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Phytochemical Composition and Antibacterial Potential of Hexane Extract from Malaysian Red Algae, *Acanthophora spicifera* (Vahl) Borgesen

[1]Nurul Aili Zakaria, [1]Darah Ibrahim, [2]Shaida Fariza Shaida and [1]Nor Afifah Supardy

[1]Industrial Biotechnology Research Laboratory,
[2]Plant Phytochemistry Laboratory School of Biological Sciences,
Universiti Sains Malaysia, Pulau Pinang, Malaysia

Abstract: The present study aims to study the antibacterial potentiality and phytochemistry constituents of hexane extract of *Acanthophora spicifera* from Malaysian water. It includes the evaluation of hexane extract for antibacterial activity using disc diffusion method, MIC, MBC and composition study using GC-MS analysis. The results revealed a potential antibacterial activity of hexane extract against MRSA and *P. aeruginosa* (27853) with low MICs of 0.125 and 0.50 mg/ml, respectively. To the best of our knowledge no details GC-MS analysis of volatile components of *A. spicifera* has been carried out from this local algae, eventhough details examination of fatty acids of *A. spicifera* from India has been performed. This prompted us to examine the preliminary phytochemical constituents of *A. spicifera* from Malaysian water. The GC-MS analysis revealed the presence of hydrocarbons, fatty acids and cholesterol. Oleic acid (14.58%) and n-hexadecanoic acid (24.73%) were the major components.

Key words: *Acanthophora spicifera* · Algae · Antibacterial · GC-MS · Hexane extract composition · Oleicacid

INTRODUCTION

Acanthophoraspicifera is more known as drift red algae that locally so called “bulung tumbung bideng” in Malaysian tropical seawater. It is an erect edible plant with growing height up to 40 mm and possesses numerous spines along the branches except for main branches. It occurs in wide range of habitats, as an epiphyte on other algae, on hard bottom or normally as drift algae due to its tolerance to high motions wave [1]. The colour can be shades of red, orange, dark brown depending on the water level or wave motion [2]. *A. spicifera* is utilized by human culture as raw foods for diets [3, 4], raw salads [5] and as flavouring and thickening ingredients in cooking [6, 7].

Having an estimated of 17% of population in Malaysian water, regardless no traditional and biological studies have locally been reported on this algal. In Malaysia the study is focusing on the ecological data [8, 9]. Several previous studies have revealed the bioactivity of active compounds isolated from the *Acanthophora* sp. such as antibacterial [10], antioxidant [11], anti-viral [12], anti-implantation [13] and anti-fouling activity [14]. Steroids and fatty acids ester of *A. spicifera* were reported to exhibit potent antitumor and antibacterial activity against human cancer lines and microorganisms [15, 16]. The purposes of this study reported here was to screen and investigate the antibacterial activity, antioxidant activity, total phenol contents and chemical constituents of the hexane extract of *A. spicifera*. The results show that the hexane extract exhibited antibacterial and antioxidant activity. Hence, *A. spicifera* might be a potential candidate for an edible antibacterial and antioxidants from natural based product.

MATERIALS AND METHODS

Collection of Algae: Field collection of algae sample was made from a flouting buoy for aquaculture at Pulau Gedung, Pulau Pinang in January, August and December 2008. The fresh sample was collected under the buoy by hand picking. The Fresh sample was kept in ice before transported to laboratory. Sample was authenticated by Associate Professor Dr. Shaida Fariza Sulaiman from

Corresponding Author: Nurul Aili Zakaria, Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Pulau Pinang, Malaysia. Tel: +60176742967, Fax: +604 6565125.

School of Biological Science. Fresh sample was cleaned under running tap water and gently brushed with soft brush to remove the sand, debris and attached epiphytes and animals [17]. The cleaned sample was oven-dried at 50-60°C until constant weight and was grounded to fine powdered (0.1-0.5 mm) using an electrical blender (3-5 min). The powdered sample was kept in sealed plastic bag in dry place to avoid deterioration.

Preparation of Extract: Oven-dried sample was extracted in solvents with increasing polarity; hexane, ethyl acetate, chloroform and methanol (1: 10; w/v) for 24-48 hr, using soxhlet apparatus as described by Nurul Aili Zakaria *et al.* (2011) [18]. The resulting extracts were concentrated to dryness under reduced pressure using rotary evaporator (40-50°C). All concentrated extracts were then left air-dried in fume hood and stored at 4°C. Solvents used are off analytically graded (Sigma, USA).

Microorganisms: Eighteen strains of bacteria were obtained from culture collection of Industrial Biotechnology Research Laboratory (IBRL), Universiti Sains Malaysia and American Type Culture Collection (ATCC) (Rockville, USA). These bacteria are 8 Gram-positive (*Bacillus subtilis*, *Bacillus cereus* (10876), *Bacillus licheniformis* (12759), *Bacillus spizizenii* (6633), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (12600), *Staphylococcus aureus, Staphylococcus epidermidis* (12228) and 10 Gram-negative (*Klebsiella pneumonia* (13883), *Klebsiella pneumonia*, *Pseudomonas aeruginosa* (27853), *Pseudomonas stutzeri* (17588), *Shigella boydii* (9207), *Acinetobacter anitratus*, *Citrotobacter freundii*, *Escherichia coli*, *Erwinia* sp. *Yersinia* sp.).

Antibacterial Assay: Evaluation of antibacterial assay was performed using disc diffusion technique [19,20]. One loopfull of each bacteria grown overnight in Nutrient agar (NA) plate at 37°C was suspended in 10 ml of sterile distilled water. The turbidity of the suspension was compare with 0.5 McFarland standard solution ($1.5x10^8$ cell/ml) and adjusted to $1.0x\ 10^5$ cell/ml. Then, 1.0 ml of the bacterial suspension was added into 20 ml of sterile molten NA and poured into sterile Petri dish (90 mm in diameter). The NA was left aside to solidify at room temperature for 15-20 min. Meanwhile, paper disc (Whatman no. 1, 6 mm) impregnated with 20 µl of 100 mg/ml of extract (corresponding to 2 mg/disc) was prepared and left air dried at room temperature. A disc impregnated with 20 µl of hexane was used as negative control and standard antibiotic of chloramphenicol (30 µg/disc) was used as positive control. The discs were then placed on the seeded agar plates and the diameter of the inhibition zones were measured after an overnight incubation at 37°C.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The determination of MICs and MBCs was performed using tube broth dilution method as described by Nurul *et al.* [19]. The extract was prepared to a series of initial concentration ranging from 0.023-6.0 mg/ml in sterile distilled water. The dilution series were performed in two folds dilution. This assay was performed aseptically in screw capped test tube. A freshly prepared bacterial suspension was prepared by suspending a 24 hr of bacterial culture in 10 ml of sterile distilled water, adjusted to $1.0x10^5$ cell/ml and was vortexed. Then, a fixed volume of the freshly prepared inocula (0.5 ml), 1.5 ml of sterile nutrient broth (NB) and respective extract (1.0 ml) differs in concentrations were pipetted into the screw cap test tube to attain final concentrations ranged from 0.007-2.0 mg/ml. The test tubes were then incubated overnight at 37°C. Turbidity of the test tubes was observed via naked eyes. MIC value was stated as the minimum concentration of the tube that shows no turbidity (no bacterial growth). Whereas for the MBCs, the test tubes that showed no turbidity were streaked on NA plate and incubated at 37°C for 24 h. The MBC value is stated as the minimum concentration that showed no visible bacterial colony growth.

Gas Chromatography-mass Spectrometry (GC-MS) Analysis: Hexane extract was dissolved in hexane and filtered over a nylon membrane filter (0.45 µm pore size) using syringe and transferred into a vial for analysis. GC-MS analysis was performed using Hewlett-Packard 6890N Network GC system equipped with Hewlett-Packard 5973 inert mass selective detector mass spectrometer. The GC-MS conditions are as follows: HP-5MS 30 m length by 0.25 mm i.d. by 0.25 µm thick capillary column (Agilent, USA): injector temp. 280°C, split ratio at 5:1 and injection vol. 1.0 µl. Oven temperature programmed at 70°C initial temperature held for 2 min, then increased from 30°C/ min to 285°C and held at this temperature for 20 min. The carrier gas was helium at a flow rate of 1.2 ml/min. Mass spectrometry zone temperature was: transfer line at 285°C and the electron impact mass scan was at the range of 35-650 amu. Ionization voltage was at 70eV. Compounds were identified by comparing the mass spectra of the peaks using computer-matching with NIST02 library.

RESULTS AND DISSCUSSION

The antimicrobial activities of the hexane extract were evaluated using disc diffusion method against 8 Gram-negative and 10 Gram-positive bacterial strains. Out of all bacterial tested, only a Gram-positive bacterium and a Gram-negative bacterium were susceptible to the extracts. The hexane extract showed antibacterial activity against both Gram-positive bacterium (MRSA) and Gram-negative bacterium (*P. aeruginosa* ATCC 27853) as seen in Table 1. Whilst, chloroform and ethyl acetate extract only showed inhibitory effect on *P. aeruginosa* ATCC 27853 with inhibition zone of 9.0 mm (Table 1). No inhibitory effect was showed by methanol extract on bacteria tested.

Table 1 : Antibacterial activity of hexane extract of *A. spicifera*

	Disc diffusion							
	Inhibition zone (mm)							
	Extract							
Bacteria	HE	C	EA	M	+ve	-ve	MIC (mg/ml)	MBC (mg/ml)
Gram positive								
Methicillin -resistant								
Staphylococcus aureus (MRSA)	9.0	-	-	-	17.5	-	0.50	> 2.0
Gram negative								
Pseudomonas aeruginosa (27859)	11.0			-	13.0	-	0.125	2.0
		9.0		-		-	0.250	>2.0
			9.0	-		-	0.250	0.50

"-"indicates no inhibition zone and no further determination of MIC and MBC conducted

"+ve" chloramphenicol was used as positive control, "-ve" methanol solvent as negative control

Table 2: Percentage composition of hexane extract of *A. spicifera*

No.	RT	Area (%)	Compounds	Matching factor (%)
1	9.46	0.28	Heptadecane	97
2	9.80	1.06	Tetradecanoic acid	99
3	10.27	1.81	2-Pentadecanone, 6,10,14-trimethyl	99
4	10.33	0.58	Pentadecanoic acid	94
5	10.44	2.76	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	86
6	10.67	0.37	Hexadecanoic acid, methyl ester	96
7	10.78	6.03	Hexadecenoicacid, Z-11-	99
8	10.89	24.73	n-Hexadecanoicacid	99
9	11.03	0.61	Eicosane	98
10	11.30	0.88	Bromoacetic acid, octadecyl ester	83
11	11.51	0.61	Heneicosane	96
12	11.76	14.58	Oleic acid	99
13	11.83	6.04	Octadecanoic acid	86
14	11.97	1.47	Docosane	97
15	12.41	2.94	1-Heptadecene	92
16	12.48	0.33	1-Docosene	93
17	12.51	0.74	9-Tricosene, (Z)-	83
18	12.61	0.41	17-Pentatriacontene	83
19	12.84	1.81	Tetracosane	99
20	13.28	2.12	Pentacosane	97
21	13.59	0.72	1,2-Benzenedicarboxylic acid, diisooctyl ester	90
22	13.77	2.40	Hexacosane	98
23	14.33	2.45	Heptacosane	99
24	14.98	2.02	Heneicosane	96
25	15.75	1.74	Nonacosane	99
26	16.68	1.34	Hexacosane	97
27	17.80	1.07	Octadecane, 1-iodo-	97
28	18.83	0.57	Cholesterol	99
29	20.48	1.64	Cholest-4-en-3-one	99
30	23.52	5.00	Cholestane-3,6-dione, (5α,17α,20S)-	86

Table 3: The chemical class distribution of the volatile components of *A. spicifera*

Compound class	Area (%)	Number of compounds
Fatty acids	54.27	7
Hydrocarbons	21.93	16
Cholesterol	7.21	3
Aromatic dicarboxylic acid ester	3.48	2
Others	1.81	12

This current finding was in contrast with Laila (2003) [16], which reported that methanolic extract of *A. spicifera* from Karachi Coast, Pakistan showed moderate antibacterial activity against several bacteria such as *B. cereus*, *E. coli* and *S. aureus* with the inhibition zone ranged from 7.5-8.5 mm. This finding might be in contrast due to several factors such as seasonal variation, environment conditions, geographical, extraction method and others. However, certain similarity was found between the present study whereby, no inhibitory activity was detected on *P. aeruginosa*, *K. pneumonia* and *S. boydii* by the methanol extract. Interestingly, the extracts showed better antibacterial activity against *P. aeruginosa* ATCC 27853 as compared with MRSA. The extracts also were found to exhibit bactericidal activity on *P. aeruginosa* ATCC 27853, whilst only bacteriostatic activity was exerted on MRSA.

Moreover, the extracts also portrayed a good antioxidant capability (15.208 to 50.098%) with high phenolic contents ranged from 5.33 to 40.583% [18]. Positive linear correlation between the antioxidant activity and the phenolic contents of the extracts has been reported by Nurul Aili Zakaria *et al.* [18] which showed that the extracts that exhibited high antioxidant activity also exhibited high level of phenolics. Thus, it suggests the contribution of the algal-polyphenols in the extract to the antioxidant activity. Among the extracts, hexane extract exhibited potent antioxidant activity (46.831±0.685%) and high total phenolics (29.917±0.382%), respectively [18]. Thus, hexane extract became the interest for further assays in identifying the phytochemical constituents using GC-MS analysis.

The relative compositions of the volatile compounds identified are represented in Table 2. The GC-MS analysis resulted in the identification of a total of 30 compounds with matching factor exceeding 80%, which representing 88.70% of the constituents of volatile components from hexane extract. The chemical class distribution of the volatile components of the algal are reported in Table 3. The volatile components were divided into five classes, which were fatty acids, hydrocarbons, cholesterol, aromatic dicarboxylic acid ester and others.

Oleic acid (14.58%)and n-hexadecanoic acid (24.73%) were the major compounds detected, along with the minor compounds of octadecanoic acid, hexadecanoic acid, Z-11-, Cholestane-3,6-dione, (5α,17α,20S)-, 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester, heneicosane, nonacosane and hexacosane. Results of our study matched with a composition study of *A. spicifera* from Pakistan in respect to the major compounds of fatty acids detected, octadecadienoic acid (36.05%) and hexadecanoic acid (8.30%) [16]. However, the fatty acids composition of *A. spicifera* from India reported that palmitic acid, arachidonic acid and eicosapentanoic acid as the dominant fatty acids [21]. This difference can be attributed to the climatic and geographical conditions from where the algal was harvested. This is the first phytochemical analysis of volatile components performed by GC-MS reported for *A. spicifera* from Malaysian water, as previous study of this local algal only focusing on the ecological and distribution pattern [9].

In this study, the alkyl chains of the hydrocarbons were ranged from C_{18} to C_{29}. Based on the GC-MS analysis of the phytochemical constituents, it can be said that unsaturated fatty acids and hydrocarbons do contributed to the inhibitory and antioxidative effects of hexane extracts. Heptadecane and hexadecane was reported as the common hydrocarbons found in seaweeds [22]. In this presence study, most of the hydrocarbons detected have been documented to exhibit inhibitory effect on *P. aeruginosa* [23, 24]. Meanwhile, the capability of oleic acids and hexadecane that exhibited antimicrobial and antioxidant capabilities have also been reported [25]. Fatty acids act as an anionic surfactants and exhibited antibacterial and antifungal properties at low pH [26]. In this study, the pure compounds and its antimicrobial and antioxidant mechanisms were not known. Thus, further studies are currently in progress to identify the active compounds.

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