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ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF FATTY ACID METHYL ESTERS OF THE BLIND-YOUR-EYE MANGROVE FROM INDIA

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ABSTRACT

Fatty acids are widely occurring in natural fats and dietary oils and they are known to have antibacterial and antifungal properties. However, little is known on the antibacterial and antifungal properties of the blind-your-eye mangrove (*Excoecaria agallocha*) and this study for the first time determines the fatty acid composition and the antibacterial and antifungal activities of Fatty Acid Methyl Esters (FAME) of the blind-your-eye mangrove plant found along the coastal areas of south India.

Key words: Blind-your-eye mangrove, *Excoecaria agallocha, a*ntibacterial activity, antifungal activity, fatty acid methyl esters

INTRODUCTION

Blind-your-eye, *Excoecaria agallocha* (Euphorbiaceae) is a typical mangrove associated species that occur along the coastal areas of Tamil Nadu State, India. Its common name refers to the damaging effect that the white milky sap can have on human eyes. This tree has religious significance for the local community and people who visit the Hindu temple at Chidambaram town revere this sacred plant. Various parts of this plant have been used in the traditional medicine for the treatment of ulcers, leprosy and also as an aphrodisiac for decades (9). In Sri Lanka, the smoke of the burning wood has been used in the treatment of leprosy, while the root pounded with ginger has been used to reduce swellings of hands and feet (6). A novel Phorbol ester, an anti-HIV principle has also been isolated from the leaves and stem of this unique plant (8).

Fatty acids are widely occurring in natural fats and dietary oils and they play an important role as nutritious substances and metabolites in living organisms (3). Many fatty acids are known to have antibacterial and antifungal properties (14). However, little is known on the antibacterial and antifungal properties of *Excoecaria agallocha*. In this paper, we present data on the Fatty Acid Methyl Esters (FAME) of leaves of *Excoecaria agallocha* and its antibacterial and antifungal properties.

MATERIALS AND METHODS

Leaves of the blind-your-eye mangrove (*Excoecaria agallocha*- herbarium # AUBOT 140) were collected from the mangrove forest at Pichavaram (11º24´ N and 79º44´ E) located in Tamil Nadu State (India) during a routine field trip conducted in November 2005. Leaves were washed with water, then surface sterilized with 10% sodium hypochlorite solution and rinsed with sterile distilled water (17). After drying at room temperature, samples were ground into a fine powder. Twenty grams of powder were refluxed with a mixture of dry methanol, benzene and sulphuric acid (200: 100: 10 v/v) for 2 h. The filtrate was transferred to a separating funnel and 60-70 cc of distilled water was added. A small amount of hexane was added and pooled. The hexane fraction was separated into two layers and the lower layer was removed. The upper layer was washed with 50 cc of 10% sodium bicarbonate, shaken twice and the lower layer removed. The upper layer was washed twice with saturated 0.9% sodium chloride solution. The upper layer was saved and passed through sodium sulphate and the extract obtained was evaporated (18). The residue was dissolved in hexane and analyzed by Gas Chromatography (Varian, Inc., USA). The capillary column used to separate the fatty acids was CP-Wax 5g (chrompack; 50 m × 0.20 mm).

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The test solution was prepared with known weight of crude extracts, dissolved in 5% dimethyl sulphoxide. Sterile filter paper discs (Whatman No. 1; 6 mm) were impregnated with 20 µl of this extract (corresponding to 10, 5 and 2.5 mg of crude FAME extract respectively) and allowed to dry at room temperature. Four strains of Gram-positive bacteria (*Bacillus subtilis* NCIM 2063, *B. pumilus* NCIM 2327, *Micrococcus luteus* NCIM 2376, and *Staphylococcus aureus* NCIM 2901) and three strains of Gram-negative bacteria (*Pseudomonas aeruginosa* NCIM 5031, *Klebsiella pneumoniae* NICM 2957 and *Escherichia coli* NCIM 2256) obtained from the National Collection of Industrial Microorganisms (NCIM), Biochemical Sciences the National Chemical Laboratory at Pune (India) were used. The stock cultures were maintained on nutrient agar medium at 4ºC. Yeasts such as *Candida albicans*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* were obtained from the Rajah Muthiah Medical College and Hospital (Annamalai University, India).

In vitro antibacterial activity was determined by using Mueller Hinton agar and Mueller Hinton broth. *In vitro* antifungal activity was determined by using antifungal assay agar, Sabouraud's dextrose agar and yeast nitrogen base (obtained from Himedia Ltd., Mumbai, India). The selected bacteria/yeasts (24 h culture) were mixed with physiological saline and the turbidity was adjusted to a Mac Farland turbidity standard of 0.5 by adding sterile physiological saline. The agar diffusion method was used for antibacterial and antifungal susceptibility tests (13). Plates were prepared by pouring freshly prepared Mueller Hinton agar for bacteria and antifungal assay agar for fungi into Petri plates and allowed to solidify, to which 0.1 ml of standardized inoculum suspension was poured and uniformly spread. The excess inoculum was drained and the plates were allowed to dry for 5 minutes, then the discs were placed on the inoculated agar. Ciprofloxacin (5 µg/disc) and Amphotericin B (100 units/disc) were used as positive control and 5% DMSO was used as negative control. The inoculated plates were incubated at 37ºC for 24 h (bacteria) and 28ºC for 48 h (yeasts).

Minimum inhibitory concentration of the FAME extract was tested in Mueller Hinton broth for bacteria and yeast nitrogen base for yeasts by two fold serial dilution method. The test extract was dissolved in 5% DMSO to obtain 4 mg stock solutions. 0.5mg of stock solution was incorporated into 0.5 ml of Mueller Hinton broth for bacteria and yeast nitrogen base for yeast to get a concentration of 2, 1, 0.5, 0.25, 0.125 and 0.06 $mg.ml^{-1}$. Fifty µl of standardized suspension of the test organism were transferred to each tube. A control tube, containing the microorganisms but not the FAME extract, was also prepared. The culture tubes were incubated at 37ºC for 24 h (bacteria) and 28ºC for 48 h (yeast). The MIC was defined as the lowest concentration of the extract that did not show any growth of the tested microorganism after macroscopic evaluation. The Minimum Bactericide Concentration (MBC) and Minimum Fungicide Concentration (MFC) of the extracts were determined by plating 100 µl samples from each MIC assay tube with growth inhibition into freshly prepared Mueller Hinton agar (for bacteria) and Sabouraud dextrose agar (for yeast) plates. The MBC and MFC were recorded as the lowest concentration of the extract that did not permit any visible bacterial and fungal colony growth on the agar plate after the period of incubation.

RESULTS AND DISCUSSION

The analysis of FAME extract of *Excoecaria agallocha* by gas chromatography revealed higher amount of saturated fatty acids than unsaturated fatty acids (Fig. 1). Among the fatty acids, palmitic acid (56.02%) and lauric acid (18.12%) were recorded in higher quantity (Table 1). Among the saturated fatty acids, higher amount of myristic acid (3.61%) followed by stearic acid (2.80%), pentadecanoic acid (2.65%), heptadecanoic acid (1.04%) and lower amount of tridecanoic acid (0.80%), behenic acid (0.80%), arachidic acid (0.13%) and nondecanoic acid (0.02%) were recorded. The presence of myristic, stearic, heptadecanoic and arachidic acids was reported previously in the roots, shoots and seeds of *Salicornia bigelovii* and in the wax esters of some mangrove leaves (11,20). Besides, the occurrence of myristic acid and pentadecanoic acid has been reported in some species of marine macro algae (18,19). Among unsaturated fatty acids, we found higher percentage of linolenic acid (7.20%), followed by linoleic acid (3.13%) and oleic acid (1.71%).

The FAME extracts possessed antibacterial and antifungal activities against a total of 11 microorganisms (7 bacteria and 4 yeast; Table 2). The mean zone of inhibition of the extract, assayed against the test organisms ranged between 7.3 and 16.6 mm. The Ciprofloxacin (5 µg/disc) antibacterial positive control produced zones of inhibition that ranged from 31 to 36

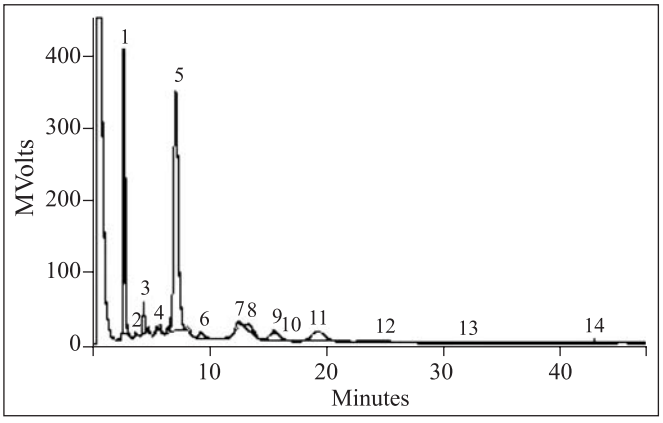

Figure 1. A chromatogram of the fatty acid methyl esters (FAME) of the leaves of the blind-your-eye mangrove, *Excoecaria agallocha* (8, 9 and 11 unsaturated fatty acids)

Table 1. Fatty acid composition of leaves of *Excoecaria agallocha*.

Peak No.	Retention time (min.)	Fatty acid	No. of carbon atom	Relative percentage
1	2.684	Lauric acid	C12:0	18.12
2	3.547	Tridecanoic acid	C13:0	0.80
3	4.347	Myristic acid	C14:0	3.61
4	5.646	Pentadecanoic acid	C15:0	2.65
5	7.034	Palmitic acid	C16:0	56.02
6	9.276	Heptadecanoic acid	C17:0	1.04
7	12.417	Stearic acid	C18:0	2.80
8	13.351	Oleic acid	C18:1	1.71
9	15.579	Linoleic acid	C18:2	3.13
10	16.847	Nondecanoic acid	C19:0	0.02
11	19.331	Linolenic acid	C18:3	7.20
12	23.17	Arachidic acid	C20:0	0.13
13	34.524	Heneicosanoic acid	C21:0	ND
14	43.272	Behenic acid	C22:0	0.80

ND – Could not be detected; Saturated fatty acids - 85.99; Unsaturated fatty acids - 12.04; Unidentified acids - 1.97; Total - 100.0.

mm. Amphotericin B (100 units/disc) antifungal positive control produced zones of inhibition that ranged from 17 to 18 mm. The MIC of the FAME extracts were 0.125 mg/ml for *Bacillus subtilis* and *Staphylococcus aureus*; 0.5 mg for *Bacillus pumilus, Micrococcus luteus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida albicans, C. krusei* and *C. parapsilosis;* and 1 mg for *Escherichia coli* and *Candida tropicalis.* These differences could be due to the nature and level of the antimicrobial agents present in the extracts and their mode of action on different test microorganisms (2).

The highest mean zone of inhibition of 16 mm and the lowest MIC value of 0.125 mg and MBC values of 0.25 mg were produced by FAME extract of *Excoecaria agallocha* against *Bacillus subtilis* and *Staphylococcus aureus*. We have reported a similar observation previously with the extracts of certain marine algae against *Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli* as well as with the extract of *Ipomoea pes-caprae* against *Bacillus subtilis, B. pumilus, Micrococcus luteus, Staphylococcus aureus, Pseudomonas aeruginoa, Klebsiella pneumoniae* and *Escherichia coli*, respectively (1, 4). Moreover, a previous study of liphophylic extracts derived from 15 different plant parts of *Pistacia vera* showed activity against *Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus* (12). Similarly, linoleic acid isolated from *Schotia brachypetala* displayed antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli* (10). The bioactive fractions, linoleic acid and oleic acid from *Pelagonium* sp. possessed antibacterial activity against *Mycobacterium aurum* and *M.*

Table 2. Antibacterial and antifungal activities of FAME extract of leaves of *Excoecaria agallocha.*

#	Microorganisms	Mean zone of inhibition [a] (mm)[b]					FAME extract	
		FAME extract			CIP	AMP		
		Concentration of the disc (mg/disc)			5 mg/disc	100 units/disc	MIC mg	MBC/ MFC mg
		200	100	50				
1.	*Bacillus subtilis*	16.6	14.0	12.6	33.0	NT	0.125	0.25
2.	*Bacillus pumilus*	13.0	10.0	8.3	36.0	NT	0.5	1
3.	*Micrococcus luteus*	14.0	12.0	10.0	32.0	NT	0.5	1
4.	*Staphylococcus aureus*	16.0	14.0	12.0	31.0	NT	0.125	0.25
5.	*Pseudomonas aeruginosa*	11.0	9.0	7.6	33.0	NT	0.5	1
6.	*Klebsiella pneumoniae*	12.0	10.0	7.3	34.0	NT	0.5	1
7.	*Escherichia coli*	10.6	9.0	8.0	31.0	NT	1	2
8.	*Candida albicans*	12.3	10.0	8.3	NT	18.0	0.5	1
9.	*Candida krusei*	12.0	10.0	8.0	NT	18.0	0.5	1
10.	*Candida tropicalis*	11.0	9.0	8.0	NT	17.0	1	2
11.	*Candida parapsilosis*	11.6	10.0	8.0	NT	17.0	0.5	1

a – Mean of three assays; b – Diameter of zone of inhibition (mm) including disc diameter of 6mm; CIP-Ciprofloxacin antibacterial standard; AMP – Amphotericin-B antifungal standard; NT – Not tested.

phlei. Furthermore, the linoleic acid was reported to be active against *Mycobacterium smegmatis* and *M. fortuitum* (19).

In the present study, the Gram-positive bacteria were more susceptible than the Gram-negative bacteria. Similar results were obtained with FAME extracts of leaves of *Ipomoea pes-caprae* and lipophylic extracts of various plant parts of *Pistacia vera* (4,12). These differences in the fatty acid sensitivities between Gram-positive and Gram-negative bacteria may result from the impermeability of the outer membrane of Gram-negative bacteria since the outer membrane of Gram-negative bacteria is an effective barrier against hydrophobic substances (5,16). In fact, Gram-negative bacteria are more resistant to inactivation by medium and long chain fatty acids than Gram-positive bacteria (7).

Lauric, palmitic, linolenic, linoleic, oleic, stearic and myristic acids are known to have potential antibacterial and antifungal agents (10, 15). Our study undoubtedly confirms that the leaves of *Excoecaria agallocha* contain higher relative percentage of the above mentioned fatty acids that has potential antibacterial and antifungal principle for clinical application.

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RESUMO

Atividade antibacteriana e antifúngica de ésteres metílicos de acidos graxos de mangue "blind-your-eye" da India

Gorduras naturais e óleos são abundantes em ácidos graxos que apresentam atividade antibacteriana e antifúngica. Entretanto, pouco se sabe sobre a atividade antibacteriana e antifúngica de ésteres metílicos de ácidos graxos de mangue "blind-your-eye" (*Excoecaria agallocha*). Esse estudo relata, pela primeira vez, a composição em ácidos graxos e a atividade antibacteriana e antifúngica de ésteres metílicos de ácidos graxos (FAME) de mangue "blind-your-eye" encontrado ao longo de áreas costeiras do sul da India.

Palavras-chave: de mangue "blind-your-eye", Excoecaria agallocha, atividade antibacteriana, atividade antifúngica, ésteres metílicos de acidos graxos

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