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Natural Product Antifoulants from the Octocorals of Indian waters

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Abstract

Natural Product Antifoulants (NPAs) have been proposed as one of the best alternatives for the globally banned toxic biocide -TBT- in antifouling coatings. In search of NPAs from Indian waters twenty nine species of Octocorals, collected from Gulf of Mannar and Lakshadweep islands, were screened for their antifouling potential against the cyprids of the cosmopolitan biofouler, *Balanus amphitrite.* The crude extracts of 8 species of these corals (*Cladiella krempfi*, *Lobophytum irregulare*, *L. sarcophytoides*, *Sarcophyton ehrenbergi*, *S. glaucum*, *Sinularia kavarattiensis*, *Melitodes* sp. and *Subergorgia reticulata*) exhibited relatively high settlement inhibition properties. The bioassay-guided purification of the crude extracts of 3 active and abundant species - *Cladiella krempfi*, *Sinularia kavarattiensis* and *Subergorgia reticulata* - yielded five NPAs, (l'E,5'E)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid **1,** (-)-6-α-hydroxy polyanthellin A **2,** (+)-(7R,10S)-2-methoxy calamenene **3**, (+)-(7R,10S)-2,5-dimethoxy calamenene **4** and (+)-(7R,10S)-2-methoxy,5-acetoxy calamenene **5**). Among these, **5** exhibited high future prospects on account of its low EC_{50} value (0.0335µg/ml) and high therapeutic ratio (799).

Keywords: Biofouling; Natural Product Antifoulants; octocorals; *Cladiella krempfi*; *Sinularia kavarattiensis*; *Subergorgia reticulata*

1. Introduction

Octocorals, like sponges, largely depend on their inherent chemical defense against predation and overgrowth (Raveendran and Limna Mol, 2009). This has evinced keen interest in chemical investigations on this group, resulting in the identification of several novel bioactive metabolites with potential biomedical applications. For example, eleutherobin, isolated from soft coral *Eleutherobia* sp., exhibited anticancer properties (Ata *et al*., 2004); the diterpene,13-*Epi*-9-deacetoxy-xenicinin, isolated from the soft coral *Asterospicularia laurae* was found to be cytotoxic (Bowden *et al*., 2003); the diterpene cembranolide, isolated from the soft-corals *Lobophytum crassum* and *L. rotundum*, exhibited antimalarial properties (Said, 2005); pseudopterosins from the gorgonian and *Psdeudopterogorgia elisabethae* exhibited potent anti-inflammatory activity (Correa *et al*., 2009). Similarly, varied structural types of compounds possessing strong antibacterial, anti-inflammatory, antioxidant, antitumour and cytotoxic activitry have been isolated from the genus *Sinularia* (Blunt *et al*., 2006; Bhosale *et al*., 2002; Faulkner, 2002). Several species belonging to the genus *Cladiella* have already been reported to have wide ranging biological activities such as antibacterial, acetylcholinesterase-inhibition, antioxidant, brine shrimp lethality and antitumour activity (Yamada *et al.,* 1997; Lan *et al.,* 2003; Ata *et al.,* 2004; Zhang *et al.,* 2005; Huang *et al.,* 2006). Also, the genus *Subergorgia* has been

associated with promising bioactivities such as cytotoxicity, antifeeding activity etc. (Bokesch *et al.,* 1996; Wang *et al.,* 2002; Qi *et al.,* 2005; Rezanka *et al.,* 2008).

Octocorals are fairly well represented in Indian waters, and are abundant in the Gulf of Mannar, Gulf of Kutch, Lakshadweep and the Andaman & Nicobar Islands. The studies on bioactive properties of octocorals from Indian waters got impetus with the Indo-U.S. project in 1984 (Thompson *et al*., 1991). Yet, the studies on antifouling properties of octocorals from India are rather limited and those carried out are only up to the crude extract level (Devi *et al*., 1998; Wisanand *et al*., 1999; Bhosale *et al*., 2002). An exception is the juncellin isolated from *Juncella juncea* having settlement inhibition against the barnacle, *B. amphitrite*. Considering the wealth of octocorals available in Indian waters, there is much scope for further studies in this avenue. This paper deals with the screening of 29 species of octocorals for their antifouling potential.

2. Materials and Methods

2.1. Collection and *Extraction of Octocorals*

Octocorals were collected from the Gulf of Mannar, Southeast coast of India (Lat. 9°5' N; Long. 79°5' E) and Lakshadweep islands (Lat. 8°–10° 13' N ; Long. 71°–74°E) by skin/SCUBA diving. After washing thoroughly with freshwater, the samples were cut into pieces and soaked in methanol/ acetone for three to four days. The extracts, so obtained, were concentrated using a rotary vacuum evaporator and subjected to antifouling assay against cyprids of the barnacle, *Balanus amphitrite* (=*Amphibalanus amphitrite*, Clare and Hoeg, 2008; Carlton and Newman, 2009). The samples were simultaneously identified and voucher specimens were deposited at the National Institute of Oceanography, Kochi.

2.2. *Purification of extracts with potential activity*

The crude extracts exhibiting high activity (Table 1) as well as relative abundance at the collection site (*Sinularia kavarattiensis* Alderslade & Prita, *Cladiella krempfi* Hickson and *Subergorgia reticulata* Ellis & Solander) were subjected to bioassay-guided fractionation and purification. The active extracts were first partitioned based on polarity into petroleum ether, ethyl acetate and aqueous fractions. The active fractions among these were subjected to column chromatography using sephadex LH-20 and/or silica gel and the fractions so obtained were tested for antifouling activity. The extent of purity of the active fractions was determined based on their TLC profile. Thereafter, these fractions were subjectd to spectroscopic analysis using NMR, IR and HRMS.

2.3. Larval Settlement Inhibition Assay

The adult barnacles were collected from their natural habitat at Vypeen beach, Kochi and maintained in the laboratory under standard conditions for obtaining the nauplii (Rittschof *et al*., 1984; Hellio *et al*., 2004). The nauplii were reared in the laboratory at 28±1°C through the six different instars on a regular diet of *C. calcitrans* on a 12:12 hour Light: Dark cycle (Anil *et al*., 2001; Limna Mol *et al*, 2009). Cyprids were collected on the sixth day and stored at 5 °C for use in the settlement inhibition assay.

The experiments were conducted in sterile 24-well polystyrene multiwell plates, Axigen (Rittschof *et al*., 1992, Marechal *et al*., 2004, Limna Mol *et al*., 2009). The crude extract was

dissolved in methanol and added to autoclaved 0.45 µ filtered sea water (FSW) to obtain a concentration of 100µg/ml. 10 competent 3-day old cyprids were added to each of the 3 replicates of 2ml of the test solution. $CuSO_4$ solution (100µg/ml) was used as positive control and wells containing only FSW with MeOH served as negative control. The plates were incubated for a 24 hour period under similar conditions at which the nauplii were reared. After the incubation period, the numbers of settled and metamorphosed cyprids were counted under a stereomicroscope and expressed as a proportion of the total number of larvae in the well. The experiment was repeated twice with different batches of larvae. The Effective Concentration (EC_{50}) and Lethal Concentration (LC_{50}) for the isolated metabolites were determined by serial dilution method.

2.4. Statistical Analysis

EC_{50} and LC_{50} were determined by Finney's Probit analysis (Finney, 1971). The cyprid settlement inhibition data was subjected to statistical analysis using Two Factor ANOVA for comparison between batches of cyprids and between the sponge species (Conover and Iman, 1981).

3. Results and Discusion

3.1. Larval Settlement Inhibition activity

Present study highlights the importance of octocorals in antifouling research. The observation that all the 29 species of octocorals exhibited some degree of larval settlement inhibition activity against cyprids of *B. amphitrite* is very encouraging. The different extracts exhibited significant difference in their activity towards barnacle cyprids ($F_{1,29}$=267.2, p<0.0001, 2-factor ANOVA). There was no considerable difference among the two batches of larvae tested (p<0.1). Of the 29 extracts screened, eight extracts (*Cladiella krempfi*, *Lobophytum irregulare*, *L. sarcophytoides*, *Sarcophyton ehrenbergi*, *S. glaucum*, *Sinularia kavarattiensis*, *Melitodes* sp. and *Subergorgia reticulata*) exhibited high activity against the settlement of barnacle cyprids (Table 1). This is further complimented by the fact that NPAs like pukalide and renillafoulin A with potential activity against *B. amphitrite* (EC_{50} 0.2µg/ml and of 0.05µg/ml, respectively) have been successfully isolated from softcorals like *Leptogorgia virgulata* and *Renilla reniformis* (Fusetani, 2004).

3.2. Isolation of active metabolites

The bioassay guided fractionation and purification of *Sinularia kavarattiensis* yielded (l'E,5'E)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid **1**(Limna Mol *et al*., 2010a); *Cladiella krempfi* yielded (-)-6-α-hydroxy polyanthellin A **2** (personal communication) and *Subergorgia reticulata* yielded three calamenene derivatives, (+)-(7R,10S)-2-methoxy calamenene **3**, (+)-(7R,10S)-2,5-dimethoxy calamenene **4** and (+)-(7R,10S)-2-methoxy,5-acetoxy calamenene **5** (Fig.1; Limna Mol *et al*., 2010b). The EC_{50} of the above metabolites ranged from 11.21 to 0.0335µg/ml (Fig.2).

3.3. Potential Natural Product Antifoulants

Of the five NPAs isolated during the present investigation, the sesquiterpene (l'E,5'E)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid **1** is isolated from *Sinularia kavarattiensis* for the first time. Incidentally, this also is the first report on the antifouling properties of **1**

against barnacle cyprids. The low EC_{50} value (11.21µg/ml) and low toxicity (therapeutic ratio of 5.16) coupled with the known synthetic route of **1** enhances its potential as a promising environmentally compatible NPA (Limna Mol *et al*., 2010a). Similarly, this is the first attempt to study the antifouling potential of cladiellin diterpenes from Indian waters. Mean EC_{50} and LC_{50} values of 9 and 36µg/ml, respectively, and a therapeutic ratio of 4 together with an LC_{50} value much greater than that of the positive control, $CuSO_4$ solution (1.9µg/ml), proves the environmental compatibility of (-)-6α-hydroxy polyanthellin A as a potential Natural Product Antifoulant. Also, this is the first report on the antifouling potential of calamenenes isolated from *Subergorgia reticulata*. Among the three calamenenes isolated during this study, (+)-(7R,10S)-2-methoxy, 5-acetoxy calamenene from *S. reticulata* is the most promising NPA. The EC_{50} value of this compound (0.0335µg/ml) is comparable to that of successfully commercialised NPAs like furanones from the red sea weed *Delisea pulchra* (0.02µg/ml; de Nys *et al*., 1995; Fusetani, 2004). Moreover, it has a therapeutic ratio (799) well beyond the recommended target ratio i.e. >1, for incorporation of NPA's in environmentally compatible antifouling coatings (Rittschof *et al*., 2003). The therapeutic ratio (LC_{50}/EC_{50}) determines the extent of environmental compatibility of an NPA. That is, compounds with high therapeutic ratio are expected to be effective at concentrations much below the lethal dose. Therefore, NPAs with high therapeutic ratio are usually selected as candidate compounds for commercial exploitation (Qian *et al*., 2010).

One of the main setbacks in NPAs research is ensuring supply commensurate with the needs of the antifouling paint industry (Raveendran and Limna Mol, 2009). The fact that synthetic routes for the sesquiterpene Furoic acid, cladiellin diterpenes and calamenene derivatives isolated during the present studies are already known provides optimism towards the possibility for synthesis of their simpler analogues. Further formulation of antifouling strategies involving a consortium of such NPAs would be highly effective in combating the problem of biofouling.

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Figure Legends:

Fig.1. Active metabolites isolated from octocorals of Indian waters: **1-** (l'E,5'E)-2-(2',6'dimethylocta-l',5',7'-trienyl)-4-furoic acid; **2-** (-)-6-α-hydroxy polyanthellin A; **3-** (+)-(7R,10S)-2-methoxy calamenene; **4-** (+)-(7R,10S)-2,5-dimethoxy calamenene; **5-** (+)-(7R,10S)-2-methoxy,5-acetoxy calamenene .

Fig.2. The effective concentrations (EC) and lethal concentrations (LC) required to inhibit/kill 50% of the tested population of *B. amphitrite* cyprids and the therapeutic ratio (TR) of the five NPAs.

Table 1. Antifouling activity of crude extracts from Octocorals against cyprids of *Balanus amphitrite* at 100 µg/ml.

Octocoral species	**Activity in test solution**
Soft corals	
Chironephthya sp.	*
Cladiella krempfi Hickson	***
Clavularia sp.	*
Dendronephthya sp. (1)	*
Dendronephthya sp. (2)	*
Juncella juncea Pallas	**
Lithophyton sp.	**
Lobophytum irregulare Tixier-Durivault	***
Lobophytum sarcophytoides Moser	***
Lobophytum sp.	**
Sarcophyton ehrenbergi Von Marenzeller	***
Sarcophyton glaucum Quoy & Gaimard	***
Sarcophyton latum Dana	*
Sarcophyton solidum Tixier-Durivault	**
Sarcophyton sp.	**
Scleronephthya sp.	*
Sinularia cruciata Tixier-Durivault	*
Sinularia inelegans Tixier-Durivault	*
Sinularia kavarattiensis Alderslade & Prita	***
Sinularia parulekari Alderslade & Shirwaiker	**
Sinularia sp.(1)	*
Sinularia sp.(2)	*
Gorgonians	
Acanthogorgia ceylonensis Thomson & Henderson	*
Acanthogorgia procera Moroff	*
Acanthogorgia turgida Nutting	*
Melitodes sp.	***
Siphonogorgia sp.	**
Subergorgia reticulata Ellis & Solander	***
Subergorgia suberosa Pallas	**

-- = No activity; * = Low activity (1- 30% inhibition); ** = Moderate activity (31-80% inhibition); *** = High activity (81-100% inhibition)

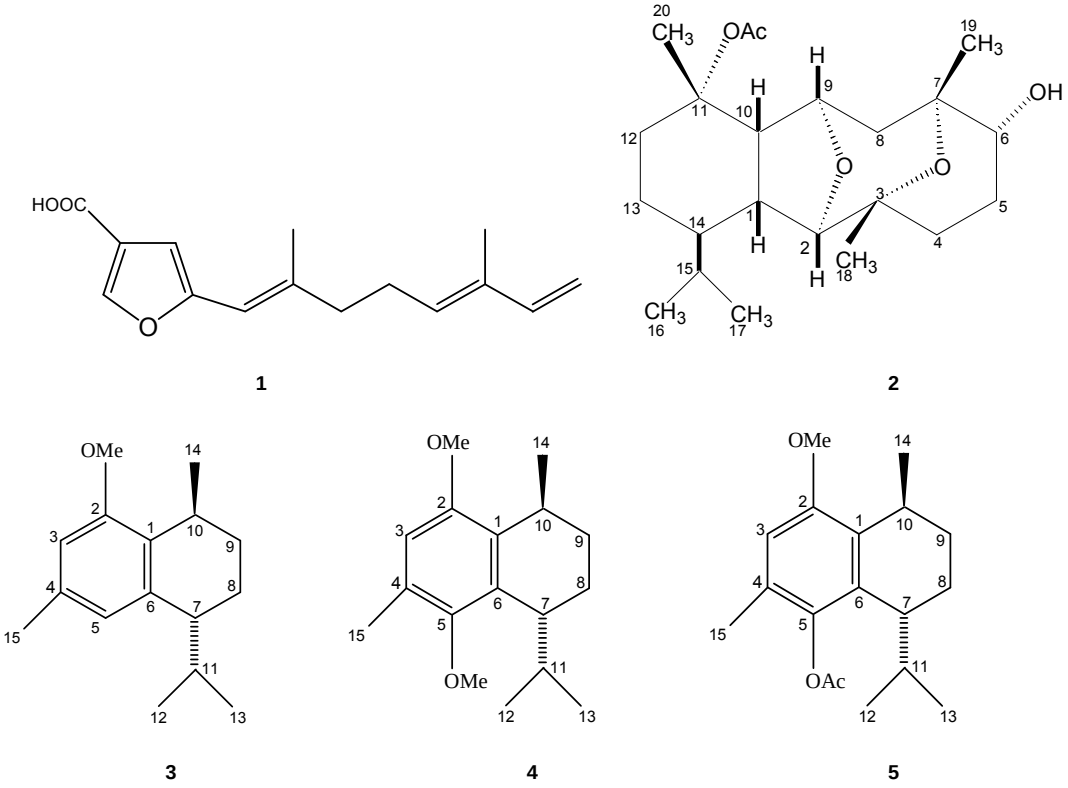

Fig.1. Active metabolites isolated from octocorals of Indian waters**:**

1 - (l'E,5'E)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid

2 - (-)-6-α-hydroxy polyanthellin A

3 - (+)-(7R,10S)-2-methoxy calamenene

4 - (+)-(7R,10S)-2,5-dimethoxy calamenene

5 - (+)-(7R,10S)-2-methoxy,5-acetoxy calamenene

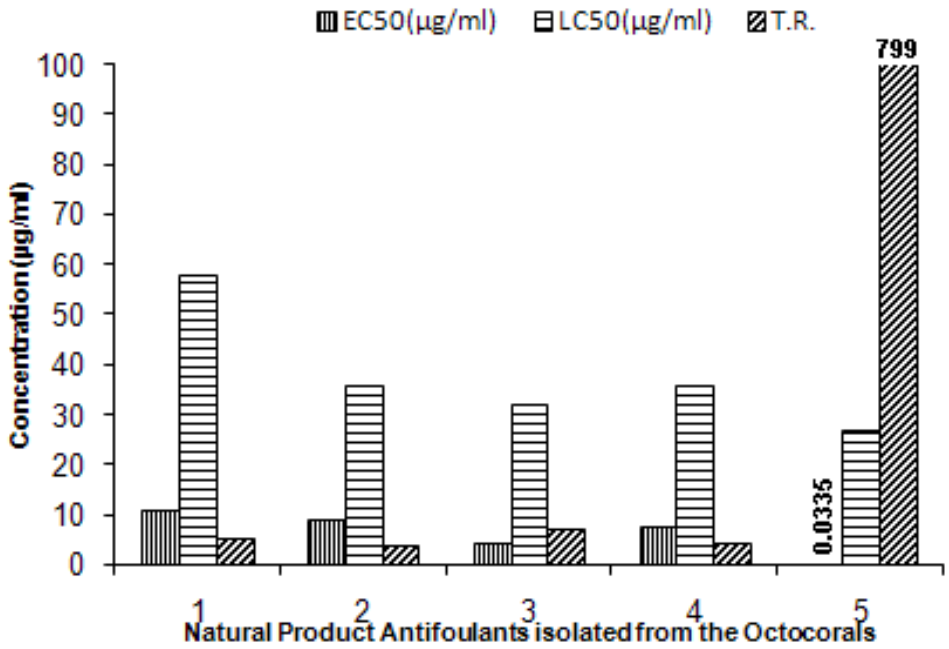

Fig.2. The effective concentrations (EC) and lethal concentrations (LC) required to inhibit/kill 50% of the tested population of *Balanus amphitrite* cyprids and the therapeutic ratio (TR) of the five NPAs