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Antibacterial and Antifouling activity of the Marine Sponge *Callyspongia diffusa* collected from south-west coast of India

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Abstract

The aqueous and methanol extract of *Callyspongia diffusa* collected from south west coast of India were screened for the antibacterial and antifouling activity. The aqueous extract showed inhibitory activity against tested bacterial pathogens *E. cloacae, V. fluvialis, V. anguillarum* and *V. harveyi, S. aureus, Bacillus subtilis, E. coli, Klebsiella pneumoniae, P. vulgaris* and *Salmonella enterica typhi.* The methanol extract exhibited inhibitory activity against *V. anguillarum, E. coli, Arthobacter* sp and *K. pneumoniae.* The various concentrations of sponge extracts (10, 100 and 1000 µg/ml) were also analysed for their ability to inhibit the formation of byssus thread by mussels at different time phases. The methanol extract of *C. diffusa* was more effective in inhibiting the byssus thread formation. TLC and GCMS analysis revealed the presence of long chain fatty acids such as 9, 12-Octadecadienoic acid (Z, Z)-2, 3-di contributing significantly to bioactivities.

Key words: *Callyspongia diffusa*, Antibacterial, Biofouling, Antifouling, Marine sponge metabolites

Introduction

The potential of Marine Sponges in producing immense collection of bioactive compounds having antitumor, antiviral, anti-inflammatory, immunosuppressive, neurosuppressive, muscle relaxant, antimalarial, antibiotic and antifouling properties is well-appreciated (Perdicaris *et al*., 2013 and Thakur *et al*., 2004). Being a primitive multicellular filter feeding organism and because of their sedentary nature, they are extensively exposed to diverse microbes, organic matters and predators which induces

the production of defensive secondary metabolites (Proksch *et al*., 1994 and 2002). The antimicrobial activity of the sponge extracts was initially reported by Nigrelli and Stempien (1963). The recent trends have seen bioprospecting of marine sponges for diverse potential compounds. Owing to the diverse bioactive compounds discovered from the sponges (Sipkemia *et al*., 2005) they are considered as an important source for getting new drugs that have potential pharmacological applications (Perdiacaris *et al*., 2013). Marine sponges are considered as rich sources of natural products and metabolites such as halogenated alkaloids, purpuroines A-J which have strong inhibitory activity against bacteria and fungi (Shen *et al*., 2012). Several studies has been carried out emphasizing the significance of marine sponge and its secondary metabolites with inevitable application in drug discovery and disease control in aquaculture (Annie *et al*., 2008; Lipton *et al*., 2014 and Mollica *et al.,* 2012) and they are proved to be potential source of antibacterial compounds (Aishwarya *et al*., 2013). The emergence of antibiotic resistant bacteria necessitates discovery of novel antibiotic compounds which can replace conventional ones that are ineffective to many pathogens.

Biofouling poses severe problems to the shipping industry by increased fuel consumption, expensive hull maintenance and cleaning with consequential economic loss (Wahl, 1989 and Champi, 2000). Tributyltin (TBT) or copper plus organic booster biocides and Triphenyltin (TPT) are being widely used as antifoulants (Yehra *et al*., 2004) that are toxic to the marine eco system. Earlier studies have shown antifouling potential of marine sponges and their activity against biofouling bacterial assemblages (Abu Sayem, 2011; Thakur *et al*., 2004; Ware, 1984). In this context, the present study is carried out to evaluate the antibacterial and antifouling potential of the marine sponge *Callyspongia diffusa* collected from South-west Coast of India.

Materials and methods

Collection of marine sponge

The specimens of the marine sponge *Callyspongia diffusa* were collected from Vizhinjam (8º22’45ºN: 76º59’29ºE), South-west coast of India at depths ranging from 6 to7 m off shore at a distance of about 1.5km and its spicule morphology was identified (Hooper, 2002).

Preparation of aqueous and methanol extract

For the extraction of secondary metabolites, aqueous and methanol extracts were prepared. The sponge sample was soaked in sterile PBS (pH 7) and kept for 2 h. After soaking and gentle shaking, the aqueous extract was squeezed out from the sponge. The aqueous extract was then filtered through Whatmann No. 1 filter paper under sterile conditions in a sterilized container and stored at 4°C for further experiments. The sponge was ascertained and cut into small pieces, soaked in methanol and kept under dark condition followed by gentle shaking for 3 to 5 days. The methanol extract was then obtained after filtering through Whatmann No.1 filter paper, fitted in a Buchner funnel using suction. Solvents were removed by rotary vacuum evaporator

under reduced pressure so as to get the crude extract. The concentrated crude extract was collected in airtight plastic containers and stored in refrigerated condition.

Antifouling activity of aqueous and methanol extract of *C. diffusa*

Effect of aqueous and methanol extract of *Callyspongia diffusa* on the inhibition of byssus thread formation in brown mussel (*Perna perna*) collected from Vizhinjam coast was studied by mussel foot repulsive assay. The mussels were kept in recirculating laboratory aquarium in constant temperature (20°C), salinity (35%) for about 12 h. The healthy mussels with 3.5 ±0.2 cm shell length were segregated and their byssus threads were removed before bioassay by using sharp scalpel. The aqueous, methanol extract of *C. diffusa* (1ml) with different concentrations (10, 100 and 1000µg/ml) were spread on sterile beakers that formed a thin uniform layer on the bottom surface. The beakers were kept at 40º C for drying. The dried beakers were allowed to cool at room temperature. The beakers were filled with filtered sterile sea water (sieve pore size 0.5µm) to half its volume. A positive control beaker with sea water alone was maintained. The mussels were carefully introduced into each beaker containing the extracts and controls. The foot repulsive action of mussel and formation of byssus thread when exposed to the aqueous, methanol extracts of *C. diffusa* monitored continuously for 24h and results were tabulated. The observations were made in IV phases, 0-6h (I-phase), 6-12h (Phase-II), 12-18h (phase-III) and 18-24h (phase 1V). Three replicates of each experiment were used.

Antibacterial activity of aqueous and methanol extract of *C. diffusa*

The antibacterial activity of aqueous and methanol extracts of *C. diffusa* was determined by well-diffusion assay (Perez *et al*., 1990). The 6 mm wells were loaded with the 30µl of sponge extracts in the Muller-Hinton agar plates seeded with 24h broth culture of the tested microorganisms of both fish and human pathogenic bacteria. The sponge extracts were tested for their antimicrobial activity against fish pathogens *viz*., *Vibrio harveyi*, *Vibrio vulnificus*, *Vibrio fluvialis*, *Vibrio pelagius*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *Enterobacter cloacae* and MTCC cultures *viz; Klebsiella pneumoniae* (MTCC 3384*), Bacillus subtilis (*MTCC 3121), *Staphylococcus aureus*, *Salmonella enterica typhi* (MTCC 98), *Proteus vulgaris* (MTCC 426), *Arthrobacter* sp., *Bacillus pumilus* and *Escherichia coli* (MTCC 40*)*. The presence or absence of inhibitory activity against the indicator organism was determined after incubating the agar plates for 24h at 37ºC and measuring the zone of inhibition (mm).

Identification of Compounds by Gas Chromatography Mass Spectroscopy (GCMS) analysis

The active crude methanol extract of *C. diffusa* and chloroform extract of *S. algae* VCDB was analysed by TLC using various solvent system and was chromatographed on GCMS system (Varian CP_{3800} GC at Centre for Scientific and Applied Research, C-SAR, Tirunelveli) equipped with a front ECD and rear PFPD detector to analyse the active compounds. The capillary column length was 30m and 0.25 mm diameter. The Column oven was programmed with an initial temperature of 70 ºC (hold for 2 min) and final temperature of 270ºC. One micro litre sample was injected and

maintained a constant flow rate of 0.1 ml/min. The inlet and interface temperature were kept at 2800º C. The scanning was done using EI-auto scanner of 25000 micro sounds. The metabolites were compared with the database from NIST and Wiley library standards. The compound identification was based on criteria with absolute Ion ratio type, Qualifier Integration using Quon ion points and Rate retention time percentage (RRT%) tolerance of ± 0.01%.

Results

Antibacterial activity of aqueous and methanol extract of *C. diffusa*

The specific sponge species selected for the study from Vizhinjam coast is identified as *Callyspongia diffusa* (Ridley 1884) based on the microscopic identification of long slender spicules present in them (Rachana *et al*., 2014). The aqueous extract of *C. diffusa* showed broad spectrum of activity against most of the fish pathogens *E. cloacae* with 17 mm zone of inhibition and *V. fluvialis* with 15 mm inhibition zone and *V. anguillarum* with 15 mm zone of inhibition and 11mm for *V. harveyi*. The bioactivity was recorded against *S. aureus* (16mm), *Bacillus subtilis* with 14mm and *E. coli, Klebsiella pneumoniae, P. vulgaris* with 10mm zone of inhibition, *Salmonella enterica typhi* with 14mm zone of inhibition. The condensed methanol extract of *C. diffusa* was observed to possess moderate activity against fish pathogens like *Vibrio fluvialis* (8mm), *Vibrio harveyi* (7mm) and *Vibrio vulnificus* with 8mm zone of inhibition and highest antibacterial activity was observed against *Vibrio anguillarum* with 12 mm zone of inhibition. The methanol extract showed 8 to 10 mm zone against *E. coli*, *Arthobacter* sp and *K. pneumoniae* (Fig.1).

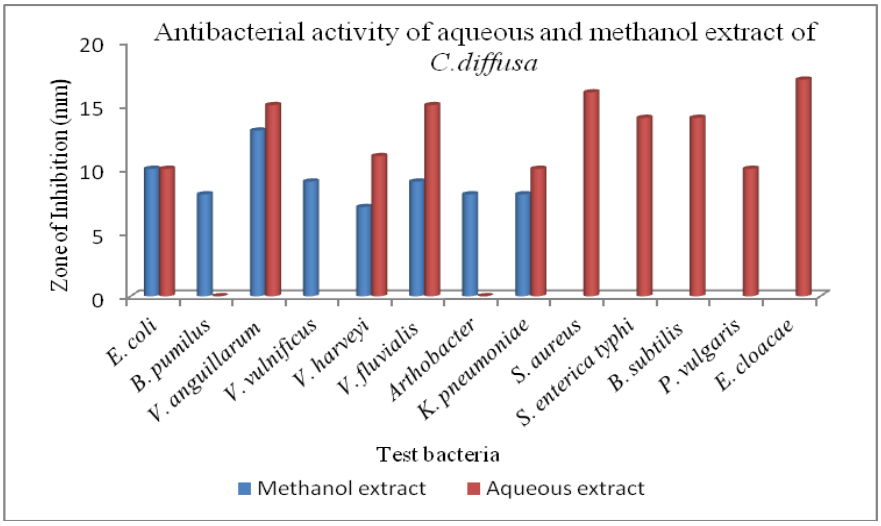
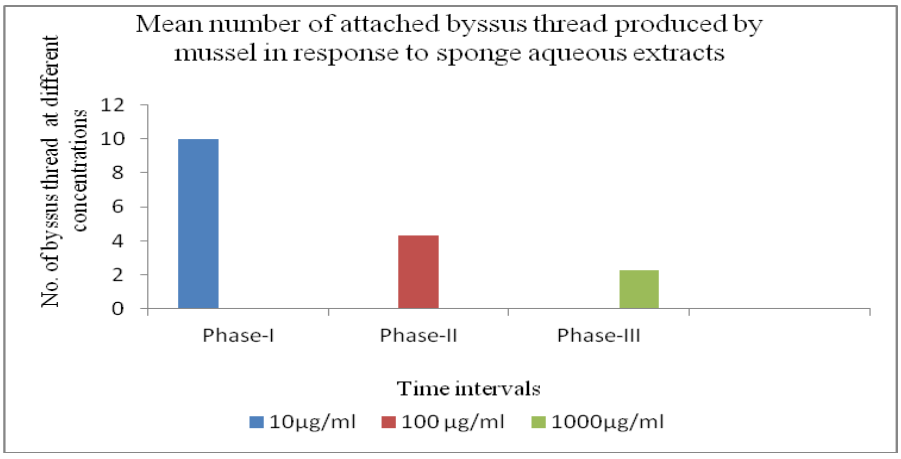
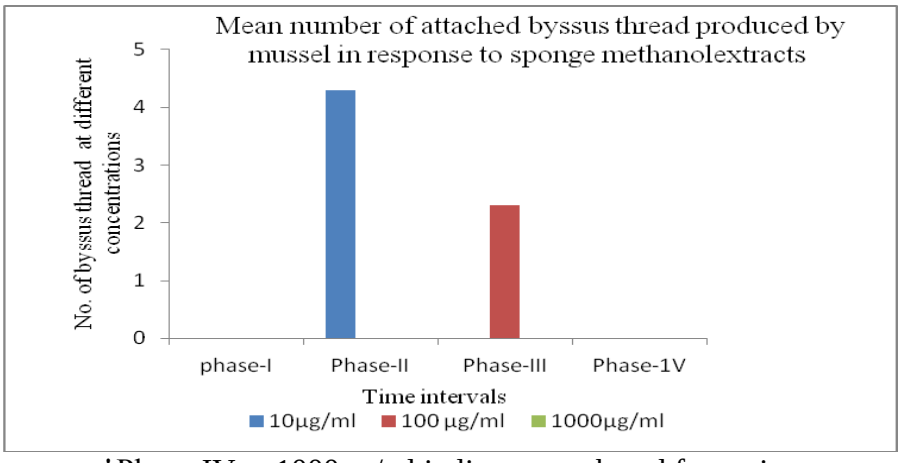


Fig. 1. Antibacterial activity of aqueous and methanol extract of *C. diffusa*

Antifouling activity of aqueous and methanol extract of *C. diffusa*

The sponge aqueous and organic extracts were tested to determine its efficacy in inhibiting the formation of byssus thread that helps the mussels to adhere firmly on the surfaces. In case of aqueous extract of *C. diffusa* the mussel always exhibited a tendency to expose its foot to the outer surface in search of a substratum for attachment. At 10µg/ml concentration ten to twelve byssus thread were formed within 0 to 6h (phase-I) and at 100µg/ml indicated phase-II, as the mussels produced three to six byssus threads within 6 to 12h. The higher concentration of aqueous extract (1000µg/ml) also favoured two to three formations of byssus thread at 12 to 18h (phase-III). At the lower concentration of 10µg/ml of methanol extract of *C. diffusa* the formation of three to four byssus thread was noted between 6 to 12h (phase-II). At 100µg/ml of methanol extract phase-III was observed, where two to three byssus threads were formed at 12 to 18h and at 1000µg/ml the formation of byssus thread was inhibited. The mussels in the control beakers produced byssus thread within few minutes and 14 to 16 byssus threads were observed (Fig. 2a&b).





†Phase-IV at 1000µg/ml indicates no thread formation

Fig.2a&b. Effect of aqueous and methanol exatrct of *C. diffusa* on byssus thread formation by *Perna perna*

The TLC analysis of methanol extract of *C. diffusa* showed four spots with RF values 0.3, 0.4, 0.8 and 0.9 in the solvent system containing Chloroform**:** methanol**:** water in the ratio 6.5**:**2.5**:**1. Gas chromatography and mass spectrometer data also confirmed the presence of long chain fatty acids 9, 12-Octadecadienoic acid (Z, Z)-2, 3-di (Fig. 3).

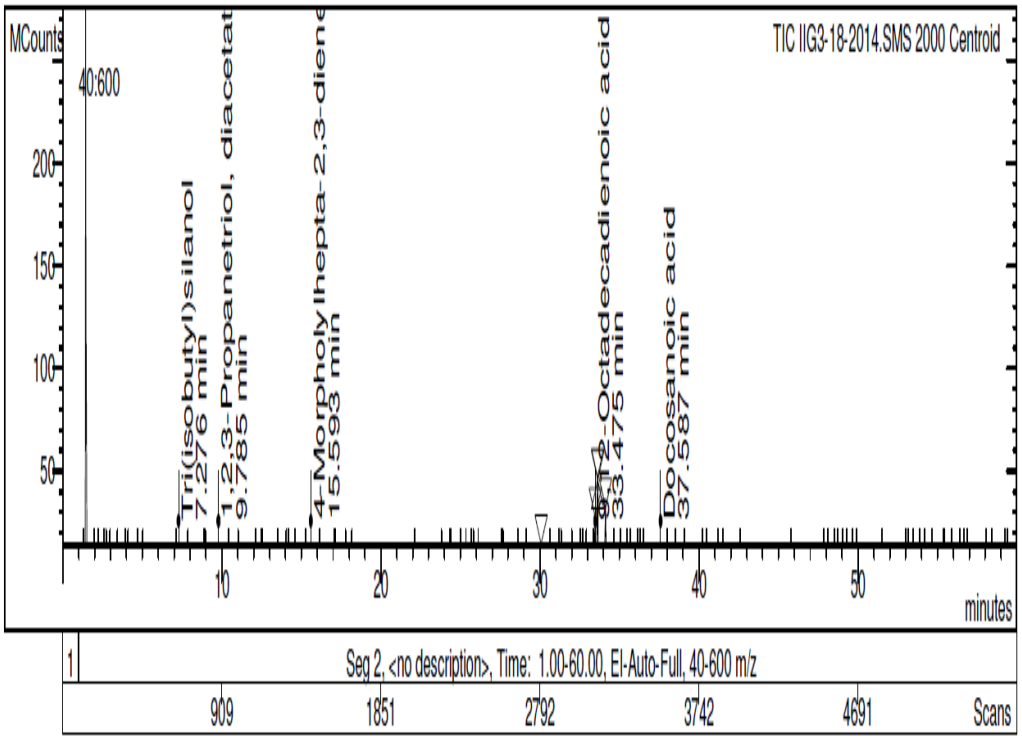


Fig. 3. GCMS spectrum of methanol extract of *C. diffusa*

Discussion

Screening of antibacterial activity of sponge extracts showed higher inhibitory activity against most of the tested pathogens. The aqueous extract of *C. diffusa* exhibited higher antibacterial activity than the methanol extract which could be attributed to the hydrophilic nature of the bioactive molecules (protein and sugars) as reported by Graca *et al*., (2015). In their studies, the aqueous extract of *Erylus deficiens* showed higher activity than organic extracts. The potential of sponge extracts in inhibiting the activity of fish pathogens evokes great interests as few human diseases are related to diseases of marine fish. Various studies highlight the efficiency of sponge extracts as an antibacterial agent. The inhibitory activity of methanol extract of *D. nigra* against shrimp pathogens like *V. harveyi* (Selvin and Lipton, 2004) and the antibacterial activity of *Axinella donnani*, *Acanthella elongata*, *Echinodictyum gorgonoides*, *Callyspongia subarmigera* and *Callyspongia diffusa* against marine fish pathogens

like *V. vulnificus, V. pelagius, V. fluvialis, V. anguillarum, V. fischeri, V. alginolyticus, P. aeruginosa* and *A. hydrophila* (Annie *et al*., 2008) emphasise the potentiality of marine sponge in aquaculture. Hutagalung *et al*., (2014) reported that the methanol and aqueous extract of *Stylotella aurantium* collected from Indonesia were able to inhibit the activity of both Gram positive and Gram negative bacteria such as *B. cereus, E. coli, S. aureus* and *S. typhii*. The extracts from *Callyspongia diffusa* were equally effective against the growth of Gram positive bacteria *S. aureus* and *B. subtilis* and Gram negative bacteria *E. coli*, *Salmonella enterica typhii* and *Vibrio* sp. Methanol extract of sponge Demosponge was reported to have a significant inhibitory activity on mussel adhesion in which the sponge extract effectively inhibited the byssus thread formation in green mussels (*Perna viridis*) (Prabhu *et al*., 2014). As cited earlier the marine sponge *D. nigra* was also has an effective antifouling activity against mollusc by inhibiting the fouler from attaching the extract coated surface as reported by Selvin and Lipton (2002). The extracts of marine sponge like *Acanthella elongata* (Ganapriya, *et al*., 2012), *Haliclona exigua*, (Limna Mol, 2009) inhibited the settlement of cosmopolitan larvae of *Balanus Amphitrite.* (Blihoghe, *et al*., 2011) evaluates the antifouling potential of structurally different compounds containing a 3-alkylpyridine moiety from the marine sponges *Haliclona* sp and *Reniera sarai* and the mollusc *Haminoea fusari* against larvae of the barnacle *Amphibalanus amphitrite*. Shaaban *et al*., (2012) studied inhibitory effects of marine sponges *Smenospongia*, *Callyspongia*, *Niphates* and *Stylissa* against carbohydrate metabolizing enzyme and they recognized the existence of di-isobutyl phthalate, di-n-butyl phthalate, linoleic acid, ß-sitosterol, cholesterol, bis-[2-ethyl]-hexyl-phthylester and triglyceride fatty acid ester and Linoleic acid; (9Z,12Z)-9, 12-octadecanoic acid by GCMS. The GCMS analysis of methanol extract of *C. diffusa* showed the presence of fatty acids and this may contribute to the various bioactivities displayed by marine sponge with related to the observation made by Bazes *et al.*, (2009), Carballeira *et al*., (2004), Keffer *et al*., (2009). Kupper (2006) suggested the possible role of free fatty acids as hydrolase in converting lipids excreted by the eukaryotic host to free fatty acids with antibacterial properties. This in turn prevents the colonization or growth of certain bacteria and produce impact on the community composition of the host microbiota. They also reported the presence of 9, 12-octadecanoic acid with RF value 0.90. The GCMS data of sea weed *L. brandeii* showed the presence of fatty acid, 9, 12-Octadecadienoic acid (Z, Z) as the potent antifouling bioactive compounds (Manilal *et al*., 2011). The presence of different metabolites of fatty acids with different function results in sponge bioactivity (Lee and Qian, 2003). The sponges, algae and bryozoans produce secondary metabolites like terpenoid, isocyanocompounds, 3-alylpyridinium compounds, brominated furanoes and brominated indole alkaloids that controls colonization of fouling organisms (Fusetani *et al*., 2011). Mora-Cristancho, *et al*., (2011) reported the importance of extracts of marine sponge *Topsentia ophiraphidites* as a significant compound in the chemical composition of antifouling paint. The GCMS analysis revealed the presence of fatty acids like 9, 12-Octadecadienoic acid Z, Z in the methanol extract of *C. diffusa* which can definitely have more application and can be further developed for antibacterial and antifouling preparations.

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