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Inhibitory activity of Terpenoid from the Medicinal plant *Andrographis paniculata* against Biofouling bacteria

Sarala V[1], M. Radhakrishnan[1] and R. Balagurunathan[2]*

[1]Department of Microbiology, Sri Sankara Arts & Science College, Kanchipuram – 631 561, Tamil Nadu,India.

[2]Department of Microbiology, Periyar University, Salem -11,Tamil Nadu,India.

*Corres. author: rbalaguru@yahoo.com
Mobile: +91 94434 46325

Abstract: Biofouling bacteria were isolated from fouling sample collected from boat and other marine structures around Parangipettai coastal area (lat.11° 29'N; long.79° 46'E) and its population was estimated as 8.15×10^6 CFU/gram. Based on the result of adherence study, five different biofouling bacterial isolates were selected, characterized and identified as *Pseudomonas sp., Alteromonas sp., Bacillus* sp., *Staphylococcus* sp., and *Serratia* sp. Crude bioactive metabolites from 15 plant samples (leaves) were collected and were extracted using methanol and tested against biofouling bacteria by disc diffusion method in which nine extracts showed inhibition against biofouling bacteria. *Andrographis paniculata* was selected for further isolation of antifouling compound, as it showed maximum of 13-21mm zone of inhibition. Of the various solvents tested for extraction methanol extract showed best activity followed by ethanol, ethyl acetate, n-hexane and aqueous extract. Antifouling compound was separated by TLC. The active compound separated in TLC was detected by bioautography in which the first spot (Rf value - 0.96) showed antifouling activity. Based on the results of phytochemical analysis the active compound was identified as terpenoids. The partially purified fraction was tested for biofouling inhibition and prevention by cover glass method, and wooden stick method. In both the studies the partially purified fraction showed good activity. Further investigation of active compound from *A. paniculata* will leads to the development of economically cheaper and ecofriendly antifouling compounds.

Key words : biofouling, antifouling compounds, *Andragraphis paniculata*, terpenoids.

INTRODUCTION

Biofouling is an undesirable phenomenon of adherence and accumulation of biotic either deposits on a submerged artificial or in contact with seawater. Microfouling organisms include bacteria, algal zoospores, diatoms and other colonizing microorganism in which bacteria secrete exopolysaccharides (EPS) to envelope and to anchor them to the substrate there by altering the local surface chemistry which can stimulate further growth and settlement of macro organisms. Antifouling technology has been currently applied wherever unwanted growth of biological organisms occurs. A number of historic antifouling techniques including mechanical cleaning, use of non-stick coating and the use of electric current have been utilized to prevent unwanted biofouling. Each method has proved useful but deleterious in practice[1].

In 1960s, the use of paints blended with copper, mercury, arsenic, organic derivatives of tin (Tributyltin (TBT) and Trifeniltina (TPT)) spread widely, but they proved to be a real risk for the marine ecosystem. On the global basis several billion US$ are being spent annually to control or prevent the problem of fouling and corrosion [2]. To overcome the problems posed by synthetic antifoulants, various steps are taken in the recent years. Natural products can be used as replacement for the chemicals commonly used in antifouling coatings. Up to date, a variety of natural products with antifouling activities have been isolated from numerous organisms, including marine bacteria, algae, sea grass, sponges, coral, bryozoans, ascidians, *etc.*, [3]. When compared to microorganisms and marine organisms, reports on antifouling compounds from plants are very few, which may be a alternate source naturally available.

Plants have been used in traditional medicine for several thousand years. Today according to the World Health Organization (WHO) as many as 80% of the world's people depend on traditional medicine for their primary health care needs[4]. India has more than one fourth (8000) of the World's known medicinal plant species (30,000), of which 90% are found in forests. Plant species still serves as rich source of many novel biological active compounds, as very few plants species have been thoroughly investigated for their medicinal properties. Thus, there is a renewing interest in phytomedicine during last decade and now many medicinal plant species are being screened for pharmacological activities[5].

From the available literature, most of the medicinal plants and their compounds were tested and applied to treat human diseases. But no research work is focused on how to use these plants extracts for other than clinical applications. To keep these lacunae in mind, the present study is initiated to use plants and their products on environmental problems like biofouling.

MATERIALS & METHODS

Isolation of biofouling bacteria

Fouling samples were collected from boats at Parangipettai coastal area (Lat. $11^0$29' N; Long. $79^0$46'E). Biofouling bacteria were isolated by standard spread plate method using Zobells marine agar 2216. After 3 days of incubation at 28^0C, morphologically different bacterial colonies were selected and maintained on nutrient agar slants supplemented with 2% NaCl. All the bacterial isolates were tested for adherence property by inoculating them in the sterilized filtered seawater in a 50 ml beaker and floating glass coverslips in a beaker [6]. After 24 hours, the coverslips were removed and stained with 0.4% crystal violet and observed under bright field microscope to check the adherence of bacteria. Bacteria that form a slimy layer on the cover slips were selected for further characterization.

Characterization and identification of biofouling bacteria

Phenotypic characteristics such as micromorphology (gram staining, capsule staining and endospore staining, motility), cultural characteristics (on basal media, differential media and selective media), biochemical characteristics (catalase, oxidase, IMViC) and physiological characteristics (salt tolerance) of the selected isolates were studied and identified by adopting standard procedures[7].

Collection of plant material and extraction

Andragraphis paniculata leaves were collected from College campus, Kanchipuram. Their taxonomic confirmation was done with the help of botanists at nearby Universities. The freshly collected leaves were dried under shade and powdered using surface sterilized Morter and pestle. One gram of dried leaf powder was mixed with 20 ml of methanol. This content was kept for extraction in air tight bottle for 72 hours. The plant material was also extracted using other solvents such as ethyl acetate, chloroform, ethanol and n-hexane[8].

Screening of plant extracts for antifouling activity

Antifouling activity of plant extracts were tested against biofouling bacteria by disc diffusion method. About 0.25 mg of crude extract was impregnated on sterile filter paper disc (5 mm diameter) and placed on nutrient agar plates swabbed with biofouling bacteria. All the plates are incubated at 28^0C for 24 hours and observed for zone of inhibition[9].

Partial purification of antifouling compound Analytical TLC

The crude extract was purified by using Thin Layer Chromatography (TLC) (Balagurunathan, 1994) using commercially available Silica gel coated chromatography sheets (50 cm x 20 cm size). To find out the best solvent system for good separation of crude compound, solvents such as methanol, chloroform, acetic acid, n-butanol, n-hexane and water were used in different proportions. After separation, the sheet was kept in room temperature for the complete drying of the plate. Then the sheet was kept in closed iodine chamber to visualize the separated compound as clear spots and Rf value was calculated.

Detection of active compound by bioautography

The bioautography method described by Rahalison *et al.,* [10] followed for the detection of active compound separated in TLC. Chromatogram developed as described above was placed over nutrient agar plate inoculated with biofouling bacteria. Activity was indicated by zone of inhibition around the spot.

Preparative TLC

Preparative TLC was performed to get partially purified compound. TLC plate was prepared by spreading the slurry of silica gel evenly on the plate. The plate was activated at 100°C for 15 minutes. Crude extract was applied on the plate as a single line and the chromatogram was performed with the solvent system n- butanol: acetic acid: water (45:30:25). After separation, the active spot band was scrapped, mixed with methanol and centrifuged at 3000 rpm for 15 minutes. Supernatant was collected in a preweighed vial and kept for evaporation

Effect of partially purified antifouling compound on biofilm formation and prevention

For biofilm experiment, each 10 ml of sterilized sea water was taken in two beakers and marked as control, inhibition and prevention. In the control and test beaker, about 1 ml of biofouling bacterial culture (OB1) was added and also two clean glass slides and wooden sticks are placed in to it. After incubation, the glass slides were removed from all the 3 beakers and stained with 0.4% crystal violet and observed under bright field microscope[11]. In addition to this, a clean wooden stick was also immersed in control and test beaker and kept for a week. Then the biofouling bacterial population in the wooden stick was calculated as CFU/gram.

Phytochemical and spectral analysis of partially purified antifouling compounds

The partially purified compound obtained from preparative TLC was subjected to qualitative phytochemical analysis for the identification of compound such as carbohydrate, proteins, phytosterol, flavanoids, terpenoids, alkaloids, saponins and tannins present in the purified extract [12]. The UV Spectral analysis of partially purified antifouling compound was carried out at Department of Biotechnology, Sri Sankara Arts and Science College, Kanchipuram.

Table 1: Characteristics of selected biofouling bacteria

Characteristics	Bacterial isolates				
	OB1	**OB2**	**OB3**	**OB4**	**OB5**
Gram staining	G^- rods	G^- rods	G^+ rods	G^+ cocci in clusters	G^+ rods
Motility	Motile	Motile	Motile	Non-motile	Motile
Endospore staining	-	-	+	-	-
Nutrient agar	Colorless	Orange	Colorless	Colorless	Pink
MacConkey agar	NLF	NLF	-	No growth	-
Cetrimide agar	Green	No growth	No growth	No growth	-
TCBS agar	No growth	No growth	No growth	No growth	-
MSA agar	No growth	No growth	No growth	Yellow	-
Catalase	+	+	+	+	+
Oxidase	+	+	-	-	+
IMViC	----	----	ND	ND	ND
Adherence	+	+	+	+	+
Salt tolerance					
0%	+	+	+	+	+
1%	+	+	+	+	+
2.5%	+	+	+	+	+
5%	+	+	+	+	+
7.5%	-	-	+	-	-
10%	+	-	-	-	-

'+' Positive, **'-'** Negative, **'ND'** Not Determined, **'NLF'** Non Lactose Fermenting.

Table 2: Screening of plant extracts for antifouling activity by disc diffusion method (Zone of inhibition in millimeter in diameter)

Sl. No	Plant name	OB1	OB2	OB3	OB4	OB5
1	*Allium sativum*	8	-	10	-	-
2	*Martynia annua*	11	12	8	-	-
3	*Phyllanthus amarus*	-	7	7	-	-
4	*Azadirachta indica*	17	-	-	-	15
5	*Achyranthes aspera*	-	-	-	-	-
6	*Eucalyptus globulus*	-	-	-	-	-
7	*Cleome viscosa*	7	-	-	-	-
8	*Cardiospermum halicacabum*	-	-	-	-	-
9	*Tridax procumbens*	15	7	8	-	-
10	*Leucas aspera*	-	-	-	-	-
11	*Citrullus colocynthis*	-	-	8	-	-
12	*Acalypha indica*	-	-	-	-	-
13	*Mimuscops elengi*	7	8	-	-	-
14	*Abrus Precatorius*	-	-	-	-	-
15	*Andrographis paniculata*	18	21	16	13	14

Table 3: Antifouling activity of different solvent extracts of *A. paniculata* leaves

Extracts	Quantity of extracts (mg/10 ml)	Activity (Zone of inhibition in millimeter in diameter)				
		OB1	OB2	OB3	OB4	OB5
Ethanol extract	17	16	15	12	8	15
Methanol extract	22	18	21	16	13	-
Ethyl acetate extract	10	12	10	7	-	-
n-hexane extract	8	-	-	-	-	-
Water extract	-	15	-	-	-	12

Table 4: Effect of partially purified compound on biofouling bacteria

Biofouling bacteria	Antifouling Activity (Zone of inhibition in millimeter in diameter)
OB1	19
OB2	22
OB3	20
OB4	18
OB5	23

RESULTS

Isolation, characterization and identification of biofouling bacteria

After incubation, morphologically different colonies were observed on Zobell's marine agar plates. Bacterial populations were estimated as 8.15×10^6 CFU/gram of sample. Totally 25 bacterial colonies were recovered from isolation agar plates from which five colonies were selected, based on the results of microscopic and colony morphology, for antifouling studies. Microscopic, cultural and biochemical characteristics of all five fouling bacteria (OB1, OB2, OB3, OB4, and OB5) were given in table 1**.** Based on the studied phenotypic characteristics the biofouling bacterial strains were identified as *Pseudomonas sp.,* (OB1), *Alteromonas sp.,* (OB2), *Bacillus sp.,* (OB3), *Staphylococcus sp.,* (OB4), *Serratia sp.* (OB5).

Screening of plant extracts for antifouling activity

Antifouling activity of selected medicinal plant extracts are given in **table 2**. Among the 15 plant extracts tested by disc diffusion method, nine plant extracts showed inhibitory activity against the biofouling bacteria. Especially *Andrographis paniculata* plant extract showed maximum zone of inhibition (13 mm to 21 mm) against all the 5 biofouling bacteria tested. Based on this result, *Andrographis paniculata* was selected for further investigations.

Optimization for extraction of antifouling compounds

Quantity and antifouling activity of different solvent extracts of *Andrographis paniculata* was given in table 3. Based on the result, methanol extract of *Andrographis paniculata* which showed maximum activity, and selected for further investigation.

Partial purification of antifouling compound by TLC

Among various solvent system tested in TLC, 3 well separated spots were observed when n- butanol: acetic acid: water (45:30:25) used as a solvent system. Rf value of all the three separated spot was calculated as 0.96, 0.93 and 0.89 respectively.

Bioautography of antifouling compound

Of three different spots separated in TLC, the first spot showed good activity against the biofouling bacteria with 18- 23 mm of inhibition.

Preparative TLC

The partially purified compound obtained from preparative TLC, showed about 18- 23 mm inhibition against biofouling bacteria.

Effect of partially purified antifouling compound on biofilm inhibition and prevention

Under bright field microscopic observation, glass slides from inhibition and prevention beaker showed less dense of biofilm formation. Wooden sticks from the control beaker showed 6.60×10^7 CFU/ml and 3.10×10^7 CFU/ml in inhibition beaker.

Phytochemical analysis of partially purified antifouling compound

Results of phytochemical analysis of partially purified antifouling compound were given in table 4. Based on the positive result for Salkowski test, the active fraction separated by TLC may be a terpenoids type of compound.

UV- Spectral analysis

It is observed that the spectral analysis of the isolated compound shows two peaks at 359.0 nm and 391.5 nm which are suspected as terpenoids as per the standard data. It is further to be confirmed with complete purified compound.

DISCUSSION

Marine fouling organisms often cause technical and economic problems by setting on artificial surfaces submerged in seawater. Although organotin compounds such as Tributyltin (TBT) and Tributyltin oxide (TBTO) have been widely use for controlling these organisms, they were also found to be toxic to many non–target marine organisms, leads to ban on the application of TBT – based antifouling paints from January 1, 2003 and the ban of the use of such paints on the surface of vessels from January 1, 2008 [13]. Currently there are some booster biocides are used for antifouling paints, but they may also pollute the aquatic environments[14]. Therefore, effective and eco friendly antifoulants are urgently needed. To date, a variety of natural products with antifouling activities have been isolated from lots of different marine organisms, including marine bacteria, algae, sea grass, sponges, coral, bryozoans, ascidians, *etc*., [15]. However, there is no work on isolation of antifouling compounds from terrestrial plants. With this view, the present study was attempted to isolate antifouling compounds from certain terrestrial plants.

Biofouling bacteria were first isolated from fouling samples and their adherence properties were confirmed. In general, biofouling bacteria secrete exopolysaccharides (EPS) to envelope and to anchor them to the substrate which can stimulate further growth and settlement of macroorganisms. Of the 25 bacteria isolated from fouling samples, 5 showed adherence on coverslips. The five isolates were characterized and identified as *Pseudomonas sp.,* (OB1), *Alteromonas sp.,* (OB2), *Bacillus sp.,* (OB3), *Staphylococcus sp.,* (OB4) and *Serratia sp.* (OB5). Bacteria such as *Bacillus, Pseudomonas, Vibrio, Alteromonas,* and *Micrococcus* are previously reported from biofouling samples[16]. The salt tolerance characteristics of biofouling isolates, further confirmed its marine nature.

Many works have been aimed at knowing the different antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of microbial infections[17]. But there is no report towards isolation of biofouling inhibitory compounds from terrestrial plants. In this study, leaves from 15 terrestrial plants were collected and were tested against

five biofouling bacteria, in which crude extracts of nine plants showed antifouling activity. Antimicrobial activities of certain plants like *Acalypha indica*, *Phyllanthus amarus, Allium sativum* (garlic) against clinical pathogens are previously reported[18]. Among the nine plants, crude extract from *Andrographis paniculata* showed maximum activity (13 to 21 mm inhibition) against all the five biofouling bacteria tested. *Andrographis* is a genus of the Acanthaceae family comprising of about 40 species, several members of which enjoy a reputation in traditional medicine. Particularly *Andrographis paniculata* is used for several applications such as antidote for snake bite and to treat dyspepsia, influenza, dysentery, malaria and respiratory infections[19]. It is an annual shrub grows abundantly in India and cultivated extensively in China and Thailand.

Extensive research of the last few decades has revealed that the herbal extract is useful as an anti-inflammatory antiviral, antithrombotic, anticancer, hypoglycemic, hypotensive[20] and antimicrobial agent[21]. About 26 different polyherbal formulations of this plant are mentioned in Ayurveda as a popular remedy for the treatment of various disorders[22]. But for the first time, the present study reported antifouling activity of *Andrographis paniculata* and further investigation on this plant was proceeded for the isolation and identification of active principle.

As on date, various works have been done on the antimicrobial activity of the Indian medicinal plants and so many solvents have been employed by various researchers in their studies and they have given varied results in each case[23]. Methanol is the most commonly used solvent for the extraction of different phytochemicals[17]. In the present study, to optimize best solvent, antifouling compound from *Andrographis paniculata* was extracted with various solvents such as methanol, ethanol, ethyl acetate and n-hexane in which methanol extract showed good antifouling activity followed by ethanol, ethyl acetate and n-hexane. In previous studies, phytochemicals from *Andrographis paniculata* such as flavanoids and andrographolides are extracted using methanol[19]. The aqueous extract showed activity against only against two strains and it may be the active compound. Based on the result, methanol was selected as best solvent and used for further extraction of antifouling compounds from *Andrographis paniculata.*

Plants are more complex chemical store houses of undiscovered biodynamic compounds[24]. Purification of crude extract in to individual compounds is a prerequisite for the further detection, characterization, identification and application of active compound. Thin Layer Chromatography (TLC) is the simple purification method for the separation of plant derived compounds. In the present study, crude extract was purified by TLC in which three separate spots were observed when n- butanol: acetic acid: water (45:30:25) used as solvent system.

Bioassays are essential for monitoring the required effects throughout activity guided fractionation. In this study TLC based bioautography method was used to detect the active antifouling compound. Bioautography is a very convenient and simple way of testing plant extracts and pure substances for their effects on human and plant pathogenic microbes[25]. This TLC based bioassay allows *insitu* detection of active compound. Further, for the first time the bioautographic assay was used for the detection of antifouling compounds.

In the present study, instead of analyzing crude extract, the active TLC fraction was subjected to phytochemical analysis. Based on the result, the antifouling compound was identified as terpenoids. Further the UV spectral data also supported that the active compound is terpenoids. To study the effect of partially purified compound on biofouling inhibition and prevention, coverglass method described by You *et al*., [11] was followed with some modifications. Satisfactory results were observed under microscopic observation. In addition to coverglass studies, inhibition of biofouling formation on wooden stick also investigated, in which biofouling bacterial population was reduced in test (3.10×10^7 CFU/ml) when compared to control (6.60×10^7 CFU/ml). The coverglass and wooden stick method is a suitable one for studying the antifouling activity of natural products, before field evaluation. Further the same extract will be tested for fouling organisms in big size wooden plates immersed with water.

The present study concluded that the widely available medicinal plant *Andrographis paniculata* can, not only provide active compound for clinical application but also for controlling fouling organisms, which pose major threat to shipping industry. As this plant can grow everywhere and also doesn't require specific climatic conditions, we can cultivate this plant in large scale for their effective use as antifouling agents. When calculating the cost of using presently using commercial antifouling paints and microbial antifouling compounds, the compounds from this plant source may be cheaper and also environmental friendly.

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