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GC-MS Analysis and Pharmacological Potential of Fixed Oil of *Eluphia dabia*

Muhammad Nisar, Shujaat Ali Khan and Ihsan Ali

Institute of Chemical Sciences, University of Peshawar, Peshawar -25120, Pakistan

Abstract: The aim of present study was to determine the fatty acids of aerial parts of *Eluphia Dabia* D. Don Hockr and investigate its biological potential. Fixed oil was extracted from *n*-hexane fraction of *E. Dabia* and analyzed by GC–MS to identify its chemical composition of the oil. Resultantly, twenty four constituents were identified which included both saturated and unsaturated fatty acids. Among analyzed F.A, tetracosanoic acid was found to be the major component (7.51%), other predominant constituents were behenic acid (4.87%), linoleic acid (3.59%), palmitic acid (3.17%), eruccic acid (3.14%) oleic acid (1.91%), arachidic acid (1.02%) and stearic acid (1.01%), while the concentration of rest of fatty acids identified remained below 1%. The oil was subsequently, screened for antimicrobial, insecticidal and phytotoxic studies. The fixed oil showed good antibacterial activity against *Klebsiella pneumonia* and *Escherichia coli* with zone of inhibition ranging between 16-18 mm. It exhibited reasonable phytotoxicity while no interesting antifungal and insecticidal activities were observed.

Key words: *Eulophia dabia* · Fatty Acids · GC/MS · Antibacterial · Antifungal · Insecticidal · Phytotoxic Activities

INDRODUCTION

Eulophia dabia belongs to family *Orchidaceae* and is a medicinally important worldly orchid used for the treatment of tumours and several health related ailments by the local healers in India. The plant extract is used in traditional medicinal system as an antitumor, anthelmintic, vermifuge and blood purifier [1, 2]. Previous studies showed that *n*-hexacosyl alcohol and lupeol are reported from the tubers were also isolated [3]. Various class of bioactive compounds have been isolated from the title plant such as phenanthrene derivative 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol which showed good antiproliferative activity against human breast cancer. Later on, same group successfully isolated and synthesized 2,7-dihydroxy-3,4-dimethoxyphenanthrene and named it as Nudol [4]. Literature survey has revealed that *E. Dabia* has not been investigated seriously for its pharmacological properties.

MATERIALS AND METHODS

Plant Material: The plant material was collected from Khwazakhela, Swat, Khyber Pakhtunkhwa Pakistan, during April 2011. *Eulophia dabia* was identified by an experienced taxonomist Dr. Hassan Sher, University of Swat, Pakistan.

Extraction of Fatty Acids: Shade-dried plant *E. Dabia* D. Don Hockr (50 g) was powdered and *kept in flask* for cold extraction in *n*- hexane for 10 days. Anhydrous $MgSO_4$ (0.5 g) was also added to remove trace amount of hygroscopic substance. The extract was filtered and concentrated *in vacuo* to obtain yellow oil (1.38 g, 2.76%). The fixed oil obtained was kept in freezer and subjected to GC-MS and biological studies.

Biological Profile

Antibacterial Activity: The Fixed oil was evaluated against various human pathogens employing agar well diffusion method as discussed previously [5-8]. Nutrient agar plates were swabbed with a 2-8 hrs broth culture of the respective bacteria. Samples (3 mg/ml) were added into the wells drug in the medium of these plates. The plates were incubated at 37°C for 14-19hrs and the activity was calculated by measuring diameter of zones of inhibition (mm). Streptomycin was used as a standard antimicrobial drug at 2 mg/ml. The analysis was performed in triplicate.

Corresponding Author: Muhammad Nisar, Institute of Chemical Science University of Peshawar, Peshawar -25120, Pakistan.

Antifungal Assay: The antifungal activity was checked by the Agar tube dilution method [9]. The fatty acids were dissolved in DMSO (24 mg/1.0ml). Sterile Sabouraud's dextrose agar medium (5 ml) was placed in a test tube and inoculated with the sample solution (400 µg/ml) kept in slanting position at room temperature (25°C)overnight. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 29°C and growth inhibition was observed. Miconazole and amphotericin B were used as standard drugs, while miconazole, amphotericin B and DMSO were used as positive and negative controls respectively.

Insecticidal Activity: The insecticidal assay was determined by direct contact application method on filter paper [10]. 3 ml of the fixed oil (1 mg/ml) was applied to filter paper of 90 mm diameter and was placed in separate Petri dish along with 10 adult's insects of each *Tribolium castaneum*, *Rhyzopertha dominica* and *Callosbruchus analis* respectively. Permethrin (235.71µg/cm^2) was used as reference insecticide. All these were kept without food for 24 hrs after which mortality count was performed.

Phytotoxicity Activity: *In vitro* phytotoxicity screening of fixed oil extracted from the title plant was carried out according to standard protocol [11]. The fixed oil was tested against *Lemna minor*. In this biological screening, three flasks were inoculated with a necessary stock solution of (20 mg/ml) to get a final concentration of 500, 50 and 5µg/ml, respectively. Each flask was added a 20 ml medium and 10 plants each one comprising rosette of three fronds. Parquet was used as a standard growth inhibitor. The whole flasks were kept in growth cabinet for incubation up to seven days. After this growth, regulation in percentage was determined with reference to the negative control.

RESULTS AND DISCUSSION

The chemical composition of fixed oil (fames) was determined by GC-MS analysis listened in Table 1. The results obtained from the GC-MS analysis showing the relative concentration of individual esterified fatty acids (Fames) based on the external standard method and the standard deviation values among the three measure results were calculated in each case. Analyses were performed in triplicate. The values of area and concentration are listed in Table 1 and represent the average of three measurement results. Quantification of FAMEs was performed using three points calibration curve with R2 value less than 0.99 (R2 > 0.99) in each case. Fig. 1 is the GC- MS chromatogram of *Eulophia dabia* seed oil with properly labeled signals of analytes detected. Both the saturated as well as unsaturated fatty acids were detected in the sample under studies. Tetracosanoic acid was found in highest concentration (7.51%), which showed broad spectrum antibacterial activity against different human and fish pathogens [12].

Behenic acid, methyl ester (4.87%), palmitic acid, methyl ester (3.17%), linoleic acid, methyl ester (3.59%), oleic acid, methyl ester (1.91%), arachidic acid methyl ester (1.02%) and stearic acid, methyl ester (1.01%), while the concentration of the rest of fatty acids identified were less than 1%. (Table1). From the results it is clear that *E. Dabia*, besides its toxicity, can also be used in various pharmaceutical products as it contains different bioactive compounds like fatty acids. The method applied is a reliable method of analyzing simultaneously many fatty acid components in a single run [13-16].

The fixed oil was also screened for antibacterial activity and showed promising results against *Klebsiella pneumonia* (18 mm) and *Escherichia coli* (16 mm). Similarly, as expected, the fatty oil exhibited good phytotoxic activity at low concentration, while no encouraging antifungal and insecticidal activities were observed.

Experimental

Chemicals and Reagents: Boron triflouride solution in methanol (10 %) was purchased from Fluka Chemie (Buchs, Switzerland). Sodium hydroxide solution (methanol; 0.5 N) and sodium chloride (analytical grade) were obtained from Merck (Darmstadt, Germany) while methanol (HPLC grade), *n*-hexane (HPLC grade) were acquired from Fischer Scientific (Leicestershire, UK). Helium gas (99.9999%) from Pak gas (United Arab Emirates) was procured. Tridecanoic acid methyl ester and Fatty acid methyl esters (FAMEs) 37 components standard mix were obtained from AccuStandard (Newhaven, Connecticut USA). These 37 components are: methyl ester of hexanoic acid, caprylic acid, capric acid, undecanoic acid, lauric acid, tridecanoic acid, myristic acid, myristoleic acid, pentadecanoic acid, pentdecenoic acid, palmitic acid, palmitoleic acid, margaric acid, heptadecenoic acid, stearic acid, oleic acid, elaidic acid, octadecenoic acid, linoleic acid, octadecadienoic acid, g-linolenic acid, linolenic acid, archidic acid, eicosenoic acid, eicosadienoic acid, 8,11,14-eicosatrienoic acid, heneicosanoic acid, arachidonic acid, eicosatrienoic acid,

Table 1: Quantification results of fixed oil isolated from *Eluphia Dabia*

ID#	Name	R. Time	Area	Conc. (%)	Std. Dev.
1	C6:0; Hexanoic acid, methyl ester	3.034	8211	0.09	0.002
2	C8:0; Caprylic acid, methyl ester	4.917	3556	0.02	0.003
4	C11:0; Undecanoic acid, methyl ester	7.608	1462	0.01	0.001
5	C12:0; Lauric acid, methyl ester	8.493	35999	0.15	0.002
6	C13:0; Tridecanoic acid, methyl ester	9.568	1757	0.01	0.002
7	C14:0; Myristic acid, methyl ester	10.910	74291	0.29	0.003
9	C15:0; Pentadecanoic acid, methyl ester	12.564	17457	0.06	0.003
10	C15:1; Pentadecenoic acid, methyl ester	12.976	9452	0.18	0.003
11	C16:0; Palmitic acid, methyl ester	14.558	895904	3.17	0.020
12	C16:1c; Palmitoleic acid, methyl ester	15.091	5332	0.10	0.002
13	C17:0; Margaric acid, methyl ester	16.851	34813	0.14	0.003
15	C18:0; Stearic acid, methyl ester	19.531	240293	1.01	0.010
16	C18:1c; Oleic acid, methyl ester	20.061	121218	1.91	0.003
17	C18:1n9T; Elaidic acid, methyl ester	20.310	10854	0.21	0.004
18	C18:2c; Linoleic acid, methyl ester	21.650	224311	3.59	0.004
19	C18:2t; Octadecadienoic acid, methyl ester	21.870	2303	0.03	0.001
21	C18:3n3; Linolenic acid, ethyl ester	24.202	10880	0.24	0.002
22	C20:0; Arachidic acid, methyl ester	27.091	240456	1.02	0.004
26	C21:0;Heneicosanoic acid,methyl ester	30.776	32089	0.15	0.003
30	C22:0; Behenic acid, methyl ester	34.244	1091669	4.87	0.005
31	C22:1; Eruccic acid, methyl ester	34.250	153179	3.14	0.002
33	C23:0; Tricosanoic acid, methyl ester	37.483	201188	0.97	0.002
34	C24:0; Tetracosanoic acid, methyl ester	40.605	1628471	7.51	0.003
36	C24:1; Tetracosenoic acid, methyl ester	41.142	3198	0.05	0.004

Table 2: Phytotoxic profile of fatty acid extracted from *E. Dabia*

Extract	Conc (µg/ml) of sample	Fronds survived	Fronds died	% Growth regulation
Fixed oil	10	10	10	50
	100	8	12	60
	1000	4	18	80

Total no. of fronds: 20. Conc of standard drug 0.015 µg/ mL, Activity results: %

Table 3: Antimicrobial activity of oil isolated from *E. Dabia*

Microorganism	Streptomycin	DMSO	Oil
Staphylococcus aurus	28	-	10
Straph Epidermis	29	-	NA
Escherichia coli	30	-	16
Klebsiella pneumonia	30	-	18
Bacillus subtilis	28	-	14

Key words: NA: not active

eicosapentaenoic acid, behenic acid, eruccic acid, docosadienoic acid, tricosanoic acid, tetracosanoic acid, docosahexaenoic acid and tetracosenoic acid. Deionized water was used throughout the experimental work.

Preparation of Standard: Internal standard was prepared by dissolving 13.7 mg of tridecanoic acid methyl ester in 1 mL of *n*- hexane. External standard was prepared by diluting 10 mg of 37 component FAMEs mix standard to 10 mL with dichloromethane. From this solution further working standard solutions were prepared.

Extraction of Oil and Preparation of FAMEs: The aerial part of *E. Dabia* (50 g) was grounded and extracted with *n*-hexane kept in conical flask (1L) for 10 days. During this period, manual shaking was performed. The extract was concentrated by recovering the solvent using rotary evaporator under reduced pressure at 40°C. Yellow oil (1.38 g, 2.76 %) was obtained after concentration *in vacuo*. Fatty acids are polar compounds and are often non- volatile. For GC analysis, it is required that the sample to be analyzed must be volatile. Hence, in order to make fatty acids present in the oil volatile, derivatizaion is

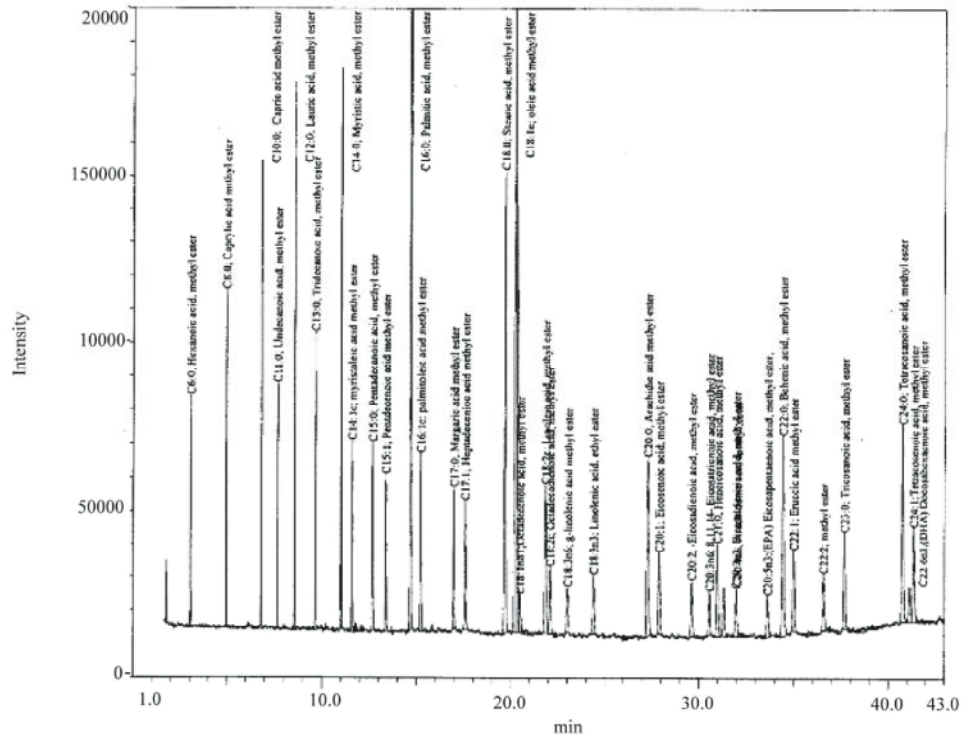

Fig. 1: GC-MS chromatogram of 37 components standard. Chromatographic conditions: inj. vol.: 1 µL, carrier gas: Helium, column: TRB-FFAP capillary column (length; 30 m, i.d.; 0.35 mm, thickness; 0.250 µm, treated with polyethylene glycol), MS scanning: 85-380 *m/z*.

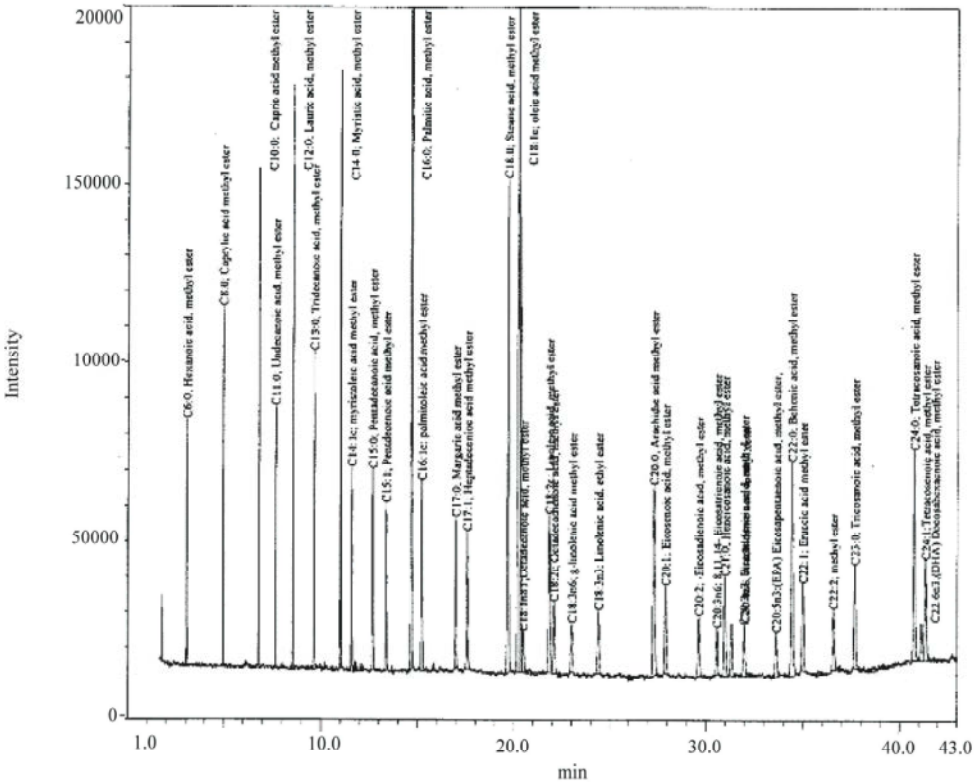

Fig. 2: GC-MS chromatogram of *Eluphia Dabia* oil extracted from aerial part

performed prior to GC-MS analysis. Methylation is the most general procedure converting non-volatile fatty acids into volatile fatty acids methyl esters (FAMEs) [17]. Methylation of fatty acids was performed with methanol stabilized boron triflouride as derivatizing reagent, which is the most accepted procedure for converting fatty acids into FAMEs [18]. Derivatization was performed according to the AOAC standard reference method [19]. To a known amount of sample (equivalent to 25 mg fat) was added 0.1 mL internal standard (1.37 mg) and 1.5 mL of NaOH solution in methanol (0.5 N), sealed and heated in boiling water bath for 5 minutes. The hydrolyzed sample was cooled and added 2.5 mL of BF_3 solution in methanol (10 %). The solution was then sealed and heated in boiling water bath for 30 minutes and cooled. To the esterified solution was added 5 mL saturated sodium chloride solution and extracted twice with 1 mL *n*-hexane. The *n*- hexane extract was filtered through 0.45 mm membrane filter and injected to GC-MS using auto injector system.

Chromatographic Separation of FAMEs: A gas chromatograph from Shimadzu hyphenated to a mass spectrometer QP 2010 plus (Tokyo, Japan) equipped with an auto-sampler (AOC-20S) and auto-injector (AOC-20i) was used. Helium was used as carrier gas. All chromatographic separations were performed on a capillary column (TRB-FFAP; Technokroma) having specifications: length; 30 m, i.d.; 0.35 mm, thickness; 0.250 µm, treated with polyethylene glycol. Other GC-MS conditions are: ion source temperature (EI); 250 C, interface temperature; 240°C, pressure; 100 KPa, solvent cut time; 1.8 min. 1 µL of sample and standard were injected into the GC column. Injector was operated in a split mode with a split ratio 1:50. Injection temperature was 240°C. The column temperature program started at 50°C for 1 min and changed to 150°C at the rate of 15°C/min. The temperature was raised to 175°C at the rate of 2.5°C/min and held for 5 minutes. Then the temperature was increased to 220°C at the rate of 2.5°C/min and kept constant for 3 minutes. Total elution time was 43 minutes. MS scanning was performed from m/z 85 to m/z 380. GC-MS solutions software provided by the supplier was used to control the system and to acquire the data. Identification of the compounds was carried out by comparing the mass spectra obtained with those of standard mass spectra from the NIST library (NIST 05).

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