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In vitro antibacterial activity of diterpene and benzoxazole derivatives from *Excoecaria agallocha* L.

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ABSTRACT

The *in vitro* antibacterial activity of column chromatographic fractions of n-hexane, benzene, chloroform, acetone, ethanol and water extracts of *Excoecaria agallocha* L. were determined against 24 localized and common bacterial pathogens. Antibacterial assay was performed by agar diffusion method against 4 specialized urinary tract pathogens, 10 antibiotic sensitive ophthalmic bacterial pathogens, 5 antibiotic resistant bacterial pathogens and 5 fish pathogens. It was found that the 11th fraction of chloroform extract and the 30th fraction of water extract exhibited broad spectrum of antibacterial activity. These 2 active fractions were further investigated through UV-Visible, NMR and FT-IR and were characterized as benzoate and diterpenes and their derivatives *viz.*, 2,3-secoatisane type diterpene and 3,4,5-trihydroxy methyl benzoate.

Keywords: 2,3-secoatisane, 3,4,5-trihydroxy methyl benzoate, Diterpenes, Mangroves.

INTRODUCTION

There is continuous and urgent need to discover new antimicrobial compounds with diverse chemical structure due to alarming increase in the incidence of emerging infectious disease. Another big concern is the development of resistant to the antibiotics in current clinical use (Rojas et al., 2003). WHO emphasized that herbal drugs from medicinal plants constitute a major part in all traditional system of medicines and utilization of medicinal plant resources in the developing countries so as to extend the health care to maximum number of population in these countries. Marine environment is an exceptional reservoir of biologically active products. It is one of the richest sources for floral wealth and diversity (Brad, 1996). Although few marine natural products are currently in the market or in clinical trials, marine plants still remain the greatest unexploited source of potential pharmaceuticals. Because of the unusual diversity of chemical structures isolated from marine organisms, there is intense interest in screening marine natural products for their biomedical potential. In the light of these evidences, an attempt was made to identify

antibacterial properties of *Excoecaria agallocha* L (Euphorbiaceae). Traditionally, E. *agallocha* was used to treat sores and stings from marine creatures, for treating ulcers, as a purgative and an emetic, and the smoke from the bark to treat leprosy (Ghani, 2003). Scientifically *E. agallocha* was reported to have antifilarial, antioxidant (Jayanta et al., 2009), cytoprotective (Bandaranayake, 2002), antifouling (Chambers et al., 2006), antinociceptive (Nusrat et al., 2008) and pisicidal activities (Konishi et al., 1999). This is due to the presence of diterpenoids (Kang et al., 2005), triterpenoids (Zou et al., 2006), flavonoid (Konishi et al., 2003), phorbole esters (Ericson et al., 1995). Moreover numerous chemical constituents *viz.,* 12-deoxyphorbol-13-(3E,5E-decadienoate) (Erickson et al., 1995), excoecarin M and N (Konishi et al., 2000) were identified by several authors without showing biological activity. The present study mainly focused on the antibacterial potential of two chemical compounds isolated from *Excoecaria agallocha* against 24 bacterial pathogens.

MATERIALS AND METHODS

Fresh, younger leaves of *Excoecaria agallocha* were collected from Pichavaram mangrove forest (Lat 11°27'N; Lan79° 47'E) South East Coast of India. The herbarium specimen was identified by Prof. Dr. K. Kathiresan, CAS, Annamalai University, Parangipettai, Tamilnadu, India. A voucher specimen has been deposited (AUOCAS008) at the department of Oceanography and Coastal Area studies, Alagappa University, Thondi campus, Thondi, Tamilnadu, India. The shade dried and chopped leaves (2 kg) was subjected to percolation with ethanol: water mixture (2:1v/v) under dark for 7 days. The extract was filtered and concentrated in rotary evaporator *in vacuo* at 70°C until become dry. The extract (420 g) was further suspended in water and defatted with diethyl ether (Kanchanapoom et al., 2001). Then the extract was subjected for fractionation through 500 g of silica column (230-400 mesh, 2X50 cm column, MERCK) by using 30 ml of n-hexane, benzene, chloroform, acetone, ethanol and water solvents. Based on the turbidity and colour of the solution totally 30 fractions were obtained which were subjected for antibacterial activity assay by standard agar diffusion method (Bose and Bose, 2008). Standard drugs *viz.,* streptomycin (10 µg), ciprofloxacin (10 µg) tetracycline (30 µg) and kanamycin (30 µg) were used as positive controls. All the fractions were dissolved in dimethyl sulphoxide and diluted in 5% Tween 20. A preliminary assay with aqueous solutions of Tween 20 up to 5% was performed to ensure that no micro-organism growth inhibition occurred. 20 ml of molten Mueller Hinton agar medium (Merck) was poured into each 12 cm Petri dish. All bacterial strains were grown in Mueller Hinton broth medium (Merck) for 24 h at 37 °C. Growth was adjusted to OD (600 nm) of 0.1 by dilution with Mueller Hinton broth medium (Merck). 100 µl of bacterial cell suspension (10^8 cells.ml^{-1}) of 4 specialized urinary tract pathogens *viz., Pseudomonas sp., Enterobacter sp., Escherichia coli, Klebsiella sp.,* 10 antibiotic sensitive ophthalmic bacterial pathogens *viz., Staphylococcus aureus, Escherichia coli, Streptococcus viridians, Staphylococcus epidermidis, Pseudomonas aeruginosa, Proteus sp., Acinetobacter sp., Streptococcus pyogenes, Klebsiella pneumoniae, Streptococcus pneumoniae,* 5 antibiotic resistant bacterial pathogens *viz., Streptococcus pneumoniae, Klebsiella sp., Pseudomonas aeruginosa, Streptococcus sp., Streptococcus aureus,* 5 fish pathogens *viz., Bacillus subtilis, Serratia sp., Aeromonas hydrophila, Vibrio harveyi* and *Vibrio parahaemolyticus* were placed in Petri dishes and dispersed over agar. Then sterile paper discs (6 mm. diameter) impregnated with different fractions (5 mg/disc) were placed on the agar. All tests were performed in triplicate and incubated for 24 hrs at 37˚C. The intensity of sensitivity was measured as the diameter of the zone of inhibition in mm. Most active fractions were

further investigated through UV-Visible, ^{13}C, ^{1}H- NMR, FT-IR for the identification of unique chemical classes.

A single peak of ultra violet spectrum (λ_{max} in nm) was recorded on Cyber UV-1 (190-1100nm) against each active fraction to find out the conjugation if any present in the bioactive compounds. ^{1}H, ^{13}C- NMR were measured on Brucker 400 MHz and 100 MHz. The samples were dissolved in dimethyl sulfide (DMSO) to provide a field frequency lock signal and contained 1% tetramethyl saline (TMS) to provide an internal chemical shift standard (Aldrich). One dimensional ^{1}H and ^{13}C-NMR spectrum were acquired using narrow bore probe. Typical experimental conditions were included two repetitions between successive acquisitions 32768 time domain points and spectral width of 8226.682 Hz for ^{1}H- NMR and 65536 time domain points and spectral width of 24038.461 Hz for ^{13}C- NMR. Data processing and peak integration were done through XWINNMR software (Bruker, Karlsruhe, Germany) running in an R-4000 workstation (Silicon Graphics, Mountain View, CA, USA). IR spectra were recorded on KBr on a JASCO FT/IR-460 plus spectrophotometer.

RESULTS

The results showed that, 15th fraction of chloroform extract showed maximum average zone of inhibition (6.3 ± 0.61) against *Pseudomonas sp.*1 (6 ± 0.67 mm. dia), *Pseudomonas sp.2* (6 ± 0.57), *Klebsiella sp.*1 (7 ± 0.39) and *E. coli* (6 ± 0.87). Moreover 30th fraction of water extract showed maximum average zone of inhibition against *Pseudomonas sp.*1 (6 ± 0.67), *Klebsiella sp.*1 (6 ± 0.62), *Klebsiella sp.*3 (7 ± 0.2) and *E. coli* (6 ± 0.31). Likewise 15th fraction of chloroform extract and 30th fraction of water extracts showed maximum zone of inhibition (7 ± 0.39 and 7 ± 0.24) against *Klebsiella sp.* 1 and *Klebsiella sp.*3 (Table 1). In addition, 30th fraction water extract showed maximum average zone of inhibition against 6 (7.2 ± 0.42 mm diameter) ophthalmic pathogens *viz.*, *Staphylococcus aureus, Streptococcus epidermidis, Proteus sp. Acinetobacter sp. Streptococcus pyogenes, Klebsiella pneumoniae.* It is also noted by the present study that, 11th fraction of chloroform extract showed maximum average zone of inhibition against (8 ± 0.47) *Proteus sp.* (Table 2). Table 3 revealed that, 11th fraction of chloroform extract showed maximum zone of inhibition (6.8 ± 0.51) against 4 fish pathogens *viz.*, *Streptococcus pneumoniae* (7 ± 0.37), *Pseudomonas aeruginosa* (7 ± 0.49), *Streptococcus sp.* (7 ± 0.57) and *Staphylococcus aureus* (6 ± 0.64). In addition, 11th fraction chloroform extract showed maximum average zone of inhibition (6.8 ± 0.61) against 4 fish pathogens (Table 4). But, none of the fractions obtained from n-hexane, benzene, acetone and ethanol showed any sensitivity against any one of the pathogens.

11th fraction of chloroform extract showed (extract was filtered and kept *in vacuo* and maintained at -20 °C and the crystalline compounds were further powdered and dissolved in chloroform solvent) white needle crystals, UV (λ max) 272 nm. The ^{13}C-NMR spectrum revealed the presence of two carbonyl groups (δ 172.1 C-2; 179.2 C-3), two methoxy carbons (δ 50.9, 51.8) three methyl carbons at δ 46.0, 46.i and 73.9, one pair of tri substituted double bond carbon at δ 131.8 and 18.6 and three quaternary carbons at δ 41.1, 42.0 and 46.1. The ^{1}H- NMR spectrum displayed six methyl as singlet, of which two were parts of the ester methyls (δ 3.52, 3.64) and four were identified as tertiary groups (δ 1.12, 1.21, 1.24 and 1.54). The olefinic proton at δ 5.83 was observed as singlet which indicated the presence of a tri substituted olefin. Its IR spectrum (υ_{max} in cm^{-1}) in KBr showed peaks of variable intensities at 3389, 2925, 2847, 2126, 1694, 1627, 1492, 1402, 1338, 1278, 1161, 1042, 933, 899, 811, 718, 632, 566, 431 and 413 indicating hydroxyl, carbonyl, ester and olefinic group respectively. The structure of the compound was identified as 2, 3-secoatisane type diterpene (Figure 1).

30th fraction of the water extract was an amorphous powder, UV (λ max) 365 nm. The ^{13}C- NMR spectrum revealed the presence of 1,3,4,5-tetrasubstituted symmetrical aromatic rings, two methylenes (δ 61.2 and δ 70.2), four methines (δ 51.8, δ 54.9, δ 83.3 and δ 83.4), methoxy carbons (δ 56.1) and one ester carbonyl carbon (δ 166.4). The ^{1}H-NMR spectrum showed the presence of ester methyl signals at δ 2.60, hydroxyl group at 4.41, 4.42, 4.92 and aromatic group at δ 7.76. The IR spectrum (υ_{max} in cm^{-1}) in KBr showed peaks of variable intensities at 3390, 1694, 1628, 1338 and 1279 indicating hydroxyl, carbonyl, methoxy and aromatic ring group respectively. The structure of compound was identified as 3, 4, 5-trihydroxy methyl benzoate (Figure 2).

Table 1: Antibacterial activity of bioactive compounds from *E. agallocha* against 12 urinary tract infectious bacterial pathogens.

Bacterial species	Zone of inhibition in mm in diameter								
	Chloroform fraction				Water fractions				Antibiotics
	11	13	14	15	26	28	29	30	-
Pseudomonas sp.1	-	-	-	-	-	6±0.24	-	6±0.67	13±1.45k
Pseudomonas sp.2	6±0.63	-	6±0.36	6±0.57	-	-	-	-	12±0.69k
Pseudomonas sp.3	-	-	-	-	-	-	-	-	14±0.89k
Pseudomonas sp.4	-	-	-	-	-	-	-	-	9±1.69k
Pseudomonas sp.5	-	-	-	-	-	-	-	-	-
Pseudomonas sp.6	-	-	-	-	-	-	-	-	7±2.54a
Enterobacter sp.	-	-	-	-	-	-	-	-	-
Klebsiella sp.1	-	6±0.74	-	7±0.39	6±0.39	-	6±0.87	6±0.62	12±2.47t
Klebsiella sp.2	-	-	-	-	-	-	-	-	15±0.96t
Klebsiella sp.3	-	-	-	-	6±0.82	-	-	7±0.24	14±0.39t
Klebsiella sp.4	-	-	-	-	-	-	-	-	-
Escherichia coli	6±0.85	6±0.98	-	6±0.87	-	-	-	6±0.31	8±1.28t
Average	6±0.74	6±0.86	6±0.36	6.3±0.61	6±0.60	6±0.24	6±0.87	6.3±0.46	-

- No sensitivity; Values are average of three replicates, k: Kanamycin 10µg; t: Tetracycline: 30 µg

Table 2: Antibacterial activity of bioactive compounds from *E. agallocha* against 10 antibiotic sensitive ophthalmic bacterial pathogens.

Bacterial species	Zone of inhibition in mm in diameter										
	Chloroform fractions					Water fractions					Antibiotics
	11	12	13	14	15	26	27	28	29	30	
Staphylococcus aureus	6±.25	6±0.87	-	6±0.96	-	6±0.17	6±0.35	6±0.57	6±0.64	8±0.38	13±0.51s
Escherichia coli	-	-	-	-	-	-	-	-	-	-	14±090s
Streptococcus viridians	7±0.21	6±0.69	-	-	6±0.19	-	-	-	-	-	9±0.74a
Streptococcus epidermidis	7±0.26	6±0.35	-	-	7±0.27	6±0.28	6±0.24	6±0.69	6±0.53	8±0.24	16±0.54s
Pseudomonas aeruginosa	7±0.68	7±0.74	6±0.36	-	7± 0.38	-	-	-	-	-	12±0.62s
Proteus sp.	8±0.47	7±0.61	-	-	-	-	6±0.59	6±0.87	6±0.24	7±0.39	8±0.31s
Acinetobacter sp.	6±0.98	7±0.62	6±0.85	6±0.85	6±0.61	6±0.39	6±0.85	6±0.89	6±0.49	6±0.21	-
Streptococcus pyogenes	7±0.54	7±0.35	6±0.52	6±0.74	7±0.91	6±0.37	6±0.62	6±0.97	-	7±0.37	15±0.25s
Klebsiella pneumoniae	6±0.68	6±0.89	-	6±0.41	-	-	6±0.94	6±0.65	6±0.76	7±0.95	-
Streptococcus pneumoniae	7±0.27	7±0.57	6±0.63	6±0.52	6±0.82	-	-	-	-	-	8±0.34s
Average	6.8±0.48	6.5±0.63	6±0.59	6±0.69	6.5±0.53	6±0.30	6±0.60	6±0.77	6±0.53	7.2±0.42	-

-- No sensitivity; Values are average of three replicates; t: Tetracycline: 30µg

Table 3: Antibacterial activity of bioactive compounds from *E. agallocha* against 5 antibiotic resistant bacterial pathogens.

Bacterial species	Zone of inhibition in mm in diameter										
	Chloroform fractions					Water fractions					Antibiotics
	11	12	13	14	15	26	27	28	29	30	
Streptococcus pneumoniae	7±0.37	6±0.28	6±0.25	-	6±0.58	6±0.97	6±0.65	7±0.70	7±0.69	7±0.38	-
Klebsiella sp.	-	-	-	-	-	6±0.24	7±0.25	-	-	-	16±0.78c
Pseudomonas aeruginosa	7±0.49	-	6±0.50	-	6±0.69	6±0.62	6±0.75	6±0.84	6±0.35	6±0.68	12±0.54s
Streptococcus sp.	7±0.57	7±0.69	-	6±0.96	-	-	-	-	-	-	14±0.37c
Staphylococcus aureus	6±0.64	-	-	-	6±0.57	6±0.43	6±0.91	6±0.39	-	-	-
Average	6.8±0.51	6.5±0.49	6±0.375	6±0.96	6±0.61	6±0.56	6.3±0.64	6.3±0.64	6.5±0.52	6.5±0.53	-

-- No sensitivity. Values are average of replicates; s: Streptomycin 30µg; c: Ciprofloxacin 10µg

Table 4: Antibacterial activity of bioactive compounds from *E. agallocha* against 5 fish pathogens.

Bacterial species	Zone of inhibition in mm in diameter										
	Chloroform fractions					Water fractions					Antibiotics
	11	12	13	14	15	26	27	28	29	30	
Bacillus subtilis	6±0.95	6±0.68	6±0.98	6±0.69	8±0.92	6±0.34	6±0.54	6±0.95	6±0.96	7±0.96	18±0.85k
Serratia sp.	8±0.62	6±0.92	6±0.67	6±0.68	7±0.64	6±0.69	6±0.69	6±0.65	6±0.68	6±0.62	15±0.94k
Aeromonas hydrophila	7±0.34	-	6±0.82	-	6±0.85	6±0.82	6±0.34	6±0.61	6±0.62	-	15±0.61s
Vibrio harveyi	6±0.58	6±0.81	-	-	6±0.28	-	-	-	-	-	17±0.45s
Vibrio parahaemolyticus	-	-	-	-	-	-	-	-	6±0.95	-	18±0.21s
Average	6.8±0.61	6±0.80	6±0.82	6±0.68	6.8±0.67	6±0.61	6±0.52	6±0.73	6±0.80	6.5±0.79	-

-- No sensitivity; Values are average of three replicates; k; Kanamycin 30µg; s: Steptomycin 30µg.

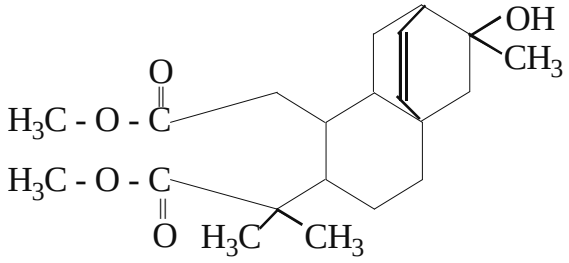

Figure 1: Chemical structure of 2, 3-secoatisane type diterpene (excoecarin N).

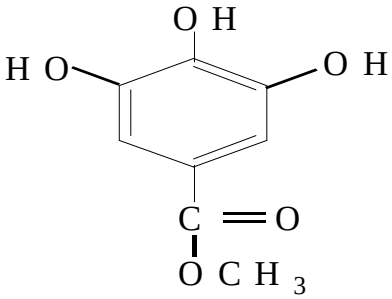

Figure 2: Chemical structure of 3, 4, 5-trihydroxy methyl benzoate.

DISCUSSION

Antibiotics provide the main basis for the therapy of bacterial infections. However, the high genetic variability of bacteria enables them to rapidly evade the action of antibiotics by developing antibiotic resistance. In recent years development of multidrug resistance in the pathogenic bacteria and parasites has created major clinical problems in the treatment of infectious diseases (Ravikumar et al., 2010a). In addition to resistance, antibiotics are sometimes associated with opposing effects such as hypersensitivity, immune suppression and allergic reactions (Ahmad et al., 1998). Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances, hence the chemical structures of secondary plant metabolites provides new and important leads against several pharmacological targets (Balunas and Kinghorn, 2005). In this connection the present study was investigated to find out the antibacterial activity of 30 fractions obtained from *E. agallocha* L. leaf extracts. The antibacterial potency, were assessed by the presence or absence of the sensitivity against the tested bacterial pathogens. Of these 30 fractions, chloroform fractions showed broad spectrum of activity against all the selected pathogens. Previously it was reported that, hexane and chloroform leaf extracts were found to show strong antimicrobial and anti-inflammatory activities (Kunle et al., 2003). The active column chromatographic fractions revealed that, the 11^{th} fraction of chloroform extract was identified as diterpene (excoecarin N). Diterpene resin acids are important defense compounds against potential pathogens, bark beetles and their associated fungi (Martin et

al., 2002). Diterpenes exhibit antibacterial (Bandaranayake, 2002) and the antibacterial activity of the 11th fraction of chloroform extract was probably resulted from the structural similarities with excoecarin A (Subrahmanyam et al., 2005). The 30th fraction of water extract was identified as 3,4, 5-trihydroxy-methyl benzoate. The antibacterial activity of 3,4,5-trihydroxy-methyl benzoate is of close structural similarity with the benzoxazole derivatives. Benzoxazole derivatives are allelochemicals occurring in a number of plant families other than the marine plants (Monocotyledonous plants: Gramineae; dicotyledonous plants: Acanthaceae, Ranunculaceae and Scrophulariaceae) (Wostmann and Liebezeit, 2008). The present study reports for the first time the presence of benzoxazole derivatives in the salt tolerant marine plants. Previous antimicrobial activities have been determined for this compound class (Bandaranayake, 2002). However these two compounds have already been reported by Konishi et al. (2000 and 2003). The reason for the different sensitivity between gram positive and gram-negative bacteria might be attributed to the membrane permeability. Cell wall of gram-negative bacteria consisted of phospholipids and lipopolysaccharides, impermeable to liphophilic solutes (Ravikumar, 2010b). In spite of this permeability barrier, the benzoxazole derivatives and excoecarin N exert strong inhibition on gram-negative bacteria. Gram positive bacteria should be more susceptible because of only an outer layer peptidoglycan. The results of the present study revealed that, *E. agallocha* possesses a promising antibacterial activity against the infectious pathogens, indicates the presence of diterpenes and benzoxazole derivatives. The results compared with the standard drugs indicate that, the isolated compounds are active; however, their activity is less than that of the standard drug. It is concluded from the present study that, the 2 important antibacterial compounds diterpene and benzoxazole derivatives from *Excoecaria agallocha* can be used effectively for the development of antibacterial agents against bacterial diseases prevalent in human beings. Further investigations are in progress to find out the effectiveness of compounds for other microbial diseases and elucidate the exact mechanism of the antibacterial activity.

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