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# Antifungal activity and investigation of bioactive compounds of marine intertidal bivalve *Gafrarium divaricatum* from West coast of India

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## ABSTRACT

*Crude methanolic extract of Gafrarium divaricatum and its five fractions viz. hexane, ethyl acetate, chloroform, acetone and water were screened against four fungal human pathogens to determine antifungal activity by using disc-diffusion assay method. Only crude methanolic extract showed consistent antimicrobial activity as compared to other fractions. Crude methanolic extract showed significant antifungal activity against Cryptococcus neoformans, Aspergillus flavus and Aspergillus niger with Minimum Inhibitory Concentration 0.1 mg/ml, 0.3 mg/ml and 0.3 mg/ml respectively. Determination of the possible chemical antifungal components from crude methanolic extract was carried out by GC-MS Technique. This analysis identified and characterized nine bioactive compounds. Four of these compounds that have known therapeutic properties include (i) Octadecane, 2,2,4,15,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl) for anticancer properties, (ii) l-(+)-Ascorbic acid 2,6-dihexadecanoate for antitumour and antibacterial properties and (iii) 2-Cyclopentene-1-tridecanoic acid for antibacterial activity (iv)17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol as drug for mineral disorders and antibacterial properties. In this study, G. divaricatum was found to be a potential source of medicinal compounds.The five other compounds obtained in the present study should be screened for their medicinal properties.*

***Key word:*** *Antifungal; Bivalve; GC-MS analysis; Minimum inhibitory Concentration; Mumbai coast.*

## INTRODUCTION

Marine invertebrates are known to possess sophisticated physiological and biochemical mechanisms, which produce unique chemical compounds, distinct from those found in terrestrial organisms. These unique chemical compounds or marine bioactive compounds show a wide range of antimicrobial, antiviral, cytotoxic, antitumour, anti-inflammatory properties and serve as a source for developing novel therapeutics against various other challenging human diseases[1].

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Since bioactive compounds are from natural sources and have been structured within living systems, they are more biological friendly than chemically synthetic molecules[2]. Marine bioactive compounds are much more effective against pathogens which start to show resistance against routinely used antibiotics[3]. These features make secondary metabolites of marine organism an ideal candidate for developing future drugs. In the year 2011, around 1152 new marine natural products have been isolated from marine organisms and 558 publications have been reported[4].

Intertidal ecosystem is amongst most harsh but productive ecosystems among all the ecosystems on the earth. To withstand seasonal, tidal and diurnal changes in the intertidal zone, its inhabitants produce a variety of secondary metabolites. Yet, studies on physiological active secondary metabolites from intertidal zone of Indian coast are limited to investigation carried out by Chatterji *et al.*[5], Annamalai *et al.*[6] and Chandran *et al.*[5,6,7]. Further, influx of sewage and microorganism from terrestrial sources too influence production of secondary metabolites in marine organisms. *Gafrarium divaricatum*, an edible clam species,is abundantly found along the Mumbai coast[8]. Its role in the intertidal ecosystem as a filter-feeder and economic value as an edible species make it a potential source of beneficial bioactive agents. We previously reported antibacterial activity of *G. divaricatum* from Mumbai coast[9]. Therefore, it is rational to investigate the antifungal potential and presence of bioactive compounds from *G. divaricatum* occurring in the intertidal area of Mumbai coast.

## MATERIALS AND METHODS

### Sampling and identification:

Samples of *G. divaricatum* were collected manually from rocky shore of Marine Drive during low tide. The samples were brought to the laboratory and immediately frozen at -20 °C. For extraction, the shells were cracked open. Whole body of the animal was collected and subsequently washed with autoclaved distilled water to remove any attached debris.

### Extract preparation:

The whole body tissue sample was chopped and soaked in 100% methanol (MeOH) for a minimum of 24 hrs to extract the most polar compounds. The supernatant was then filtered and concentrated under vacuum on a Rotary Evaporator at low temperature and reduced pressure to get crude methanolic extract. This procedure was repeated two more times to get the maximum yield. The crude MeOH extract was further fractioned by Hexane, ethyl acetate, chloroform, acetone and water (increasing polarity of solvent).

### Antifungal assay:

Antifungal activity of each extract was tested against four fungal strains *Aspergillus flavus*, *A. niger*, *Cryptococcus neoformans* and *Candida albicans.* Antifungal activity testing was carried out using standard agar disc-diffusion assays[10]. A spore suspension prepared at a concentration of $2x10^5$ CFU/ml in sterile distilled water was inoculated on the surface of a Sabouraud's dextrose agar (HiMedia, Mumbai). Inoculated suspension was uniformly spread on agar using a sterile glass spreader. Previously sterilized Whatman No. 1 filter paper discs (6 mm in diameter), impregnated with 0.5 mg/ml solution of each crude extract were placed on the surface of inoculated plate. Disc of serially diluted concentrations (0.05-0.5mg/ml) of crude extract were used to determine Minimum Inhibitory Concentration (MIC) against each fungal spore suspension. Disc of Fluconazole (0.1 mg/ml) and absolute methanol were used as positive control and negative control respectively in each plate. MIC of Fluconazole was also carried out with discs of its serially diluted concentration (0.001 - 0.1 mg/ml) against each fungal spore suspension. Plates were incubated for 72 hrs at 25°C in the dark. All the assays were carried out in triplicates. The bioactivity of the extracts was measured by calculating the diameter (mm) of the growth inhibition halos. Zones of growth inhibition greater than 7 mm were considered susceptible to crude extracts[11].

### Determination of compounds by Gas Chromatography-Mass Spectroscopy (GC-MS):

The GC-MS procedure was standardized from the procedure developed by Ramasamy and Balasubramanian (2012)[12]. GC-MS analysis of the extract was performed using GC SHIMADZU QP2010 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column (Length : 30.0 m, Diameter : 0.25 mm, Film thickness : 0.25 µm

Composed of 100% Dimethyl poly siloxane). For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.51ml/min and an injection volume of 2µl was employed (split ratio: 20). The column temperature was maintained initially at 400°C for 3.5 min followed by increase to 600°C at a rate of 50°C/min from 60° to 120°C at a rate of 60°C/min and from 120 to 2300°C. The electron impact energy was 70ev and the ion source temperature was set at 2300°C. Electron impact (EI) Mass spectra were recorded in the range of 10-600 m/z at 1s intervals. Total GC running time was 35 min. The identification of compounds was carried out using GC-MS library National Institute of Standards and Technology (NIST08s) by comparing the spectrum of unknown component with the spectrum of known components stored in the NIST08s. Synonym compounds were also obtained from NIST08s library.

## RESULTS AND DISCUSSION

### Antifungal assay:

Totally six samples including one crude extract and its five fractions were screened against four fungal human pathogens. During the assay, only crude methanolic extract showed consistent antifungal activity even though all other samples contained fractions of crude extract. Bioactive compounds in marine invertebrates are often present in minute concentrations, sometimes accounting for less than $10^{-6}$ % of the wet weight[13]. Also sometimes single compound is not responsible for bioactivity, but it's a synergism of several compounds[14]. Thus, only crude methanolic extract was selected for further analysis and identification of the active molecule present in the *G. divaricatum*.

The results of the *in vitro* antifungal assay of the methanolic extract are presented in Figure 1. Considerable activity was observed against fungal strain *A. niger* with an inhibition zone of 16 ± 0.7 mm. Comparatively less but distinct activity was also observed against *A. flavus* and *C. neoformans* with inhibition zones of 13±1.73 mm and 11±1 mm respectively. However, insignificant activity was observed against the fungal strain *C. albicans.* This specificity may be due to the chemical composition of the outer cellular layers and differences in stress responses of microorganism[15]. Positive controls Fluconazole are significantly effective against all the selected fungal strain. It showed highest inhibition zone of 19 mm against *A. niger* and lowest of 15 mm against *A. flavus*. Typically commercial antibiotics are in pure form and hence significantly effective against all the pathogens. Whereas crude extracts contain many impurities including non bioactive compounds that enter during extraction. Because of this, the MIC of crude bioactive extracts even required higher concentration as compared to the pure antibiotics. The MIC values obtained from crude methanolic extract and Fluconazole against fungal spore suspension are presented in Figure 2. Lowest MIC value of crude methanolic extract was obtained against *C. neoformans* (0.1 mg/ml) and highest (0.3 mg/ml) against *A. flavus* and *A. niger*. Moreover, lowest MIC value of Fluconazole obtained against *C. albicans* (0.035 mg/ml) and highest against *A. flavus* (0.075 mg/ml). MIC value of Fluconazole obtained against *A. niger* and *C. neoformans* was 0.07 mg/ml, and 0.05 mg/ml respectively.

### GC-MS analysis:

The chromatogram obtained by crude methanolic extract of *G. divaricatum* is presented in Figure 3. Ten peaks representing nine bioactive compounds were detected in Gas Chromatography. Total ionic chromatogram obtained from GC-MS library showing active compounds, peak area, Concentration (%) and Retention Time (RT) is presented in Table 1. 2-Cyclopentene-1-tridecanoic acid eluted at 6$^{th}$ and 8$^{th}$ peak was the most abundant compound (42.77%) present in the extract. It is commonly known as Chaulmoogric acid and is often found in plants and may have got introduced into the clam through its planktonic diet. Much of the bioactivity in marine invertebrates occur because of ingestion and metabolism of secondary chemicals derived from plants[16]. 2-Cyclopentene-1-tridecanoic acid is potent antibacterial compound against *Mycobacterium leprae* and *Bacillus tuberculosis*[17]. Compounds present in crude methanolic extract and their bioactive potential is presented in Table 2. Bioactivity of following five eluted compounds from crude methanolic extract of *G. divaricatum* is still unknown (1)1$^{st}$ peak (2,4-Bis [4-chloro-

trans-styryl]-6- [(3-pyrrolidinomethyl-4-hydroxyphenyl)amino] pyrimidine), (2) 3rd peak (7-Chloro-1,3,4,10-tetrahydro-10-hydroxy-1-[[3-[1-piperidinyl]propyl]imino]-3-[3,4,5(trimethoxy)phenyl]-9(2H)-acridinone), (3) 4th peak (4,7-Benzofurandione, 3-acetyl-3a,7a-dihydro-2-methyl-3a,5,6,7a-tetrakis[(trimethylsilyl) oxy], (4) 7th peak Cobalt(I), bromotris (trimethylphosphite)) and (5) 9th peak (2,2-Dimethylpropanoic acid, heptadecyl ester) Compound, Octadecane, 2,2,4,15,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl)- eluted with second peak. It was present in 5.67% of total crude methanolic extract and has not been reported earlier from any marine invertebrate. However, its synonym compound Benzenesulfonyl fluoride, 3-chloro-4-(4-(2-chloro-4-(4,6-diamino-2,2-dimethyl-1,3,5-triazin-1(2H)-yl) phenyl) butyl)-, monoethanesulfonate is a potent anticancer compound[18] and currently under clinical trials for developing anticancer drug[19]. Another synonym compound Stigmasterol trimethylsilyl ether to compound eluted in second peak is one of the prominent sterols in bivalve[20]. Compound eluted in fifth peak contains l-(+)-Ascorbic acid 2, 6-dihexadecanoate. It is a potent antitumor and antibacterial compound[21]. It inhibits enzyme hyaluronidases which is responsible for tumour growth in mammals. In the current study, 13% of crude methanolic extract was accounted by l-(+)-Ascorbic acid 2,6-dihexadecanoate. Presence of 3.59% concentration of its synonymous compound Octadecanoic acid reported in clam *Anadara granosa* from Muthupet estuary, South East coast of India[12]. High concentration of this l-(+)-Ascorbic acid 2,6-dihexadecanoate in *G. divaricatum* than *Anadara granosa* could be due to the variation in geographic location, food availability and climatic conditions of both organisms. Also interspecies variability in bioactivity has been documented in marine invertebrates[22].

Tenth eluted compound 17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta [a] phenanthren-3-ol found 6.23% in crude methanolic extract. It is commonly known as Cholest-5-en-3.beta.-ol or Provitamin D. It is the principal sterol in all higher animals, distributed in body tissues, especially in the brain and spinal cord. They are of potential therapeutic use in various mineral disorders[23]. Presence of synonymous compound Cholest-5-en-3beta-ol has been reported in bivalve *Mytilus edulis* (34%), *Atrina fragilis* (31.1%) and *Ostrea edulis* (33.2%) collected from Netherlands and France[24]. Comparatively Cholest-5-en-3.beta.-ol was found in very less quantity (mention %age) in *G. divaricatum.* This difference could be attributed to variation in ecological conditions at the study sites.

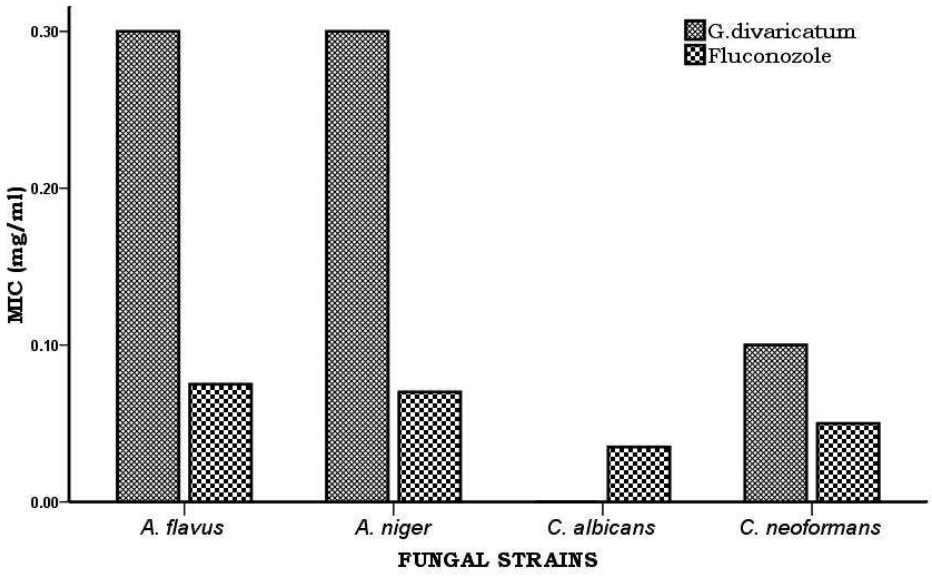


**Fig. 1**: Antifungal activity of the crude methanolic extract (0.5 mg/ml) compared to the positive control of Fluconazole (0.1 mg/ml) fungal spore suspension

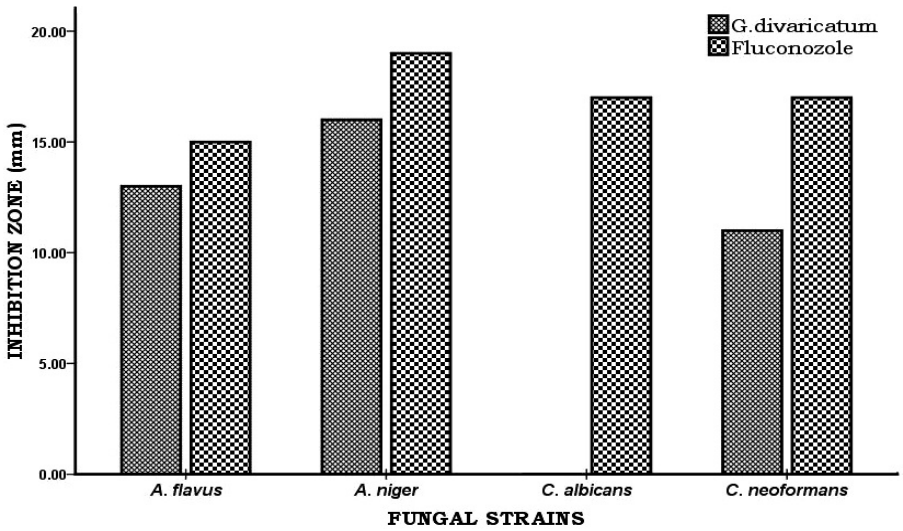


**Fig. 2**: Minimum inhibitory concentration (mg/ml) of the crude methanolic extract and positive controlled Fluconazole against fungal spore suspension

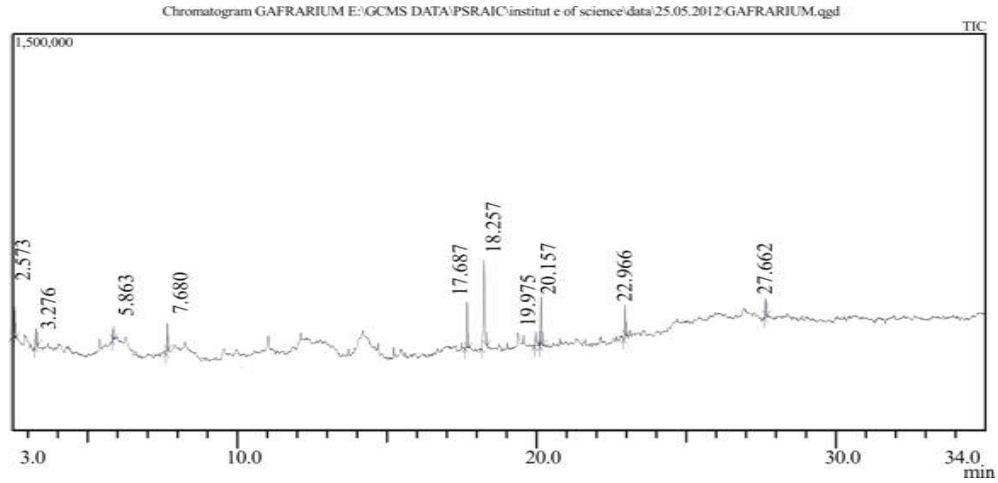


**Fig. 3**: Gas chromatography chromatogram of crude methanolic extract of *G. divaricatum*

**Table 1.** Total ionic chromatogram showing the compounds present in crude methanolic extracts obtained from GC-MS library

| Peak | Retention Time | Area | Area% | Height | IUPAC Name of compound |
|---|---|---|---|---|---|
| 1 | 2.573 | 59351 | 7.64 | 32812 | 2,4-Bis[4-chloro-trans-styryl]-6-[(3-pyrrolidinomethyl-4-hydroxyphenyl)amino] pyrimidine |
| 2 | 3.276 | 44058 | 5.67 | 19088 | Octadecane,2,2,4,15,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl)-$$ 2,2,4,15,17,17- Hexamethyl-7,12-di(3',5',5'-trimethylhexyl)octadecan |
| 3 | 5.863 | 21318 | 2.74 | 11327 | 7-Chloro-1,3,4,10-tetrahydro-10-hydroxy-1-[[3-[1-piperidinyl]propyl]imino]-3-[3,4,5(trimethoxy)phenyl]-9(2H)-acridinone $$ (1Z)-7-Chloro-1 |
| 4 | 7.68 | 60292 | 7.76 | 29591 | 4,7-Benzofurandione,3-acetyl-3a,7a-dihydro-2-methyl-3a,5,6,7a-tetrakis[(trimethylsilyl) oxy]- |
| 5 | 17.687 | 101034 | 13 | 48681 | l-(+)-Ascorbic acid 2,6-dihexadecanoate |
| 6 | 18.257 | 221303 | 28.47 | 87393 | 2-Cyclopentene-1-tridecanoic acid |
| 7 | 19.975 | 44396 | 5.71 | 12473 | Cobalt(I), bromotris(trimethylphosphite) |
| 8 | 20.157 | 111186 | 14.3 | 52218 | 2-Cyclopentene-1-tridecanoic acid |
| 9 | 22.966 | 65939 | 8.48 | 35203 | 2,2-Dimethylpropanoic acid, heptadecyl ester |
| 10 | 27.662 | 48396 | 6.23 | 19746 | 17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol |
| **Total** | | 777273 | 100 | 348532 | |

**Table 2.** Compounds present in crude methanolic extr act and their bioactive potential

| S. No. | IUPAC Name of compound | Bioactive Potential |
|---|---|---|
| 1 | 2,4-Bis[4-chloro-trans-styryl]-6-[(3-pyrrolidinomethyl-4-hydroxyphenyl)amino]pyrimidine | Not known |
| 2 | Octadecane, 2,2,4,15,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl)- $$ 2,2,4,15,17,17-Hexamethyl-7,12-di(3',5',5'-trimethylhexyl)octadecan | Anticancer[18,19] |
| 3 | 7-Chloro-1,3,4,10-tetrahydro-10-hydroxy-1-[[3-[1-piperidinyl]propyl]imino]-3-[3,4,5(trimethoxy)phenyl]-9(2H)-acridinone $$ (1Z)-7-Chloro-1 | Not known |
| 4 | 4,7-Benzofurandione, 3-acetyl-3a,7a-dihydro-2-methyl-3a,5,6,7a-tetrakis[(trimethylsilyl)oxy]- $$ 3-Acetyl-2-methyl-3a,5,6,7a-tetrakis[(trimeth | Not known |
| 5 | l-(+)-Ascorbic acid 2,6-dihexadecanoate | Antitumour and antibacterial activity[21] |
| 6 | 2-Cyclopentene-1-tridecanoic acid | Antibacterial[17] |
| 7 | Cobalt(I), bromotris(trimethylphosphite) | Not known |
| 8 | 2,2-Dimethylpropanoic acid, heptadecyl ester | Not known |
| 9 | 17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol | Drug for mineral disorders[23] |

## CONCLUSION

The current work testifies the bioactive potential of clam *G. divaricatum* and provides a baseline data for further research into the drug development against various human diseases. Bioactivity of some of the compounds found in this study is still not known and needs detailed investigation. *G. divaricatum* is traditionally used as a food source on the Mumbai coast by the local fisher communities which underline the importance of a thorough investigation into its activity against common pathogens.

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