Antimicrobial Activity of Fatty Acid Methyl Esters of Some Members of Chenopodiaceae

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Z. Naturforsch. **63c**, 331–336 (2008); received November 26/December 28, 2007

Fatty acid methyl ester (FAME) extracts of four halophytic plants, *viz. Arthrocnemum indicum*, *Salicornia brachiata, Suaeda maritima* and *Suaeda monoica* belonging to the family Chenopodiaceae, were prepared and their composition was analyzed by GC-MS. The FAME extracts were also screened for antibacterial and antifungal activities. The GC-MS analysis revealed the presence of more saturated fatty acids than unsaturated fatty acids. Among the fatty acids analyzed, the relative percentage of lauric acid was high in *S. brachiata* (61.85%). The FAME extract of *S. brachiata* showed the highest antibacterial and antifungal activities among the extracts tested. The other three extracts showed potent antibacterial and moderate anticandidal activities.

Key words: Chenopodiaceae, Fatty Acid, Antimicrobial Activity

Introduction

Bacterial infections are prevalent in the developing countries due to factors such as inadequate sanitation, poor hygiene and overcrowded living conditions (Rasoanaivo and Ratsimamanga-Urveg, 1993). Microorganisms can become resistant to antibiotics and chemotherapeutic agents by various mechanisms: inactivating drugs by hydrolysis (*e.g.* via β-lactamase) or modification (*e.g.* aminoglycoside resistance), by altering targets (*e.g.* by mutating DNA gyrase in fluoroquinolone resistance) or by preventing the access of drugs to the target (Nikaido, 1998). Therefore, actions must be taken to reduce this problem such as controlling the use of antibiotics, carrying out research to understand the genetic mechanisms of resistance in a better way and continuing investigations aimed at the development of drugs from natural sources (Dash *et al.*, 2001). The development of resistance by a pathogen to many of the commonly used antibiotics leads to the finding of new antimicrobial agents to combat infections and overcome problems of resistance and side effects of the currently available antimicrobial agents (Ali-Shtayeh *et al.,* 1998; Primo *et al.*, 2001).

Lipids and fatty acids are constituents of all plant cells, where they function as membrane components, storage products, metabolites and as a source of energy (Wada *et al.*, 1994). Fatty acids are widely occurring in natural fats and dietary oils, and they are also important nutritious substances and metabolites in living organisms (Chen and Chuang, 2002). Linolenic acid and linoleic acid are essential fatty acids for human health (Burton *et al.*, 2004). They are like arachidonic acid, known as vitamin F, polyunsaturated fatty acids and necessary for growth and protection of the skin (Cakir, 2004). Interestingly, many fatty acids have bactericidal properties. Fatty acids such as oleic, palmitic, stearic, myristic, linoleic and linolenic acids have been demonstrated to have activity against *Clostridium perfringens* and *Streptococcus pyogenes* (Kabara, 1978). Free saturated and unsaturated fatty acids of seaweeds showed antitubercule and antibacterial activities (Katayama, 1962). The bacteriostatic and bactericidal effects of 30 straight-chain fatty acids and their derivatives on a range of Gram-positive and Gram-negative bacteria were investigated by Kabara *et al.* (1972). Ouattara *et al.* (1997) reported that the fatty acids lauric and palmitoleic acid possess antibacterial activity. Fatty acid methyl esters (FAMEs) from ten species of marine macroalgae belonging to the classes Chlorophyceae, Phaeophyceae and Rhodophyceae possess antibacterial activity (Anantharaj *et al.*, 2004).

In the present study, FAMEs of four salt marsh halophytes, namely *Arthrocnemum indicum, Sali-*

cornia brachiata, Suaeda maritima and *Suaeda monoica*, were chosen for antimicrobial assays. The salt marsh halophytes are small herbs and sedges that are able to tolerate salinity and can grow in salt-rich habitats. *A. indicum* is alexipharmic in Ayurveda, and the ashes of the plant are prescribed for the treatment of snakebites. The ashes of *S. brachiata* are used for the manage and for the itch. They are considered as emmenagogue and abortive. *S. monoica* is used in an ointment for wounds (Kirtikar and Basu, 1991).

Material and Methods

Plant material

The shoots of two salt marsh halophytic species, *viz*. *Arthrocnemum indicum* (Willd.) Moq. and *Salicornia brachiata* Roxb., and the leaves of two salt marsh halophytic species, *viz*. *Suaeda maritima* (L.) Dumort. and *Suaeda monoica* Forsk., belonging to the family Chenopodiaceae were collected from the mangrove forest of Pichavaram (11° 24′ N and 79° 44′ E) in the Vellar-Coleroon estuarine complex and used for the present investigation.

Healthy and well-grown leaves/shoots from the representatives of the salt marsh halophytic plants were collected and brought to the laboratory using separate poly bags. First they were washed with tap water and surface-sterilized in 10% sodium hypochlorite solution to prevent the contamination of any microbes. Then they were rinsed with sterile distilled water and air-dried in the shade at room temperature. The samples were ground into a fine powder.

Preparation and analysis of fatty acid methyl esters (FAMEs)

20 g of plant powder were refluxed with a mixture of dry methanol/benzene/concentrated sulfuric acid (200:100:10 v/v) for 2 h. The filtrate was transferred to a separating funnel, and 60–70 ml of distilled water were added. Then 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 ml of 10% sodium bicarbonate solution and shaken two times vigorously, and the lower layer was removed. The upper layer was washed two times with saturated 0.9% sodium chloride solution. The upper layer was saved, passed through sodium sulfate and saved for further analysis. The extract so obtained was evaporated under reduced pressure. The residue was dissolved in hexane and analyzed by gas chromatography (Varian GC# 1). The capillary column used to separate the fatty acids was a CP-Wax 5 g (Chrompack) (50 m × 0.20 mm) column. The temperature of the injector was 210 °C, and of the detector it was 220 °C. The temperature of the oven was programmed from 180 °C and the carrier gas was N_2, H_2 and zero air; attenuation was 3^{-11} A/mV. A small quantity of methyl ester solution (2 μl) was introduced onto the column. The constituents of the FAME extract were identified by comparison of their relative retention times with those of authentic standards from Sigma Chemical Company.

Microorganisms used

The antimicrobial activity of four salt marsh halophytes was investigated against four strains of Gram-positive bacteria, *viz*. *Bacillus subtilis* (NCIM 2063), *Bacillus pumilus* (NCIM 2327), *Micrococcus luteus* (NCIM 2376) and *Staphylococcus aureus* (NCIM 2901), and against three strains of Gram-negative bacteria, *viz*. *Pseudomonas aeruginosa* (NCIM 5031), *Klebsiella pneumoniae* (NCIM 2957) and *Escherichia coli* (NCIM 2256). These standard strains were obtained from National Collection of Industrial Microorganisms (NCIM), Biochemical Sciences Division, National Chemical Laboratory, Pune, India. Ten isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and reference standard of MRSA (NCTC 6571) were obtained from Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar, India. The stock cultures were maintained on nutrient agar medium at 4 °C. Four human pathogenic yeast type fungi (*Candida albicans, Candida krusei, Candida tropicalis* and *Candida parapsilosis*) and three mould fungi (*Aspergillus niger, Aspergillus flavus* and *Aspergillus fumigatus*) were also obtained from Rajah Muthiah Medical College and Hospital. The stock cultures were maintained on Sabouraud dextrose agar medium at 4 °C.

Antibacterial and antifungal assays

Disc diffusion method

The agar diffusion method (Bauer *et al.*, 1966) was followed for antibacterial and antifungal susceptibility tests. Petri plates were prepared by pouring 20 ml of Mueller Hinton agar, Mueller Hinton agar supplemented with 4% sodium chlo-

Table I. Relative percentages of fatty acids in some salt marsh halophytes.

S. No.	Fatty acid	No. of atoms	*Arthrocnemum indicum*	*Salicornia brachiata*	*Suaeda maritima*	*Suaeda monoica*
1	Lauric acid	C12:0	19.5	61.85	23.88	10.78
2	Tridecanoic acid	C13:0	0.04	1.33	0.39	0.22
3	Myristic acid	C14:0	1.42	1.24	1.64	2.61
4	Pentadecanoic acid	C15:0	1.15	0.49	0.86	1.36
5	Palmitic acid	C16:0	44.74	19.08	41.93	43.12
6	Heptadecanoic acid	C17:0	0.24	0.04	0.31	0.23
7	Stearic acid	C18:0	0.27	0.21	1.13	0.03
8	Nonadecanoic acid	C19:0	ND	0.01	ND	ND
9	Arachidic acid	C20:0	0.25	0.01	0.74	0.58
10	Heneicosanoic acid	C21:0	0.01	0.01	0.06	0.01
11	Behenic acid	C22:0	1.59	0.01	3.19	4.19
12	Oleic acid	C18:1	3.11	0.01	3.90	5.31
13	Linoleic acid	C18:2	11.01	10.62	14.26	22.17
14	Linolenic acid	C18:3	13.16	4.28	6.23	8.38

ND, not detected.

ride and Sabouraud dextrose agar and allowed to solidify for susceptibility tests against bacteria, MRSA and fungi, respectively. Ciprofloxacin (Cip) (5 μg/disc) for bacteria, methicillin (5 μg/disc), oxacillin (1 μg/disc) and vancomycin (30 μg/disc) for MRSA, and amphotericin-B (Amp) (100 units/disc) for fungi were used as positive controls, and 5% DMSO was used as blind control in these assays. Finally, the inoculated plates were incubated at 37 °C for 24 h (bacteria), 35 °C for 24–48 h (MRSA), 28 °C for 24–48 h (yeasts) and 28 °C for 72–96 h (mycelial fungi). The zone of inhibition was observed and measured in millimeters. Each assay in this experiment was repeated four times.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of the FAME were tested in Mueller Hinton broth for bacteria, Mueller Hinton agar supplemented with 4% sodium chloride for MRSA, yeast nitrogen base for yeasts and Sabouraud dextrose broth for mycelial fungi to get concentrations of 16–0.06 mg/ml by the broth macrodilution method (Ericsson and Sherris, 1971). The culture tubes were incubated at 37 °C for 24 h (bacteria), 35 °C for 24–48 h (MRSA), 28 °C for 48 h (yeasts) and 72 h (mycelial fungi).

Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The MBC and MFC of the extracts were determined (Kartnig *et al.*, 1991) by plating 100 μl samples from each MIC assay tube with growth inhibition into freshly prepared Mueller Hinton agar (for bacteria), Mueller Hinton agar supplemented with 4% sodium chloride (for MRSA) and Sabouraud dextrose agar (for fungi). The plates were incubated at 37 °C for 24 h (bacteria), 35 °C for 24–48 h (MRSA), 28 °C for 48 h (yeasts) and 28 °C for 72 h (mycelial fungi).

Identification of methicillin-resistant *Staphylococcus aureus*

The MRSA isolates were identified and confirmed by conventional methods, *viz.* Gram's stain, catalase test, mannitol fermentation test and coagulase test (Colle *et al.*, 1996). Methicillin resistance was determined by a disc diffusion technique using 1 μg oxacillin per disc. Retesting was done using 5 μg methicillin per disc. A zone of inhibition less than 10 mm or any discernable growth within the zone of inhibition was the indication of methicillin resistance.

Results and Discussion

The fatty acid composition (as methyl esters) of some salt marsh halophytes, *viz. A. indicum, S. brachiata, S. maritima* and *S. monoica,* was determined and the relative percentages are presented in Table I. In *A. indicum,* the relative percentage of palmitic acid (44.74%) was high followed by the lauric acid (19.5%), linolenic acid (13.16%) and linoleic acid (11.01%). In *S. brachiata* lauric acid content (61.85%) was high followed by that of palmitic acid (19.08%), linoleic acid (10.62%) and linolenic acid (4.28%). Palmitic acid

Table II. Effect of fatty acid methyl ester (FAME) extracts of some halophytes[a] on some microorganisms.

Microorganism	Mean zone of inhibition[b] [mm]				Reference standard Cip/Amp	Minimum inhibitory concentration [mg/ml]				Minimum bactericidal/fungicidal concentration [mg/ml]			
	1	2	3	4		1	2	3	4	1	2	3	4
Bacillus subtilis	17.3 ± 1.3	20.5 ± 1.3	18.3 ± 1.3	18.5 ± 1.3	31.5 ± 2.4	0.125	0.06	0.125	0.125	0.25	0.125	0.25	0.25
Bacillus pumilus	14.3 ± 1.0	17.5 ± 1.3	16.3 ± 1.3	15.3 ± 1.0	34.3 ± 2.1	0.25	0.125	0.25	0.5	0.5	0.25	0.5	1
Micrococcus luteus	16.3 ± 1.3	18.3 ± 1.3	18.3 ± 1.0	17.3 ± 1.0	30.5 ± 2.4	0.25	0.125	0.125	0.125	0.5	0.25	0.25	0.25
Staphylococcus aureus	17.5 ± 1.3	20.3 ± 1.5	18.5 ± 1.3	18.5 ± 1.3	29.0 ± 1.8	0.125	0.06	0.125	0.125	0.25	0.125	0.25	0.25
Pseudomonas aeruginosa	12.3 ± 1.0	13.3 ± 1.0	12.3 ± 1.0	12.5 ± 1.0	30.0 ± 1.8	0.5	0.5	0.5	0.5	1	0.1	1	1
Klebsiella pneumoniae	12.3 ± 1.0	13.8 ± 1.0	12.8 ± 1.0	12.5 ± 1.0	32.5 ± 1.7	0.5	0.5	0.5	0.5	1	1	1	1
Escherichia coli	10.5 ± 0.6	12.0 ± 0.8	12.0 ± 0.8	11.0 ± 0.8	30.3 ± 1.7	0.5	0.5	0.5	0.5	1	1	1	1
MRSA	17.3 ± 1.0	20.5 ± 1.3	18.3 ± 1.0	18.3 ± 1.3	25.0 ± 1.7	0.125	0.06	0.125	0.125	0.25	0.125	0.25	0.25
Candida albicans	14.3 ± 1.0	18.5 ± 1.3	14.3 ± 1.0	12.0 ± 0.8	17.5 ± 1.3	0.5	0.125	0.5	0.5	1	0.25	1	1
Candida krusei	12.0 ± 0.8	18.3 ± 1.3	14.0 ± 0.8	12.3 ± 0.8	18.8 ± 1.3	0.5	0.5	0.5	0.5	1	1	1	1
Candida tropicalis	9.5 ± 0.6	17.3 ± 1.3	12.3 ± 1.0	11.0 ± 0.8	17.3 ± 1.0	2	0.125	0.5	2	4	0.25	1	4
Candida parapsilosis	14.3 ± 1.0	19.3 ± 1.3	14.0 ± 0.8	13.5 ± 1.0	18.3 ± 1.0	0.5	0.125	0.5	0.5	1	0.25	1	1
Aspergillus flavus	7.8 ± 0.5	12.0 ± 0.8	9.5 ± 0.6	8.8 ± 0.5	16.5 ± 1.3	8	2	8	8	16	4	16	16
Aspergillus fumigatus	6.8 ± 0.5	12.0 ± 0.8	7.8 ± 0.6	7.8 ± 0.5	15.5 ± 1.0	8	4	8	8	16	8	16	16
Aspergillus niger	7.8± 0.5	12.3 ± 0.5	7.8 ± 0.6	7.8 ± 0.5	15.3 ± 1.0	8	4	8	8	16	8	16	16

[a] 1, *A. indicum*; 2, *S. brachiata*; 3, *S. maritima*; 4, *S. monoica.*

[b] Diameter of zone of inhibition (mm) including disc diameter of 6 mm; mean of four assays ± standard deviation.

(41.93%) followed by lauric acid (23.88%), linoleic acid (14.26%) and linolenic acid (6.23%) were recorded in *S. maritima.* In *S. monoica,* the palmitic acid content was high (43.12%) followed by that of linoleic acid (22.17%), lauric acid (10.78%) and linolenic acid (8.38%).

In the present study, the saturated fatty acid content was found higher than the unsaturated fatty acid content. Similar results were reported from some marine macroalgae and the halophyte *Ipomoea pes-caprae* (Venkatesalu *et al.*, 2003, 2004; Chandrasekaran *et al.*, 2005).

Among the FAME extracts of the four halophytic species screened for antimicrobial activity, the extract of *S. brachiata* showed the highest activity against all the microorganisms tested (Table II). However, a potent antibacterial and a moderate anticandidal activity were recorded with the extracts of the other three plants screened. The highest mean zone of inhibition (20.5 mm) and the lowest MIC (0.06 mg/ml) and MBC (0.125 mg/ml) values were obtained for the extract of *S. brachiata* against *B. subtilis.* The extract showed good antimicrobial activity against *S. aureus, C. parapsilosis, C. albicans, C. krusei* and *M. luteus.*

Kabara (1978) reported that fatty acids such as oleic, palmitic, stearic, myristic, linoleic and linolenic acids show activity against *Clostridium perfringens* and *Staphylococcus pyogens.* Some *in vitro* studies have indicated that the fatty acid composition could either directly or indirectly affect the aflatoxin contamination (Passi *et al.*, 1984; Doehlert *et al.*, 1993; Burrow *et al.*, 1997). The report of Ouattara *et al.* (1997) which showed that lauric acid possesses antibacterial activity supports the present study. Similarly, long-chain unsaturated fatty acids, including linoleic acid, are well known to inhibit bacteria like *E. coli* (Dilika *et al.*, 2000; Sun *et al.*, 2003). Galbraith and Miller (1973) reported that long-chain fatty acids have higher antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria. The difference in the fatty acid sensitivity between Gram-positive and Gram-negative bacteria may result from the impermeability of the outer membrane of Gram-negative bacteria since it is an effective barrier against hydrophobic substances (Sheu and Freese, 1973; Sheu *et al.*, 1975).

Linolenic, linoleic and palmitic acids isolated from *Schotia brachypetala, Pelargonium* sp. and *Pentanisia prunelloides*, respectively, were found to have antibacterial activity (McGraw *et al.*, 2002; Seidal and Taylor, 2004; Yff *et al.*, 2002). In our study, all four plants contained one or more of the above said fatty acids in higher amount. There were only a few earlier studies carried out on the antimicrobial activity of FAME extracts of marine plants, though there were many studies on the antimicrobial activity of individual fatty acids (Anantharaj *et al.*, 2004; Chandrasekaran *et al.*, 2005). Since the halophytes screened for antimicrobial activity showed potential activity against most of the microorganisms tested in the present study, the FAME extracts of these plants can be used as potential antimicrobial agents.

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