observed as a clear zone around the well after 24 hrs. Antimicrobial activity was measured with the help of a scale, and the diameter of inhibition zone was recorded. The tests were done in triplicates.

Statistical analysis
The values of antibacterial activity of the extracts of sponge *C. diffusa* are expressed as the mean ± standard deviation (SD) (n=3) [Table 1].

Zoochemical analysis
Zoohemical analysis was performed on extracts of *C. diffusa* [16,17].

Test for alkaloids
One ml of the extract was treated with Mayer's reagent. Yellow color precipitate formation indicates the presence of alkaloids.

Test for terpenoids
The extract was dissolved in an equal amount of chloroform and evaporated to dryness. Then, concentrated sulfuric acid is added and heated for about 2 minutes. Gray color formation indicates the presence of terpenoids.

Test for flavonoids
The extract was mixed with a few fragments of magnesium ribbon and to this concentrated hydrochloric acid was added. Pink color formation indicates the presence of flavonoids.

Test for sterols
The crude extract was mixed with chloroform and concentrated sulfuric acid. Red color formation indicates the presence of sterols. [Table 2]

Thin layer chromatography (TLC)
TLC aluminum sheets coated with silica gel were used for the TLC study. The methanol extract suspended in water was then spotted on the TLC plate using a capillary tube. The plates were then developed in closed glass chambers saturated with the developing solvents (petroleum ether:hexane:Chloroform - 60:20:20). After a plate had run, it was removed from the developing chamber and dried in air. The separated compounds were then visualized using long wavelength ultraviolet light. The components separated were then scraped from the plates and collected in Eppendoff® microfuge tubes.

Determination of antibacterial activity of the separated compounds
The compounds separated by TLC were then extracted with methanol. The extract was then dried, and 50 µg of the extracted compound was reconstituted in 50 µl of water. Wells of 8 mm diameter were punched using sterilized cork borer. 1 ml exponential phase culture of the test bacteria corresponding to 0.5 ml McFarland opacity was swabbed with a sterile cotton on the surface of the medium. The suspension of different concentration 25 µl, 50 µl, and 100 µl was then loaded into the wells on Muller-Hinton agar seeded with the bacterial test culture. Solvent control and the standard antibiotic disc ciprofloxacin (5 mcg) were maintained as a control for each test organism. The plates were incubated at 37°C for 24 hrs to determine the antibacterial activity of the separated compounds. Antimicrobial activity was measured using a scale, and the diameter of inhibition zone was recorded. The tests were carried out in triplicates [Table 3].

GC-MS analysis
The GC-MS used in the present study was Perkin Elmer made with GC model as Clarus 680 and Mass Spectrometer model as Clarus 680 (EI). The instrumental acquisition parameter was as follows. Oven: Initial temperature 60°C for 2 minutes, ramp 10°C/minutes to 300°C, hold 6 minutes, total run time = 32.0 minutes. Carrier gas was Helium and the column was Elite 5MS. The mass condition (EI) was kept as follows. Solvent delay = 2.00 minutes, transfer temperature = 230°C, source temperature = 230°C, and scan = 50-600 Da.

Identification of components
The chromatograph showing peaks corresponding to different compounds were obtained and interpreted. Interpretation was carried out using the database of National Institute Standard and Technology (NIST). The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and molecular formula of the components of the test materials were ascertained [Table 4].

RESULTS AND DISCUSSION

The trend of antibacterial activities of the extracts of *C. diffusa* against human pathogenic bacteria indicates that the solvent extracts of *C. diffusa* possess antibacterial activity against all the test bacteria such as *E. coli*, *P. aeruginosa*, and *S. aureus* and the results are expressed as mean ± SD (n=3). A maximum diameter zone of inhibition (19.33±0.57 mm) for *E. coli* was observed with methanol extract. Maximum diameter zone of inhibition (20.00±1.00 mm) for *P. aeruginosa* was observed with petroleum ether extract. A maximum diameter zone of inhibition (16.66±1.52 mm) for *S. aureus* was observed with methanol extract. There was no zone of inhibition for the control DMSO against all the test bacteria. The result indicates that the solvent extracts exhibited greater antibacterial activity.

Results of Zoochemical analysis revealed the presence of alkaloids, terpenoids, and sterols. It was observed that alkaloids were detected in the methanol, ethanol, petroleum ether, chloroform, and n-butanol extracts. Terpenoids were detected in the methanol, ethanol, petroleum

Table 1: Antibacterial activities of extracts of *C. diffusa*

Zone of inhibition (mm)			
Extracts (500 µg/ml)	**Bacteria**		
	E. coli	***S. aureus***	***P. aeruginosa***
Methanol	19.33±0.57	16.66±1.00	19.66±0.57
N-Butanol	17.33±0.57	15.00±1.52	18.33±1.52
Ethanol	16 .00±1.00	15.33±0.57	17.00±1.00
Chloroform	Nil	12.66±1.52	11.66±1.52
Petroleum ether	18.66±1.52	16.33±0.57	20.00±1.00
Water	Nil	Nil	Nil
DMSO	Nil	Nil	Nil

Values are expressed as mean±standard deviation of the three replicates.
C. *diffusa: Callyspongia diffusa, E. coli: Esherichia coli, S. aureus: Staphylococcus aureus, P. aeruginosa: Pseudomonas aeruginosa*, DMSO: Dimethyl sulfoxide

Table 2: Zoochemical analysis of extracts of *C. diffusa*

Sponge	Extract	Alkaloids	Terpenoids	Flavonoids	Sterols
C. diffusa	Methanol	+	+	−	−
	N-Butanol	+	+	−	−
	Ethanol	+	−	−	+
	Chloroform	+	+	−	+
	Petroleum ether	+	+	−	+
	Water	−	−	−	−

C. diffusa: Callyspongia diffusa

Table 3: Antibacterial activities of TLC scrapings of *C. diffusa*

Name of the pathogen	Zone of inhibition				
	25 µl	50 µl	100 µl	Ciprofloxacin disc (5 mcg)	N-butanol
E.coli	12.00±1 mm	18.00±1 mm	22.33±0.57 mm	Nil	Nil
P. aeruginosa	16.66±1.52 mm	20.00±2 mm	23.66±1.52 mm	24.33±0.57 mm	Nil
S. aureus	16.33±0.57 mm	21.00±1 mm	23.00±1 mm	Nil	Nil

Values are expressed as mean ± standard deviation of the three replicates. *E. coli: Esherichia coli, S. aureus: Staphylococcus aureus, P. aeruginosa: Pseudomonas aeruginosa, C. diffusa: Callyspongia diffusa,* TLC: Thin layer chromatography

Table 4: GC-MS analysis

Retension time (minutes)	Molecular formula	Molecular weight	Name of the compound
12.69	$C_9H_8O_2$	148	2 (3H)-benzofuranane, 3-methyl
	$C_9H_8O_2$	148	2H-1-benzopyran- 2 –one, 3,4-dihydro
	$C_9H_8O_2$	148	2H-1-benzopyran- 2 –one, 3,4-dihydro
	$C_{10}H_{12}O_3$	180	Methyl 3-(2-hydroxyphenyl) propionate
13.81	$C_9H_6O_2$	146	2H-1-benzopyran-2-one
	$C_{10}H_6O_3$	174	1,2-naphthalenedione,6-hydroxy-
	$C_9H_6O_2$	146	2H-1-benzopyran-2-one
	$C_9H_6O_2$	146	2H-1-benzopyran-2-one
20.00	$C_9H_{10}N_2$	146	1H-benzimidazole,1-Ethyl
	$C_{16}H_{13}C_2N_3$	317	Acetamide, N-(1H-1,3-benzimidazol-2-ylmethyl)-2-(2,4- difluorophenoxy
	$C_9H_{10}N_2$	146	1H-benzimidazole,5,6-Dimethyl-
	$C_{11}H_{14}ON_2$	190	Benzo(F)-1,5-diazabicyclo(3.2.2) nonen-3-ol
21.85	$C_9H_6O_3N_2$	190	Quinazoline-4-carboxylic acid,1,2-dihydro-2-Oxo
	$C_8H_7N_3$	145	1H-1,2,3-triazole,4-phenyl-
	$C_{10}H_6O_4$	190	Coumarin-3-carboxylic acid
	$C_{10}H_{10}O$	146	1,2 napthalenone,3,4-dihydro
23.70	$C_9H_6O_3N_2$	190	Quinazoline-4-carboxylic acid,1,2-dihydro-2-Oxo
	$C_{10}H_{28}O_4S_{13}$	296	Silicic acid, diethyl bis (trimethylsilyl) ester
24.65	$C_{26}H_{29}ON$	371	Ethanamine,2-(4-(1,2-diphenyl-1-butenyl)phenoxy)-N,N-dimethyl-,(Z)
	$C_{26}H_{29}ON$	371	Ethanamine,2-(4-(1,2-diphenyl-1-butenyl)phenoxy)-N,N-dimethyl-,(Z)
	$C_{19}H_{21}ON$	279	Doxepin
	$C_{19}H_{21}ON$	279	Doxepin
27.06	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-
	$C_{24}H_{36}O_2Si_2$	412	4-Methyl-2,4-Bis(4'-trimethylsilyloxyphenyl)pentene-1
	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-
	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-
28.93	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-
	$C_{17}H_{30}OSi_3$	278	Trimethyl(4-(1,1,3,3,-tetramethylbutyl) phenoxy) silane
	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-
	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-
30.29	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-
	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-
	$C_{17}H_{30}OSi_3$	278	Trimethyl(4-(1,1,3,3,-tetramethylbutyl) phenoxy) silane
	$C_6H_{18}O_3Si_3$	222	Cyclotrisiloxane, hexamethyl-

GC-MS: Gas chromatograph-mass spectroscopy

ether, and chloroform extracts. Sterols were detected in ethanol, chloroform, and petroleum ether extracts. None of the extracts tested had shown the presence of flavonoids.

In TLC, spots were visualized under ultraviolet light. Spots corresponding to a Rf value of 0.67 were found to possess antibacterial activity against the test bacteria. The standard antibiotic (ciprofloxacin) was effective against *P. aeruginosa* but it was resistant to *E. coli* and *S. aureus*. The solvent control showed no zone of inhibition against all the test bacteria.

The GC-MS analysis of potential extract revealed the occurrence of different compounds corresponding to the peaks obtained at different retention times including 2(3H)-benzofuranane, 3-methyl, 2H-1-benzopyran- 2 –one,3,4-dihydro, methyl 3-(2-hydroxyphenyl) propionate, 2H-1-benzopyran-2-one, 1,2-naphthalenedione,6-hydroxy,

1H-benzimidazole, 1-ethyl, acetamide, N-(1H-1,3-benzimidazol-2-ylmethyl)-2-(2,4-difluorophenoxy, 1H-benzimidazole,5,6-dimethyl, benzo(F)-1,5-diazabicyclo(3.2.2) nonen-3-ol, quinazoline-4-carboxylic acid,1,2-dihydro-2-Oxo, 1H-1,2,3-triazole,4-phenyl, coumarin-3-carboxylic acid, 1,2 napthalenone,3,4-dihydro, Silicic acid, diethyl bis (trimethylsilyl) ester, ethanamine,2-(4-(1,2-diphenyl-1-butenyl) phenoxy)-N,N-dimethyl-,(Z), doxepin, cyclotrisiloxane, hexamethyl, 4-methyl-2,4-Bis(4'-trimethylsilyloxyphenyl) pentene-1,trimethyl(4-(1,1,3,3,-tetramethylbutyl) phenoxy) silane.

DISCUSSION

Drug discovery has become a very important field of study within the past few decades. With the rate of drug resistance by pathogenic microbes that cause human infection, it has become increasingly important to investigate natural products for novel antimicrobial compounds. Marine organisms consist of a valuable source of new compounds. In this study, the extracts of the sponge *C. diffusa* showed effective antibacterial activity against *E. coli*, *P. aeruginosa*, and *S. aureus*.

Zoochemical analysis revealed the presence of alkaloids, terpenoids, and sterols in the sponge subjected to study. Alkaloids are nitrogenous organic compounds which have a wide range of applications in pharmacology and cosmetic industries [18]. The marine sponge hexane extract of a *A. Globe stellata* is reported to have antioxidant properties due to the presence of phenolic and flavonoids content in the sponge [19]. It is also reported that sponge associated bacterial extracts have cytotoxicity effect on MOLT 4 cell lines through apoptosis [20].

The bioactive compounds were confirmed by GC-MS analysis. Although most of the compounds isolated in the present study have been known for various biological activities, the compound 2H-1-Benzopyran-2-one exhibit wide range of biological activities such as antimicrobial, antiviral, antidiabetic, anticancer activity, antioxidant, antiparasitic, anti-helminthic, antiproliferative, anticonvulsant, anti-inflammatory, and antihypertensive activities. [21]. Cyclotrisiloxane, hexamethyl has been demonstrated to exhibit significant pharmacological effects such as antimicrobial, anti-inflammatory, antioxidant etc. [22]. Cyclohexasiloxane dodecamethyl isolated from marine sponge *S. pumila* is reported to have many industrial applications and in household care products [23]. 1,2 Napthhalenedione-6-hydroxy has biological activities such as Larvicidal, antibacterial, antifungal, antiviral, insecticidal, anti-inflammatory, and antipyretic activity. [24]. By isolating and identifying these bioactive compounds, new drugs can be formulated to treat different diseases.

CONCLUSION

The present study indicates the presence of an active antibacterial compound in marine sponge *C. diffusa*. The results support that sponge *C. diffusa* could be a valuable source of novel substances for future drug discovery. A detailed investigation with the objective of isolating biologically active molecules along with the search for novel compounds is currently under study.

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