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Biological activity of the red alga *Laurencia brandenii*

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Abstract – The marine red alga *Laurencia brandenii* collected from the southwest coast of India (Indian Ocean) was extracted and fractioned using column chromatography. The individual fractions were evaluated *in vitro* via antimicrobial activity against six species of Microbial Type Culture Collection and three species of clinical human pathogens, antipest activity on *Sitophilus oryzae*, maggoticidal activity against 2nd instar larvae of *Sarcophaga* sp. and termiticidal activity against *Microtermes obesi.* It was found that the fraction eluted using petroleum ether:chloroform (6:4) exhibited broader biological activities. The phyco-constituents of the active fraction were identified by gas chromatography--mass spectrometry (GC-MS) analysis. The GC-MS profile of the active fraction revealed that the main constituent was octadecadienoic acid (49.75%) followed by n-hexadecanoic acid (14.24%), which might have a functional role in the biological activities. The overall activity profile envisages that these bioactive compounds from *L. brandenii* could be utilized as a renewable natural resource for the development of novel environmental-compatible formulations for the control of human pathogens, pests, termites and maggots.

Keywords: Alga*, Laurencia brandenii*, antimicrobial activity, antipest activity, maggoticidal activity, termiticidal activity, larva, *Sarcophaga*, octadecadienoic acid, n-hexadecanoic acid

Introduction

Natural marine products are in great demand due to their prolific biological activities and serve as source for the discovery of novel bioactive compounds (BLUNDEN 2001). During the past few decades, over 14,000 novel natural products from marine organisms have been isolated and described (PROKSCH 2002). Such biogenic compounds exhibit unique structural and functional properties that are not found in terrestrial natural products (FENICAL 1982). Among the diverse group of marine flora, seaweeds are the most primitive vegetation, surviving over a large range of environmental conditions and being continu-

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ously exposed to high density of harmful micro and macrofauna. The survival of this sedentary vegetation has primarily relied on efficient defence mechanism against diverse invaders. Seaweeds from varied locales have been evaluated for a wide range of biological activities, e.g., antibacterial (TUNEY et al. 2006), antiviral (SERKEDJIEVA 2004), antifungal (TANG et al. 2002) and antialgal activities (HELLIO et al. 2002). Over 2,400 secondary metabolites have been isolated and described from the divisions Rhodophyta, Phaeophyta, and Chlorophyta, many of which have been reported to have excellent biological activity (FAULKNER 2001 and references therein).

Of the three groups of seaweeds, the class Rhodophyta produces a plethora of structurally diversified novel halogenated natural products, which in fact symbolize the extraordinary wealth of biogenic compounds principally for pharmaceutical leads (FAULKNER 2001). Among the red algae, the genus *Laurencia* is known to produce the largest number and diversity of secondary metabolites, ultimately making it the world's most chemically complex seaweed genus (PEREIRA et al. 2003). Moreover, the anticandidal, antibacterial (MANILAL et al. 2009a, SHANMUGHAPRIYA et al. 2008), nematicidal and mosquito larvicidal activity, as well as the ichthyotoxicity and brine shrimp cytotoxicity (MANILAL et al. 2009b) of crude extracts of different red algal species from the Indian coast has already been reported. Therefore, the present study was aimed to evaluate the biological activity of *L. brandenii* against human pathogens, pests, maggots and termites and to detect the bioactive compounds from *L. brandenii* that could be useful for the development of novel environmental compatible formulations for the control of human pathogens, pests, termites and maggots.

To this day, information regarding biological activities of flora and fauna from the southwest coastline of India (Kollam coast) remains scanty.

Materials and methods

Study area and collection of samples

The study area was located along the 45 km long southwest coast of India (Indian Ocean), between latitude 08°54' N and longitude 76°38' E. Seaweed specimens were collected from the intertidal and subtidal habitat of Kollam prefecture (Thirumullavaram) located on the southwest coast. The area is rich in flora and fauna (MANILAL et al. 2009a). Samples were collected during December 2007 to February 2008 when red algal diversity remains dominant. Cleaned plant materials were shade-dried under a stream of air for one week to prevent photolysis and thermal degradation. The completely dried material was weighed and ground coarsely in a mechanical grinder. In the present study we evaluate the biological potencies of a column-purified fraction of the red alga *Laurencia brandenii,* on the following bioassays: 1) antimicrobial activity on nine species of human pathogens; 2) antipest activity on *Sitophilus oryzae*; 3) maggoticidal activity on 2nd instar larvae of *Sarcophaga* sp. and 4) termiticidal activity against *Microtermes obesi*.

Extraction of seaweed bioactives

The shade-dried samples (5 kg) were extracted using methanol and purified in column chromatography to yield eleven fractions (F1-F11). The individual fractions were evaluat-

ed for biological activities (data not shown). The fraction eluted using petroleum ether: chloroform (6:4), fraction F8, showed a wide spectrum of activity in all the bioassays. For the extraction of bioactive compounds, 100 g of the dried seaweed powder was weighed and immersed in a flask containing 1000 mL of methanol (purity grade 99%) and placed at 35 °C in a shaker at 120 rpm for 7 days for the extraction of active ingredients. The algal material was re-extracted with methanol in a 1 L capacity round bottom flask in a water bath at 60 °C for 3 h. The individual crude mixture was pooled and filtered using paper filter fitted with a Buchner funnel using suction pressure followed by centrifugation (Eppendorf) at 6000 × g for 5 min at 20 °C. The supernatant was collected in a round-bottomed flask and the remaining solvent was concentrated up to 15–20 mL in a rotary vacuum evaporator (Yamato). The residue collected was evaporated to dry completely in a vacuum desiccator and stored in the refrigerator.

Chromatography of *Laurencia brandenii*

The methanolic extract of *Laurencia brandenii* (200 g) was applied in a silica gel (60–120 mesh) column developed with petroleum ether and eluted with petroleum ether and chloroform (9:1 to 1:9 and 100% chloroform) followed by chloroform and methanol (9:1 to 1:9 and 100% methanol) and yielded eleven fractions. The individual fractions were screened for biological activity (data not shown). The fraction that was eluted using petroleum ether: chloroform (6:4), which exhibited activity was used for Hewlett Packard gas chromatography-mass spectrometry (GC-MS) analysis. Peak identification was carried out by comparison of the mass spectra with those available in the NIST Version 2 (2005).

Test microorganisms

To characterize the antimicrobial activity, the algal fractions were evaluated against a battery of human pathogenic bacteria. Antimicrobial activity (inhibition area) was determined against six species of type cultures from Microbial Type Culture Collection (MTCC) of human Gram-positive pathogens, such as *Staphylococcus aureus* (MTCC 2940), *Bacillus subtilis* (MTCC 1306), *Micrococcus luteus* (MTCC 106), *Rhodococcus rhodochrous* (MTCC 265), and the Gram-negative bacteria *Escherichia coli* (MTCC 739) and *Pseudomonas aeruginosa* (MTCC 2453), as well as three species of clinical isolates, *Vibrio cholerae*, *Salmonella typhi*, and *Streptococcus pneumoniae*. The resistant patterns of these human pathogenic isolates were confirmed using selective antibiotics: streptomycin, oxytetracycline, ampicillin and erythromycin, in a preliminary experiment.

Antimicrobial assay

The antibacterial assay was carried out following the methodology of SELVIN and LIPTON (2004).

Anti-pest activity

Pesticidal activity of the algal fraction was evaluated by a modified area preference test (MCDONALD et al. 1970) using the common rice weevil *Sitophilus oryzae* (L.). The organism was collected from infested rice obtained from a local market and reared in glass bottles under standard insectaria conditions at ambient temperature (28 ± 3 °C) and the relative humidity 70 ± 5%. A uniformly saturated filter paper disc of different concentrations of al-

gal extracts (2, 4, 6, 8 and 10 mg cm^{-2}) was pasted in the bottom of each petri dish. Ten unknown-sex insects of same age were released in the petri dish and kept in a dark room. Mortality was observed after 24 h of exposure. All the experiments performed in the present study were repeated six times to validate the findings statistically. The mortality percentage was converted into probit scale to determine the LD50 values.

Maggoticidal activity

For the determination of maggoticidal activity, 2nd instar maggots of the flesh fly, *Sarcophaga* sp. (Miegen) were used as test organism. A stock colony of the insect was established using mature flies collected from local slaughter house. A couple of adults were released into a wide-mouth glass chamber covered with a copper mesh screen and a piece of chicken meat was placed for the oviposition. The whole setup was left for 72 h with constant monitoring. Ten successfully hatched larvae were picked using a blend end probe and placed in a 40 mm disposable petri dish filled with boiled egg yolk uniformly mixed seaweed extracts (20, 40, 60, 80 mg g^{-1}). Petri dishes containing no seaweed extracts and methanol were used as positive and negative control. Since this was the active feeding stage of the maggots, they could consume the appropriate quantity of the algal fraction mixed with boiled egg yolk, so the effect of the algal extracts could be evaluated effectively. Mortality rate of treated maggots was recorded after 24 and 48 h of exposure. Based on the percent mortality, the LD50 value was determined using probit scale (WARDLAW 1985).

Termiticidal activity

The test termite *Microtermes obesi* (Holmgren) worker was collected from the thickets of Bharathidasan University. The collected organisms were maintained for three days on a degraded log in a glass chamber at 30 °C ± 2 °C, 75 ± 5% relative humidity and a 12:12 h photoperiod. Ten worker termites were transferred to a 30 mm Petri dish uniformly lined with different concentration of algal fractions which dried overnight to remove the solvent completely. The Petri dishes were covered and kept in a dark cabinet maintained at 25 °C for 24 h. The survivors were counted under mild light and the percentage of survivors was calculated. All the experiments were repeated six times to validate the findings statistically.

Results

In antimicrobial assay, the algal fraction exhibited a growth inhibition range of 213 mm^2 to 87 mm^2 against the nine species of Microbial Type Culture Collection and clinical isolates (Fig.1). The results of the antipest activity indicated that the algal fraction was toxic to *Sitophilus oryzae* which produce an LD50 value of 3.7 mg cm^{-2} (Fig. 2). Maggoticidal assay showed the algal fraction was active against the 2nd instar maggots of *Sarcophaga* with an LD50 of 43 mg g^{-1} (Fig. 3). Concerning the termiticidal assay, the active fraction of *L. brandenii* was found to be highly lethal at 2.6 mg mL^{-1} causing 50% mortality at a 6 h exposure (Fig. 4).

The fraction F8 exhibited biological activity and proceeded to GC-MS analysis, to show the relative percentage of identified active compounds in *Laurencia brandenii* (Tab. 1). The main phycoconstituents of the active fraction were octadecadienoic acid (49.75%) followed by n-hexadecanoic acid (14.24%).

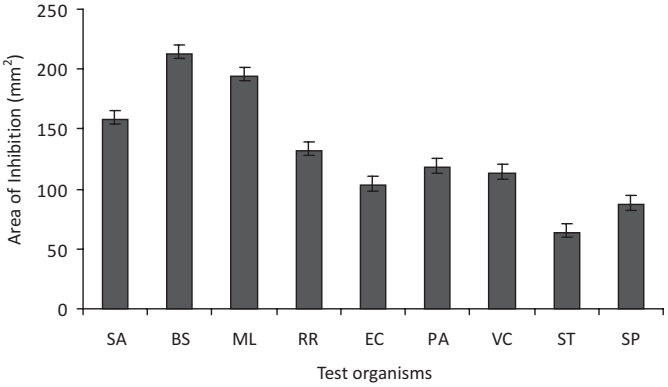

Fig. 1. Antibacterial potentials of *Laurencia brandenii* against Microbial Type Culture Collection and clinical isolates. BS – *Bavillus subtilis*, EC – *Escherichia coli*, ML – *Micrococcus luteus*, RR – *Rhodococcus rhodochrous*, PA – *Pseudomonas aeruginosa*, SA – *Staphylococcus aureus*, SP – *Streptococcus pneumoniae,* ST – *Salmonella typhi,* VC – *Vibrio cholerae.*

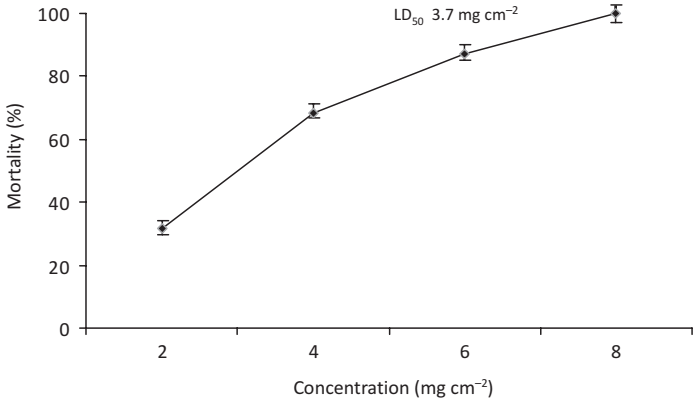

Fig. 2. Antipest activity of *Laurencia brandenii* against *Sitopzhilus oryzae*

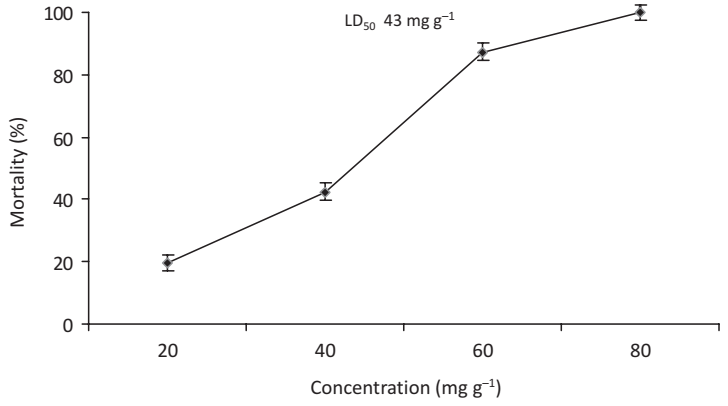

Fig. 3. Maggoticidal activity of *Laurencia brandenii* against *Sarcophaga albiceps* larvae

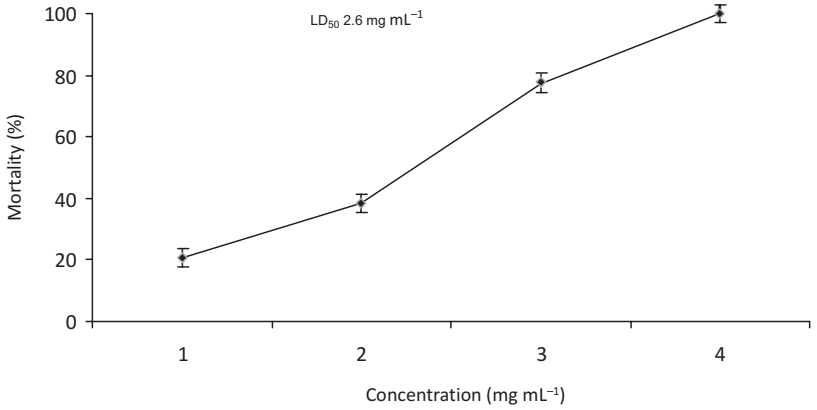

Fig. 4. Termiticidal activity of *Laurencia brandenii* against *Microtermes obesi*

Tab. 1. GC-MS data of active fraction of *Laurencia brandenii*

No	RT	Name of the Compound	Molecular Formula	Molecular Weight	Peak Area %
1	5.01	Cyclohexasiloxane – dodecomethyl	$C_{12}H_{36}O_6Si_6$	444	1.07
2	8.20	Trisiloxane 1,1,1,5,5,5-hexamethyl-3,3--bis[(trimethyl silyl)oxy]-	$C_{12}H_{36}O_4Si_5$	384	0.89
3	9.36	3,5-Dimethyl-5-hexan-3-ol	$C_{18}H_6O$	128	0.11
4	11.64	3,5-Dimethyl-5-hexan-3-ol	$C_{18}H_6O$	128	0.11
5	13.59	4-Dodecanol	$C_{12}H_{26}O$	186	0.08
6	16.04	2-Decanone. 5,9-dimethyl-	$C_{12}H_{24}O$	184	0.13
7	18.65	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	14.24
8	20.90	9-Dodecanoic acid, methyl ester,(E)-	$C_{13}H_{24}O_2$	212	0.48
9	21.11	Oxalic acid, allyl hexadecyl ester	$C_{21}H_{38}O_4$	354	0.40
10	21.85	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	49.75
11	24.33	Cyclohexanecarboxylic acid, decyl ester	$C_{17}H_{32}O_2$	268	2.70
12	25.96	9,12-Octadecadienoyl chloride (Z,Z)-	$C_{18}H_{31}ClO$	298	3.50
13	28.26	Oxalic acid, allyl pentadecyl ester	$C_{20}H_{36}O_4$	340	1.23
14	28.75	cis- 9,10-Epoxyoctadecan-1-ol	$C_{18}H_{36}O_2$	284	0.61
15	29.79	Heptanoic acid, 9-decen-1-yl ester	$C_{17}H_{32}O_2$	268	0.65
16	30.63	9-Octadecenal	$C_{18}H_{34}O$	266	5.91
17	34.00	9,12-Octadecadienoic acid (Z,Z)-, phenylmethyl ester	$C_{25}H_{38}O_2$	370	0.44
18	34.51	Oxalic acid, allyl hexadecyl ester	$C_{21}H_{38}O_4$	354	1.11
19	37.64	Oxirane, tetradecyl-	$C_{16}H_{32}O$	240	3.49
20	38.61	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	254	3.01
21	40.00	7,11-Hexadecadienal	$C_{16}H_{28}O$	236	5.99
22	40.40	Thunbergol	$C_{20}H_{34}O$	290	4.11

Discussion

Data obtained from the antimicrobial activity of the column purified fraction (F8) of *Laurencia brandenii* exhibited considerable activity against all the tested microbial type culture collection and clinical isolates. The magnitude of inhibition against organisms was on the decreasing order of *Bacillus subtilis* (213 mm^2) > *Micrococcus luteus* (194 mm^2) > *Staphylococcus aureus* (158 mm^2) > *Rhodococcus rhodochrous* (132 mm^2) > *Pseudomonas aeruginosa* (118 mm^2) > *Escherichia coli* (103 mm^2). A high activity range was extended against one of the Microbial Type Culture Collection human pathogens, *B. subtilis* with 213 mm^2, whereas the activity was moderate against *E. coli* 103 mm^2. Regarding the clinical isolates, *Salmonella typhi* was found to be the most resistant bacteria, producing an inhibition area of 64 mm^2 whereas the fraction was moderately able to inhibit the growth of *Streptococcus pneumoniae* (87 mm^2). The bactericidal potency of the algal fraction was high against the clinical pathogen, *Vibrio cholerae* to the extent of 113 mm^2 at 37 °C. In the present study, algal fraction of *L. brandenii* showed maximum antibacterial activity against the gram positive bacteria, whereas the activity was found to be moderate against gram negative bacteria. The resistant mechanism of gram negative bacteria could be due to the permeability provided by the cell wall or to the membrane accumulation tactics (ADWAN and ABU-HASAN 1998). Antimicrobial potentials of red algae have already been reported from different locales, but their efficacy against clinical and human pathogens have scarcely been investigated. Concerning the prevalence of microbial resistance to conventional antibiotics, our study provides a new insight into the development of novel phytotherapeutics for the management of infectious diseases. Data pertaining to the susceptibility of gram positive strains to algal extracts were reported by many authors (PESANDO and CARAM 1984, ROSETT and SRIVASTAVA 1987). Our result is in accordance with the antibacterial activity of *Laurencia* spp. reported from various geographic regions (DE NYS et al. 1996, HELLIO et al. 2001, TASKIN et al. 2007) against marine, human and fish bacterial pathogens (VAIRAPPAN et al. 2001, 2003; BANSEMIR et al. 2006). Based on the present findings *L. brandenii* could be utilized as renewable bioresource for the development of potential antimicrobials.

In the present study, the algal fraction of *Laurencia brandenii* exhibited considerable effects on the rice pest, *Sitophilus oryzae* showing an LD50 value of 3.7 mg after 24 h, significantly higher than that of solvent control, clearly demonstrating the direct contact toxicity as the rationale. The mode of action of this seaweed might be due to the shutdown of different biosynthetic routes of the pest's metabolic pathways. These insecticidal compounds can inhibit the feeding behaviour and growth regulators, disrupting the endocrinological balance of the insects (BALANDRIN et al. 1985). Marine flora and fauna with their broad chemical diversity are still an untapped resource for the development of new agro-chemical agents (CROMBIE 1990) and insecticides (SAXENA 1987). The findings of the present study indicate that red alga *Laurencia* could be employed as a biological grain protectant to combat invading pests.

The maggoticidal activity of *Laurencia brandenii* was studied by feeding the maggots on an artificial diet containing different concentrations of the active fraction of the seaweed. It showed only a moderate toxicity effect on maggots. Response varied with different concentrations of the algal fraction i.e., the toxicity of the extracts varies with an increase in concentration. Maximum mortality (100%) was recorded at 60 mg g^{-1}, whereas at

20 mg g^{-1} extract showed a decrease in activity. The results indicate that the *L. brandenii* fraction contains compounds that are toxic to maggots since the LD50 value is moderately low. The literature proved that the present study would be the first report on the maggoticidal property of seaweeds. The mortalities of the larvae were 100% and 50% after 24 h of exposure to 60 and 43 mg g^{-1} of algal extract respectively. At higher concentrations, the seaweed fraction was an annoyance to maggots, which showed feed aversion, avoidance, and repellence. The present findings proved that a direct feeding trial assay would be useful to prove the maggoticidal property of seaweed. According to this finding, *L. brandenii* could be used for developing a novel natural veterinary grade maggoticides, since these natural products would likely be biodegradable, thus posing no threat to the environment.

The inexorable use of synthetic termiticides is a major threat to the natural environment, and as a result there is an urgent need to explore and utilize naturally occurring products for combating harmful termites (CARTER 1976). Historically, plants are well-known for their various bioactivities. The toxicity of plants in termites has been reported since 1947 (WOLCOTT 1947). The bioactive compounds have evolved in plants for defense against phytophagous insects. The sesquiterpenes from *Cryptomeria japonica* reported to possesses termiticidal activity (ARIHARA et al. 2004). Similarly, essential oils from *Melaleuca* species can suppress the growth of termites (SAKASEGAWA et al. 2003).

The termiticidal assay represents a rapid, inexpensive and simple bioassay for testing seaweed bioactivity. In the present study, a fatal effect was observed when 3 mg mL^{-1} concentration of algal fraction of *L. brandenii* was applied to the petri dish, causing 90% mortality at a 6 h exposure. The mortality rate for the positive control experiment was much lower (<10) while that of negative control gave a mortality of 15%. The study showed that the termite population declined drastically relative to the concentration and time of exposure. The termiticidal potential of *L. brandenii* has not been investigated or reported in the literature. The tested seaweed fraction may be a valuable source of biorational compounds and could be employed for the development of novel natural termiticide.

Spectral data by GC-MS showed that a mixture of fatty acids characterised the chemistry of the active fraction. In all, 22 peaks with different retention times were observed. It is possible that bioactive compounds primarily consisting of 9, 12-Octadecadienoic acid (Z, Z) – (49.75 %) may be involved in the biological activity. The fatty acid composition of the active fraction revealed that the main acid was octadecadienoic acid (49.75 %) followed by n-hexadecanoic acid (14.24 %). Seaweeds exhibit a high level of fatty acid diversity and many of them possess potential bioactivity. Therefore in the present study, the biological activity of *L. brandenii* might be attributed due to the presence of the fatty acid octadecadienoic acid (49.75 %) in a high percentage.

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