British Journal of Pharmaceutical Research
11(1): 1-10, 2016, Article no.BJPR.24740
ISSN: 2231-2919, NLM ID: 101631759

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Comparative Evaluation of Bioactivities of two Marine Sponges, *Zygomycale parishii* and *Callyspongia diffusa* from Southwest Coast of India

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Authors' contributions

This work was carried out in collaboration between the authors. Author KAAK designed the study, wrote the protocol, managed the experimental process and wrote the first draft of the manuscript. Author TRK managed the literature searches and analyses of the study performed. Both the authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2016/24740
Editor(s):
(1) N. Alyautdin Renad, Chair of the Department of Pharmacology (Pharmaceutical Faculty), I. M. Sechenov MSMU, Moscow, Russia.
Reviewers:
(1) Hedi Indra Januar, Bogor Agricultural University, Indonesia.
(2) Eliton da Silva Vasconcelos, Federal University of Sao Carlos, Brazil.
Complete Peer review History: http://sciencedomain.org/review-history/13822

Original Research Article

Received 30th January 2016
Accepted 10th March 2016
Published 24th March 2016

ABSTRACT

Aims: The present study was done to evaluate and compare the bioactivities of methanol and dichloromethane extracts of two marine sponges, *Zygomycale parishii* and *Callyspongia diffusa* from Southwest coast of India.
Place and Duration of the Study: At the Microbial Biotechnology Laboratory, School of Biosciences, Mahatma Gandhi University between May 2013- August 2014.
Methodology: The methanol and dichloromethane extracts of the two selected species were prepared and were subjected to various bioactivities. Antioxidant activity was assayed using DPPH radical scavenging activity assay and total antioxidant activity assay. Antibacterial activity was assayed by the disc diffusion method using the selected pathogens such as *E. coli, Salmonella typhi, Salmonella paratyphi, Staphylococcus aureus* and *Vibrio cholerae.* Immunomodulatory activity was analyzed by calculating the phagocytic index and by Nitro Blue tetrazolium assay. Acetylcholinesterase inhibitory activity was examined by modified Ellman's method. The chemical constituents of these extracts were analyzed by routine phytochemical methods.

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Results: Antioxidant activity was assayed using DPPH radical scavenging activity and total antioxidant activity indicated that both sponge extracts possessed antioxidant activity and the dichloromethane extract of Zygomycale was more active with an IC50 value of 486µg. Immunomodulatory activity was analyzed by calculating phagocytic index and Nitro Blue tetrazolium assay indicated that the extract of Zygomycale was more immunomodulatory. Acetylcholinesterase inhibitory activity assay revealed that Zygomycale extract was more active with an inhibitory potential in the range of 49-61%. The chemical constituents of these extracts were analyzed by routine phytochemical methods showed the presence of alkaloids, phenols, triterpenoid, and aromatic acids in both the sponge extracts.
Conclusion: The extracts of *Zygomycale parishii* was more active in all the assays performed compared to that of *Callyspongia diffusa*. So it can be concluded that further fractionation and purification will yield potent compounds for pharmaceutical leads.

Keywords: Zygomycale parishii; Callyspongia diffusa; organic extracts; bioactivity; acetylcholinesterase inhibition.

1. INTRODUCTION

Oceans have great biodiversity and potential for discovery of novel compounds with biomedical significance. Natural products continue to be a major source of pharmaceuticals and for the discovery of new molecular structure [1]. Over 60% of the potentially bioactive compounds discovered so far from living organisms have been obtained from marine environment, 70% of which comes from sponges [2].

Marine sponges are the oldest known metazoans of the Phylum Porifera. They are sedentary sessile filter feeders and are immense source of novel bioactive entities. These compounds comprised of diverse structural classes, including polyketides, terpenes, steroids and peptides with a wide range of biologically relevant properties such as anticancer, antibacterial, antifungal, antiviral, antifouling and anti-inflammatory [3]. Large numbers of secondary metabolites have been reported from the class Demospongiae and the orders Halichondrida, Poecilosclerida and Dictyoceratida. The two sponges selected in this study were abundant in the collection site. They belong to the class Demospongiae - *Zygomycale parishii* and *Callyspongia diffusa*. Of these, Zygomycale belong to the order Poecilosclerida and Callyspongia belong to the order Haplosclerida.

2. METHODOLOGY

2.1 Sample Collection

The marine sponge samples were collected from southwest coast of India with the help of SCUBA divers at depth of 8-10 feet and were identified as *Zygomycale parishii* and *Callyspongia diffusa* with the help of Dr. P. A Thomas, Retd. Principal Scientist, CMFRI, Vizhinjam. The samples were collected in sterile plastic bags kept in ice box and transferred to lab and stored at -20℃.

2.2 Organic Extraction

Extraction was carried out using methanol and dichloromethane as solvents by some modification of method of Sepcic et al. [4]. The sponge samples were lyophilized and then 1g of sponge tissue was soaked in 30ml of methanol in sealed container and dichloromethane in another container and was shaken overnight at 37℃. The extracts were filtered and remaining material was repeatedly extracted for three days at 37℃ with constant shaking. The solvents were evaporated and the extracts were dissolved in dimethyl sulfoxide (DMSO).

2.3 Screening for Chemical Constituents

Preliminary screening for chemical constituents (Table 1) in the extracts was performed with slight modifications of the method of Harborne [5].

2.4 Antioxidant Activity

2.4.1 Total antioxidant activity assay

The total antioxidant activities of the extracts were evaluated by the phosphomolybdenum method by Prieto et al. [6]. This assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the subsequent formation of a green phosphate /Mo (V) complex at acidic pH. A 0.3 mL extract solution was dispensed into screw capped test tubes. A 3 mL reagent solution (6M H_2SO_4, 28 mM sodium phosphate, 4mM ammonium molybdate) was

added and the tubes were capped and incubated at 95℃ for 90 minutes. After cooling to room temperature, the absorbance was measured at 695 nm using a spectrophotometer. A blank test was done using the solvent used. The antioxidant activity was expressed as ascorbic acid equivalents using ascorbic acid as reference standard.

2.4.2 DPPH radical scavenging activity test

The stable 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging activity was determined using the method of Mensor et al. [7]. The samples and the reference were then mixed with DPPH solution. Remaining DPPH amount was measured at 517 nm using spectrophotometer. Ascorbic acid was employed as the reference. Inhibition of DPPH in percentage was calculated as given below:

I%= [(A blank- A sample)/ A blank] ×100, where A blank is the absorbance of the control reaction and A sample is the absorbance of the extracts.

2.5 Phagocytic Activity Assay

Immunomodulatory activity was analyzed by a slide method by Wadekkar et al. [8] through *in vitro* phagocytosis of *Saccharomyces cerevisiae.* This assay was performed using blood from healthy donors and placing 100 µl of it into each glass slide. The slide was incubated for the cells to get attached and then the clot was removed and the slide was flood with test sample and incubated for half an hour. The slide was then drained and flooded with a suspension of heat killed opsonised yeast cells and incubated for 1 hour. After draining the yeast suspension, slides were fixed in absolute ethanol. Then it was stained with Giemsa stain.

The phagocytic index was calculated as the number of yeast cells phagocytosed by 100 granulocytes and the percentage of phagocytosis was calculated using the equation:

[PI (test) - PI (control)/PI (control) × 100]

Table 1. Phytochemical screening tests

Sl. no.	Test	Positive reaction
1	Test for steroids Extract in 0.5 mL acetic acid was warmed and cooled under tap water. Then H_2SO_4 was dropped sidewise.	Green colour
2.	Test for terpenoids 5 mL of chloroform was added to 1 mL of ice cold extract and con. H_2SO_4 was dropped sidewise.	Yellow layer in acid layer
3.	Test for reducing sugar To 2 ml of ice cold extract, 2 drops of α-naphthol was added and con. H_2SO_4 was dropped sidewise.	Purple colour
4.	Test for alkaloids Equal volumes of ice cold extract and acetic acid was taken and aqueous layer was decanted.1 mL Dragendorff's reagent was added.	Orange/red orange precipitate
5.	Test for phenolics 0.8 mL neutral $FeCl_3$ was added to 2 mL of the ice cold extract.	An intense blue/violet colour
6.	Test for Xanthoprotein To 1 mL of the the ice cold extract, 0.5 mL of 3N HNO_3 was added followed by addition of 2-4 mL of ammonia	Red orange precipitate
7.	Test for aromatic acids To the 1 mL of ice cold extract 2-3 mL of saturated $NaHCO_3$ was added	Brisk effervescence
8.	Test for flavonoids Cold extract+ 1-2 mg of Mg^{2+} + 1-2 ml of con. HCl To 1 mL of cold extract, 1-2 mg of Mg^{2+} was added followed by addition of 1-2 mL of 2N HCl.	Red/ orange colour

2.6 NBT Assay

The assay was performed with a modification of the method of Weiss et al. [9]. For the assay, $1x10^4$ leucocytes/ ml were incubated with 0.1% Nitro Blue Tetrazolium dye in phosphate buffered saline (pH-7.2) and the test samples for 30 minutes at 37℃ with 5% CO_2. A set of cells treated with crude filtrate of *E. coli* broth culture was used as a positive control. After incubation, a drop from each sample was carefully transferred onto a grease free glass slide and a coverslip smear was made. The smear was allowed to dry and fixed with ethanol for three minutes. Then the slides were stained with saffranin for three minutes, air dried and observed under 40X objective for calculating percentage of neutrophils with formazan granules.

2.7 Acetylcholinesterase Inhibition Assay

The acetylcholinesterase assay was performed according to the method of Ellman et al. [10] with slight modifications. Briefly acetylcholinesterase (AChE) from electric eel (Sigma, USA), was dissolved in 100 mM phosphate buffer (pH 7.3) to achieve 500EU/mL. Prior to the test, the enzyme was 100 fold diluted in the same buffer. To each microtiter plate well 140 µL of the Ellman reagent (5,5-dithiobis-2-nitrobenzoic acid) in 25 mM phosphate buffer (pH 7.0), 10 µL acetylcholine (ACh) in 1 mM final concentration, 20 µL of sponge sample (aqueous or organic), and finally 50 µL of AChE was added to start the reaction. Deionized (20 µl) water was used as controls. The time course of the enzymatic reaction was monitored for 12 minutes at 412 nm at 25℃.

2.8 Antibacterial Activity

Antibacterial activity was determined against cultures of *E. coli, Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi* and *Vibrio cholerae* using disc diffusion assay method by El- Marsy et al. [11]. Sterile disks of 6 mm diameter were impregnated with extracts (1 mg/ml). Tetracycline (2 mg/ml) was used as positive control. Agar plates were surface inoculated uniformly from broth cultures of test organisms. The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 37℃ for 24 hour. The diameter of the zones of inhibition obtained was measured in mm.

2.9 Statistical Analysis

The experiments were done in triplicates and error bars in graphs represents standard error of means per triplicate samples. The results were analyzed by Student's t test and were considered statistically significant when $P<0.05$. IC 50 values were calculated in MS Excel.

3. RESULTS AND DISCUSSION

Sponges provided the largest number of marine derived secondary metabolites including some of the most important pharmacological drug candidates [12]. The sponges are naturally in close association with a variety of microorganisms and this symbiotic relation provides a rich source of biologically active secondary metabolites. Though many compounds have been reported from the sponges, commercialization of only a very few has been possible. This is mainly due to the problem of supply, collection difficulties and low concentration of active compound.

3.1 Screening of Chemical Constituents

Triterpenoids, aromatic acids and phenolics were present in both the extracts of the two sponge species (Table 2). In addition to these, methanolic extracts of both species contains steroids and Zygomycale extracts contained flavonoids. But steroids are absent in dichloromethane extracts. Dichloromethane effectively extracts all except the most polar steroids. So the steroids present in the tested sponge species may be of highly polar nature which was extracted using the polar solvent methanol. All these chemical constituents singly or in combination contribute to the bioactivities exhibited by the extracts. Several compounds such as ceramides, heptadecane, eicozane, dodecane phenol, 2, 4- bis (1, 1- dimethylethyl), dibutyl phthalate etc. were reported from *Zygomycale parishii* [13,14]. Sponges belonging to the genus Callyspongia have proven to be rich source of various substances such as polyketides, polyacetylenes, alkaloids and cyclic peptides. Callyspongiolide, a macrolide have also been reported from the genus Callyspongia [15]. Kohamaic acids A and B are known to be constituents of Ircinia species from Okinawa [16]. Cytotoxic activity of triterpenoids from various marine sponges has been reported [17].

3.2 Antioxidant Activity

3.2.1 Total antioxidant activity assay

This assay is employed for quantitative determination of antioxidant capacity through the formation of phosphomolybdenum complex. The results are expressed as mg/g equivalence of ascorbic acid.

The results (Table 3) show that dichloromethane extract of Zygomycale is more potent.

3.2.2 DPPH radical scavenging activity

DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to deep violet colour and absorption is measured at 517 nm.

This assay was performed for different concentrations (62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml) of the extracts. Both the extracts exhibited significant percentage inhibition (Fig. 1). IC50 values (Table 3) were calculated and it showed that dichloromethane extract has very low IC50 which indicates its higher antioxidant capability. Zygomycale was more active than Callyspongia.

Table 2. Screening of chemical constituents

Sample		Phytochemicals							
		Alkaloid	Triterpenoid	Xanthoprotein	Phenolics	Reducing sugar	Aromatic acids	Steroid	Flavonoids
Zygomycale parishii	Methanol extract	+	+	-	+	-	+	+	+
	Dichloromethane extract	+	+	-	+	-	+	-	+
Callyspongia diffusa	Methanol extract	+	+	-	+	-	+	+	-
	Dichloromethane extract	+	+	-	+	-	+	-	-

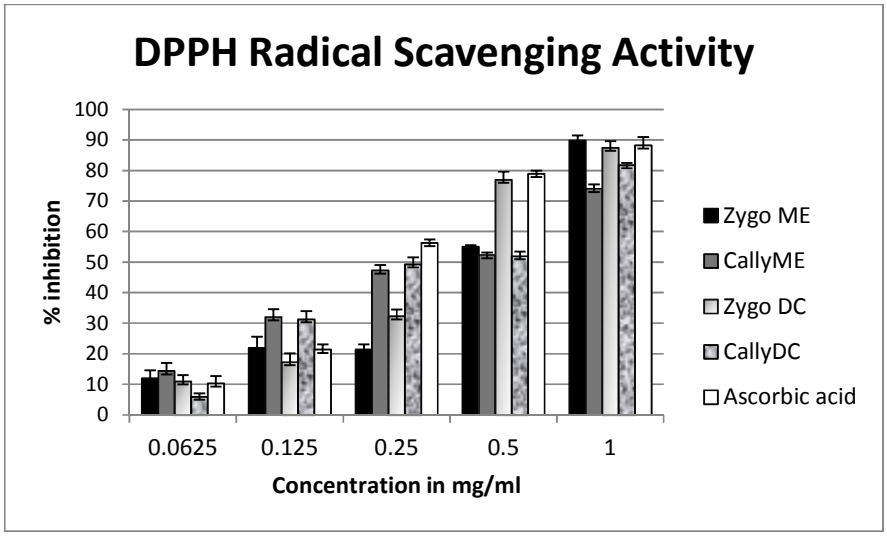


Fig. 1. DPPH radical scavenging activity of the extracts

Zygo ME- Zygomycale parishii methanol extract; Cally ME- Callyspongia diffusa methanol extract; Zygo DE- Zygomycale parishii dichloromethane extract; Cally DE- Callyspongia diffusa dichloromethane extract. Mean ± standard error from the tested triplicate samples were represented as mean error bars. P value by students test: P<0.05

Table 3. Antioxidant activity of the extracts

Sample	Total antioxidant activity (mg/g equivalents of ascorbic acid)	DPPH radical scavenging activity assay (IC50 value in µg)
Zygomycale parishii methanol extract	52.5±0.04	527.78
Zygomycale parishii dichloromethane extract	42.5±0.76	486
Callyspongia diffusa methanol extract	85±2.33	569.76
Callyspongia diffusa dichloromethane extract	52.5±01.44	535.28

Total antioxidant activity expressed in milligram per gram equivalents of ascorbic acid. IC 50 of DPPH Radical scavenging activity expressed in microgram

Antioxidant assays revealed that organic extracts of both the species possessed significant activity. Total antioxidant activity and DPPH Radical scavenging activity was more for dichloromethane extract of Zygomycale than for Callyspongia. Recent studies reported that the methanol extract of *Sigmadocia carnosa* exhibited good DPPH radical scavenging activity [18]. There were reports of good DPPH activity exhibited by the extracts of Mediterranean sea sponges *Dysidea avara*, *Axinella cannabina, Axinella damicornis, Agelas oroides* and *Ircinia fasciculata* [19]. Phenolic metabolites from marine sponges have been reported to possess antioxidant activity [20]. Antioxidant activity of Callyspongia species has been reported from Persian Gulf [21].

3.3 Phagocytic Activity

Phagocytic activity (Table 4) was more for dichloromethane extracts of both the sponges.

3.4 NBT Assay

Immunomodulatory effects of the extracts were further confirmed by NBT assay. Nitro Blue Tetrazolium is an electron acceptor used to detect indirectly the production of superoxide by stimulated neutrophils. Superoxide reduces the yellow soluble NBT to the blue black formazan, an insoluble material that precipitate which can be seen microscopically within the cell.

Both the extracts exhibited significant reduction (Fig. 2). Dichloromethane extracts were more potent and among them the extract of Zygomycale is more active.

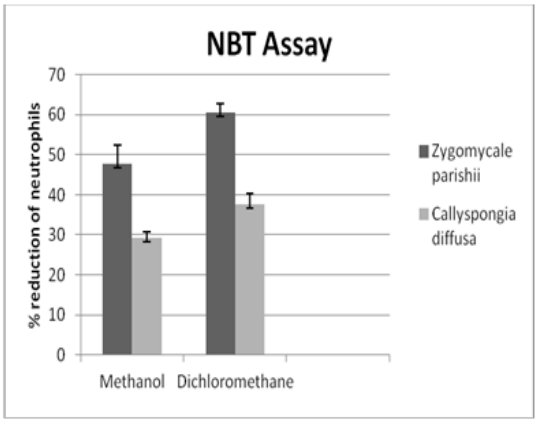


Fig. 2. NBT reduction

Percentage of neutrophils by Nitroblue tetrazolium reduction assay Mean ± standard error from the tested triplicate samples were represented as mean error bars. P value by students test: P<0.05

Table 4. Phagocytic activity

Sample		Phagocytic index	% of phagocytosis
Zygomycale parishii	Methanol extract	1206	27.18±1.93
	Dichloromethane extract	1629	71.9233±0.90
Callyspongia diffusa	Methanol extract	1114	13.89±0.76
	Dichloromethane extract	1226.33	25.36±0.51

Phagocytic index and percentage of phagocytosis were calculated and represented as mean ± SEM (P<0.05)

Immunomodulatory activities of the extracts were analyzed by phagocytosis assay and Nitroblue tetrazolium reduction assay. Assays indicate that both the extracts possess immunostimulatory activity. Percentage of phagocytosis and phagocytic index and NBT reduction percentage was greater in dichloromethane extracts. This may be because the varying concentration or high polarity of the chemical constituents in the methanol extracts may be interfering with the assays or the intermediate polar compounds in the dichloromethane extracts may be stimulatory. Extract of Zygomycale was more active in both assays. Both the extracts possessed considerable immunomodulatory activity. Since these assays are qualitative, we can just confirm the presence of activity of the extracts. Structural elucidation of the active principle will be needed for confirmation of the reason for higher activity of the dichloromethane extracts in the immunomodulatory assays. Significant immunostimulant activity of low concentrations of extract of sponge *Halichondria panacea* was reported [2].

3.5 Acetylcholinesterase Inhibitory Activity

The extracts of both the sponges exhibited significant percentage inhibition (Table 5 and Fig. 3). Methanol extract of Zygomycale was more active.

Table 5. Acetylcholinesterase inhibitory activity

Sample	IC 50 in µg
Zygomycale parishii methanol extract	581.13
Zygomycale parishii dichloromethane extract	750.22
Callyspongia diffusa methanol extract	826.7
Callyspongia diffusa dichloromethane extract	985.47

Percentage inhibition per triplicate samples were calculated and expressed as mean ± SEM. The values were significant by Student's t-test (P<0.05). IC 50 values were expressed in µg

AChE inhibiting compounds may act as anticancer compounds affecting cholinergic system expressed in a variety of cancer cell lines. AChE inhibitors have a significant role in the treatment of Alzheimer's disease. Methanol extracts of both the species possessed significant inhibitory activity. Methanol extract of Zygomycale showed a low IC 50 value of 581.13 µg. Dichloromethane extracts were not as effective as methanol extracts. AChE inhibitory activity exhibited by both the extracts indicate that these extract may serve as good candidates for development of drugs against cancer and Alzheimer's disease since AChE inhibitors act on cholinergic system. Acetone, butanol and methanolic extracts of marine sponge *Agelas clathrodes* exhibited substantial AChE inhibitory activity [10]. Methanol extracts of *Ircinia* and *Dysidea* species displayed promising results in AChE inhibition test over 50% [19].

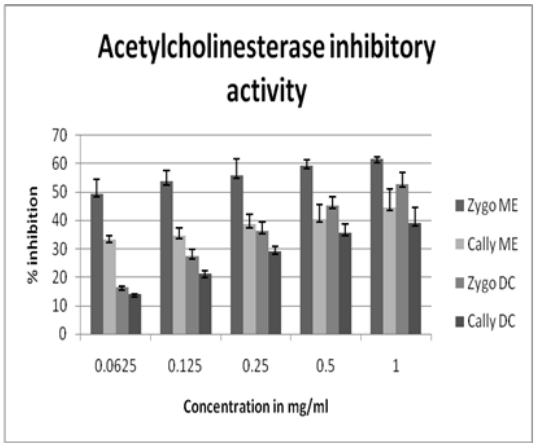


Fig. 3. Acetylcholinesterase inhibitory activity
Zygo ME- Zygomycale parishii methanol extract; Cally ME- Callyspongia diffusa methanol extract; Zygo LDE- Zygomycale parishii dichloromethane extract; Cally DE- Callyspongia diffusa dichloromethane extract . Mean ± standard error from the tested triplicate samples were represented as mean error bars. P value by students test: P<0.05

3.6 Antibacterial Activity

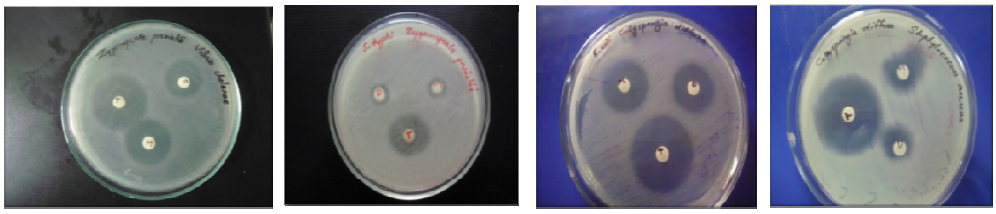
Antimicrobial activity is the principal functional important immune defense mechanism in marine animals. Marine sponges have numerous tiny pores on their surface through which they filter microorganisms and organic particles. So it is not surprising that it produces a wide variety of antimicrobial compounds. Both the extracts possessed broad and significant antibacterial activity (Table 6 and Fig. 4). Methanol extract was more active compared to the dichloromethane extracts.

The methanol extracts of both the species exhibited greater antibacterial activity against the tested strains. Methanol and dichloromethane extracts of the two sponge species exhibited

Table 6. Antibacterial activity

Sample		Pathogens				
		***Staphylococcus aureus* (MTCC 96)**	***Escherichia coli* (MTCC 443)**	***Salmonella typhi* (MTCC 734)**	***Salmonella paratyphi* (MTCC 735)**	***Vibrio cholerae* (MTCC 3906)**
Zygomycale parishii	Methanol extract	+++	++	++	**++**	**+++**
	Dichloromethane extract	+++	+	+	++	+=
Callyspongia diffusa	Methanol extract	+++	+++	++	++	+++

The results of the zones of inhibition were recorded according to the following scale: (+++)> 18 mm, (++) = 11-17 mm and (+) <10 mm



(A) **(B)** **(C)** **(D)**

Fig. 4. Antibacterial activity

M- Methanol extracts; D- Dichloromethane extracts
A)Vibrio cholerae B) Salmonella typhi C) Escherichia coli D) Staphylococcus aureus

significant antibacterial activity against the five pathogens – *E.coli, S. typhi, S. paratyphi, S. aureus and Vibrio cholerae*. The extracts of *Callyspongia* sp, *Clathria* sp., *Sigmadocia* sp and *Zygomycale* sp. possess different intensities of antibacterial activity against the tested bacteria [22].

The screening of chemical constituents revealed that both the extracts have similar constituents except the presence of steroids in methanol extract. The greater activity of the methanol extracts in the bioassays may be due to the greater concentration of the chemical constituents or due to the presence of polar steroids in it.

This study confirmed the medical potential of the two species. Though many reports were there on the antibacterial, antifungal, larvicidal and insecticidal activities of these two species, reports on antioxidant and anticholinesterase activities were scanty. This is the first report on the antioxidant and anticholinesterase activities of the two species from the Southwest coast of India.

4. CONCLUSION

The results indicate that both the extracts of the selected sponge species possess significant bioactivity. The species *Zygomycale parishii* exhibited greater potency in all the assays performed. This may be attributed to the chemicals present in it. Although both species possess similar chemical constituents, their concentration may vary with species to species. In addition to all the chemical constituents present in the extracts of Callyspongia, extracts of Zygomycale contained flavonoids which may have contributed greater potency to these extracts. Thus the bioactivities of both the species have been confirmed and it became clear that the species Zygomycale would yield highly potent bioactive compound on further bioassay guided fractionation and purification studies.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENT

The authors would like to thank DST-INSPIRE Fellowship (IF120633) scheme for constantly supporting the work and we would like to express our deep gratitude to the DBT-SERB instrumentation facility for providing us the technical support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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