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Fatty Acid Extracts of Sea Pen (*Virgularia gustaviana*) and Their Potential Applications as Antibacterial, Antifungal, and Anti-Inflammatory Agents

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Abstract**—**In this study, the crude extracts of *Virgularia gustavina* were examined as antibacterial, antifungal and anti-inflammatory agent. To assess inflammation, Xylene was applied to the ear of mice. The mice of the experimental group were fed with doses of 10 mg/kg, 20 mg/kg, and 40 mg/kg of lipid extract of chloroform and hexane as a separate group and then statistical analysis was performed on the results. Chloroform and hexane extracts of sea pen have strong anti-inflammatory effects even at low doses which is probably due to 54% arachidonic acid. Antibacterial and antifungal effects of hexane and chloroform extracts were measured with MIC and MBC methods and it is shown that chloroform extract has best activity against *Staphylococcus aureus* on 125 µg/ml doze in MIC method.

Keywords**—**Sea pen (*Virgularia gustaviana*), lipid extract, anti-inflammatory, antibacterial activity.

I. Introduction

THE ocean is considered to be a great source of potential drugs. As marine environment contains ecological, chemical and biological diversity, several studies are underway in this regard. The recent studies on the bioactive compounds isolated from marine organisms have shown that they have anticancer, antimicrobial, antifungal or anti-inflammatory and other pharmacological activities [1], [26]. Marine sessile invertebrates are the important source of natural products. Although marine sessile invertebrates do not have any physical mechanisms for defense; they have special chemical mechanisms to protect themselves against other animals [1], [13]-[16]. Soft corals elaborate a large variety of bio active chemical compound such as terpenoids, sesquiterpenoids, diterpenoids, alkanoids and fatty acids like arachidonic acid.[5], [6].

Pennatulacea are a group of Anthozoa, animals that baffled to the ground of water living in all regions of the world usually regions above 6100 meters of tide [4], [7]. Sea pens are the colonial feathery animal which stand upright in the sand [2]. They are benthic animals and their appearance is feather-like. They are found at water depth ranging from 15-200 m or more. They have 300 species belonging to order Pennatulacea, and suborder Sessiliflorae and subsessiliforae. Sea pens belong to the class Anthozoa (phylum Cnidarians) [3], [4].

Fatty acids are involved in many biological reactions. Fatty acids in the sea pen belong to the essential fatty acids which are a series of chemical compounds called eicosanoids in the body. These materials possess some anti-inflammatory, antibacterial and antifungal activities and prevent the release of compounds that play a role in coagulation of platelets and vasoconstriction [5].

Inflammatory properties of lipid extract of sea pen *Virgularia gustavina* was performed on mice as described in [8] and antibacterial and antifungal activities of this compound are investigated by disk diffusion method and Minimum inhibitory concentration (MIC), Minimum Fungicidal concentration (MFC) and MBC Minimum Bactericidal concentration in this study [9].

II. Materials and Methods

A. Animal Materials

Virgularia gustavina were collected by searching in low inter tidal zoon from Suru estuary in Bandar Abass and stored on freezer -20° until extraction.

B. Extraction

For extraction, the Blight and Dyer method was used [10]. In order to obtain lipid extract, samples were chopped in small pieces and then solution of chloroform-methanol (1:9) and methanol-hexane (1:9) were applied and kept in the refrigerator at 8 °C for 24 hours. The resultant was filtered by Whatman No. 1 paper filter. Finally, the solvent was removed by evaporation using rotary evaporator. After complete solvent evaporation, extracts were poured in closed small glass containers and were kept in the refrigerator at 8 °C [8], [11], [12].

C. Identification of Fatty Acids

Fatty acids obtained from lipid extract of *Virgularia gustavina* were separated by GC-MS. To identify fatty acids, two methods were carried out. First of all, to analyze the peak achieved from GC-MS, retention index formula: $RI = T_n + 100(\frac{T_x - T_n}{(T_n + 1) - T_n})$ was used and RIs was used and RIs

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were obtained by comparing them with RI database. Finally, identification was approved by the Eight peak book [13].

D. Sterols Profile

Cold extracting with chloroform and methanol was used to determine the sterols profile [14], [15]. The goal was to measure sterols in lipid extract of sea pen using remote gas chromatography [16], the same method described on [8].

E. Mouse Ear Inflammation Test

Small male NMRI mice with weight of 25-20 g purchased from the Pasteur Institute were used in this study. Animals were kept with normal clock period at temperatures from 24 to 22 °C with enough food and water. In each series of the experiments, 3 mice were evaluated. Also for inflammation in the ears of mice, 0.03 µl of Xylene was applied to the dorsal and anterior surface of the right ears of animals just 15 minutes after treatment by Fatty acid extracts [8], [17].

F. Anti-Inflammatory in vivo Assay

Mouse ear edema method was used for anti-inflammatory assay which has been explained on last study [8], [18].

$$\% \text{ Inhibition} = (\Delta Pc - \Delta Pt) / 100 \times \Delta Pc$$

where ΔPc: mean weight variation in the control group; ΔPt: mean weight variation in the treated group [18].

G. Antibacterial Test

For microbiological tests, the antimicrobial properties of hexane extract and chloroform by marine *Virgularia gustaviana* were studied against four strains of bacteria including gram positive bacteria *Bacillus subtilis* (Atcc6633) and (Atcc25923) *Staphylococcus aureus*, and gram negative bacteria *Pseudomonas aeruginosa* (Atcc27853) and *Escherichia coli* (Atcc25922) in the laboratory of Marine Science and Technology. Consequently, the anti-fungal properties of both extracts were studied against a yeast *Candida albicans* (Atcc 10231).

Three tests were used to evaluate the antimicrobial and antifungal effects. The disk diffusion test was performed, and then the extract, which displayed the desired effect (diameter more than 9 mm) in the disk diffusion test, was evaluated by MIC test and MBC took place [9], [19].

H. Agar Diffusion Disc Test

Diffusion agar test is developed for testing the susceptibility of rapidly growing bacteria. Prepared paper disk was placed on agar surface plates. After an overnight incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disk. The size of the circle is a measure of the combined effect so that the larger circle around the disk demonstrates the more effective combination [20]. For this test, certain strains of bacteria were used, including both Gram-positive, *Bacillus subtilis* and *Staphylococcus aureus,* and gram-negative, *Escherichia coli* and *Pseudomonas aeruginosa.* Then, each bacteria suspension was prepared in sterile physiological serum containing 108 × 5/1 cfu/ml of opacity standard 0.5 McFarland. Then, suspension applied to Mueller Hinton agar medium that was prepared 24 hours before the plates were incubated at 37 °C. After that 0.1 g of dry extract dissolved on 10 ml solvent and then 100 µl of this solution was added to disk so each disc contains 1 mg. Disks prepared in this way were placed on the surface of the medium. The disc containing antibiotics amoxicillin, ampicillin, cephalexin and erythromycin were used as positive control. To evaluate anti-fungal test, 1 mg extract applied on disks and they were placed on the surface of the medium (Dextrose agar). Nystatin was used as positive control [21].

I. MIC

Hexane and chloroform extracts of marine sea pen in different concentrations were prepared [21]. Eight sterile tubes for each strain were chosen that each tube contains 0.5 ml culture medium for bacteria nutrient broth (Merck, Germany) and hexane and chloroform extract of concentrations (2000 µg/ml), (1000 µg/mg), (500 µg/ml), (250 µg/ml), (125 µg/ml) (62.5 µg/ml) Finally, same concentrations of hexane solvent and water are used as control group. A sterile suspension of 108 × 5/1 cfu/ml is equal to 5.0 McFarland turbidity standard for bacteria in each prepared tube. The tubes incubated for 24 hours at 37 °C for bacteria and 48 hours at 24 °C were placed in an incubator for fungi. [22]. After that, the turbidity tubes were examined, representing growth of microorganisms. The lowest concentration of antimicrobial agents that inhibit bacterial growth and the absence of turbidity was determined as MIC was placed [21].

J. MBC

MBC test is used to determine the minimum concentration for antibiotics or antifungal to kill microorganisms. The MIC test demonstrates the lowest level of antimicrobial agent that inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent that results in microbial death.

To determine the minimum concentration for antibiotics, the dilutions representing the MIC and two dilutions which indicate the more concentrated test product were plated to nutrient agar medium (Merck, Germany). Then, plates are incubated for 24 hours at 37 °C and incubated for 48 hours at 24 °C were for growth of *Candida albicans* [22], [23].

K. Statistical Analysis of Data

Data from the ear edema test were analyzed by One-way ANOVA and in case of significant difference, Tukey - Kramer was used.

III. Results

Virgularia gustaviana is the one of the species of sea pens in the Persian Gulf which the extract of this animal contains fat about 96/21%, 57/95% cholesterol and fatty acids such as: pentadecanoic acid, ethyl arachidonic acid, octadecanoic acid, heptadecanoic acid. These compounds were potentially responsible for the pharmacological properties of this animal.

Characterization of hexane and chloroform extract of sea pen represents that compounds such as arachidonic acids and Heptadecanoic acid have anti-inflammatory properties which are shown in Tables II and III. Fig. 1 shows the sterol of hexane and chloroform extracted isolated from sea pen *V. guastavina.*

TABLE I
MEASUREMENT OF STEROLS IN LIPID EXTRACT OF SEA PEN

Result(mg/kg)	Sterols
1910/38	Cholesterol
139/59	Brassicasterol
1381/22	24-methylen-Cholesterol
645/72	Campesterol
310/21	Campestanol
58/59	Stigmasterol
1050/93	Beta-sitosterol
3291/6	Total Closterole
5860/31	*Total sterols*

TABLE II
RESULT OF FATTY ACIDS FROM HEXANE EXTRACT OF SEA PEN LIPIDS USING GC-MS AND RI

NO	Fatty acid	%	RI
1	Octadecanoic acid methyl ester	15.6	1100.78
2	Ethyl arachidonate	54.2	1500.10
3	Total fatty acid of hexane extract	69.8	

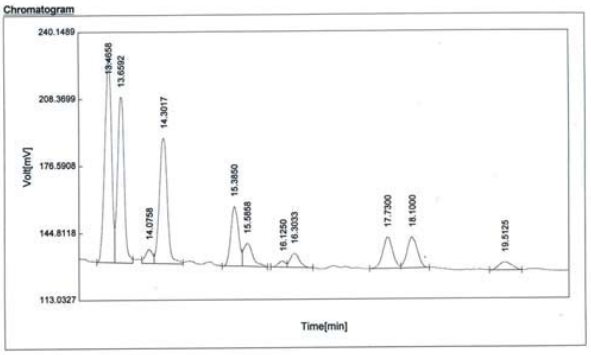
The results of inhibition of hexane lipid infusion of sea pen extract with doses of 10, 20 and 40 mg/kg to male mice caused a significant reduction in inflammation compared to control group that received only normal saline ($P < 0.001$) as shown in Table I [8].

$$\{RI = T_n + 100(\frac{T_x - T_n}{(T_n + 1) - T_n})\}$$

GC-MS analyze of Methanol-Hexane extract showed that *arachidonic* acid in retention time 17:00 with 54.2% and identification proved using Retention Index formula(RI) (Figs. 2 and 3).

The results of the antimicrobial properties showed that the Hexane and chloroform extracts had the desired effects. In Table IV, the results of the antimicrobial effect on the four bacteria were shown. Hexane and chloroform extract on gram-negative and gram positive bacteria had the significant effects.

The extract which has favorable effects on bacteria on disk diffusion method were chosen for MIC and MBC test. Results of MIC and MBC tests were shown in Tables IV and V.



Result

Num	RT[min]	Area	Type	Width[sec]	Area%
1	13.4658	662.0594	BV	16.5000	26.5328
2	13.6592	526.6864	VB	16.9500	21.1075
3	14.0758	48.3772	VV	10.9000	1.9388
4	14.3017	478.6764	BV	26.6500	19.1835
5	15.3850	223.7809	VV	18.3000	8.9683
6	15.5858	107.5076	VB	23.5500	4.3085
7	16.1250	20.3056	BV	14.7500	0.8138
8	16.3033	63.6509	BV	24.0000	2.5509
9	17.7300	153.9438	BV	28.1500	6.1695
10	18.1000	157.4349	VB	26.6500	6.3094
11	19.5125	52.8301	BV	30.7000	2.1172
Sum		2495.2530			

Fig. 1 The Hexane and Chloroform Extracts Sterol Profile of Sea Pea

TABLE III
HEXANE EXTRACT OF SEA PEN ON INFLAMMATION CREATED BY XYLENE THE IN THE EAR OF SMALL LABORATORY MICE

Level of inhibition%	Ear inflammation	Treatment mg/kg
	0/001±0/0084	Control group
94/12	0/017±0/00123	Dexamethasone15 mg/kg
60	0/0068±0/00206	hexane extract 10 mg/kg
96/48	0/0006±0/00293	hexane extract 20 mg/kg
80/159	0/0033±0/00151	hexane extract 40 mg/kg

TABLE IV
THE RESULTS OF THE ANTIBACTERIAL EFFECTS OF EXTRACT MARINE SEA PEN *V. GUSTAVINA* BY DISK DIFFUSION METHOD

Treatment	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*B. subtilis* ATCC 6633	*P. aeruginosa* ATCC 27853
Chloroform Extract Disk/ 1 mg	13 ± 0/3 cm	20 ± 0/5 cm	7/3± 0/3 cm	7/5± 0/1 cm
Hexane Extract Disk/ 1 mg	14±0/5 cm	12± 0/7 cm	13±0/7 cm	7/5±0/1 cm
Streptomycin 30 mg in each disc	13±0/1 cm	15±0/1 cm	17±0/5 cm	R
Ampicillin 30 mg in each disc	16±0/1 cm	27±0/4 cm	8±0/1 cm	R
Hexane	-	-	-	-
Chloroform	-	-	-	-

The numbers indicate the diameter and standard deviation is in millimeters
R: Symptoms of the bacterial extract desired impact.

TABLE V
MIC AND MBC OF HEXANE EXTRACT OF SEA PEN (*VIRGULARIA GUSTAVIAN*) WITH GOOD EFFECT (THE DIAMETER OF 9 MM) IN THE DIFFUSION

Test's	MBC		MIC	
Bacteria	Hexane	Choloroform	Hexane	Choloroform
Escherichia coli	1 mg/ml	1 mg/ml	µg/ml 500	µg/ml 500
Staphylococcus aureus	250 µg/ml	1 µg/ml	µg/ml 125	µg/ml 500
Bacillus subtilis	none	1 µg/ml	µg/ml 500	µg/ml 500

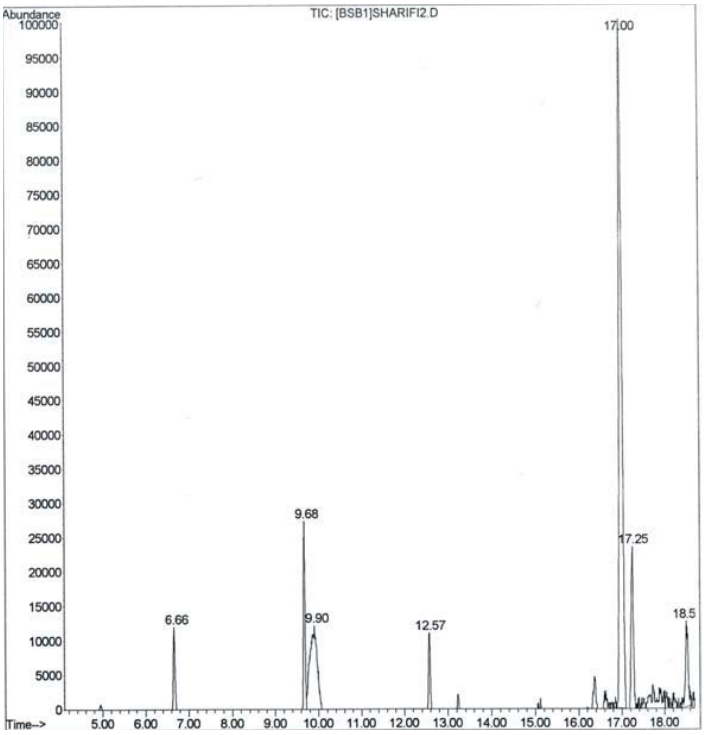


Fig. 2 Measurement of Fatty Acids Related to Hexane-Methanol Extract Gc-Ms Analyzed

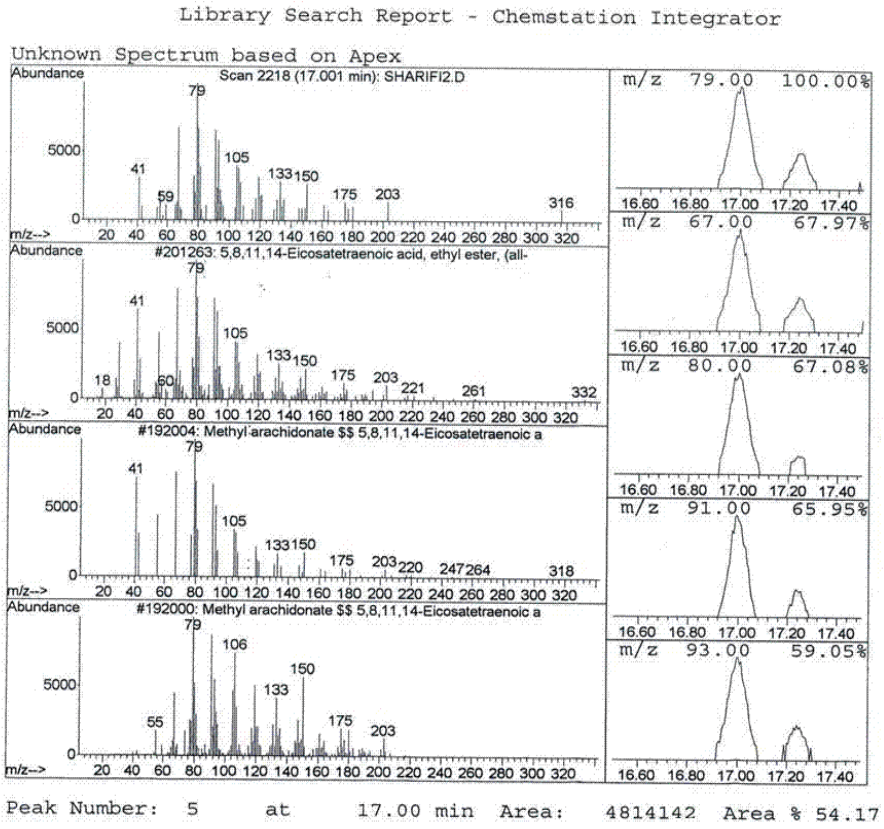


Fig. 3 The Peak of Methyl Arachidonate, Peak Number 5 in Retention Time 17:00 min

Fig. 4 *S. aureus* Disc Diffusion Test, Plate A treated by 1 mg/disc of Chloroform Extracted and Plate B treated by 1 mg/disc of Hexane Extract

IV. Discussion

The findings of this study proved the biological activity of fraction isolated from the sea pen. The results are comparable with Al-Hindawi et al. about hydro alcoholic extract of celery. As chromatography results showed, arachidonic acid obtained from hexane extract stands as the most abundant and perhaps most important facilitators of eicosanoids. In order to make eicosanoids, arachidonic acid must first be released. The substance is important in making anti-inflammatory drugs [24].

Arachidonic acid is a fatty acid consisting of 20 carbons (C20) and involved in inflammation [25]. In order to make eicosanoids, when cell damaged, the enzymes such prostaglandin, thromboxane and leukotrienes which transform arachidonic acid will be activated and eicosanoid cause inflammation [26].

Based on the study, the application of fatty acids isolated from fish and plants are to supplement food [27]. Due to the large amount of arachidonic acid extracted from hexane and since it is a fatty acid important for the body and due to its mechanism of action, this extract can be used in anti-inflammatory drugs. [28]. The study on lipid extract of sea pen *Virgularia gustavina* showed hexane extract containing 54.17% arachidonic acid which could be useful in the biological effect. In a previous study, anti-inflammatory properties were mentioned [8].

The antibacterial tests such as disk diffusion, MIC and MBC were evaluated and the results showed that ethyl acetate extract of *L. pauciliforum* on *E. coli* and *S. aureus* ATCC 6358 comparing to the control group indicated significant differences [19].

(±)-pestalachloride D is an antibacterial compound derivative from marine fungus *Pestalotiopsis* sp. and exhibited moderate antibacterial activity [29].

In this study, the maximum diameter of the extract on *Staphylococcus aureus* was 20 cm and also the MIC test results showed that the extract at a concentration of 125 mg/ml of the bacteria was more effective. Therefore, the effect of the extract on gram-positive bacteria is higher than gram-negative bacteria.

V. Conclusions

As a result, all the compounds extracted from marine sea pen *Virgularia gustavina* had anti-inflammatory effect on mouse ears. Inflammation may be because of large quantity of arachidonic acid of hexane extract. The biological properties of fatty acid extracted compound of *Virgularia gustavina* collected from Bandar Abbas are studied for the first time.

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