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Research Article

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Constituents and Antimicrobial Activity of Sudanese *Haplophyllum tuberculatum* A. Juss (Rutaceae) Oil

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Abstract Phytotherapy is still playing a vital role in primary health care in developing countries and intensive literature reports on the impact of secondary metabolites on human physiology potentiated the applications of medicinal plants**.** *Haplophyllum tuberculatum* is a plant of many medicinal attributes. The plant is used traditionally against a wide range of diseases. In this study, the oil from *Haplophyllum tuberculatum* seeds was analyzed by GC-MS. The GC-MS analysis showed 22 components. Major constituents are: i) 9,12-octadecadienoic acid methyl ester (49.60%); 9,12,15-octadecatrienoic acid methyl ester (17.91%); hexadecanoic acid methyl ester (11.70%) and methyl stereate (8.07%). oil was assessed for antimicrobial activity. It exhibited good antibacterial activity against *Bacillus subtilis*.

Keywords *Haplophyllum tuberculatum*, Flavonids, Oil, GC-MS Analysis, Antimicrobial Activity

Introduction

Haplophyllum tuberculatum A. Juss is a perennial plant in the family Rutaceae. The plant is native to North Africa and some Middle East countries. Many reviews [1-10] reported on the traditional uses of *Haplophyllum tuberculatum*. All parts of this plants and especially leaves find some applications in ethnomedicine [11-16]. *Haplophyllum tuberculatum* contains some phytochemicals like alkaloids, flavonoids, coumarins and volatile oil known for their potential bioactivity [17,18]. In Sudanese system of medicine *Haplophyllum tuberculatum* is used against gynecological disorders, asthma, rheumatic pain and allergic rhinitis [13-16]. In Iraq folklore the plant is used for stomach-ache, convulsions and nervous disorders [11,19]. In Oman leaves of *Haplophyllum tuberculatum* are used traditionally against arthritis and headache, while in Saudi Arabia the plant is a natural remedy for skin discoloration, infections and parasitic deseases [13-16]. In Algeria, local healers use this plant for ulcers, injury, diabetes, hypertension, fever, constipation, menstrual pain, tonsillitis, cough and rheumatism [12]. It has been reported that the plant has antioxidant and cytoprotective potential [20]. The ethanol extract of the aerial parts exhibited efficient antimicrobial potency [21]. An alkaloid isolated from this plant species(tuberine) showed significant antimicrobial activity against some miroorganisms [22]. The antimicrobial activity of *Haplophyllum tuberculatum* polyphenols has also been investigated [23-24].

Materials and Methods

Plant material

Seeds of *Haplophyllum tuberculatum* were collected from Kassala, west Sudan. The plant was identified and authenticated by The Medicinal and Aromatic Plants Research Institute, Khartoum, Sudan.

Instruments

For GC-MS analysis a Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m, length; 0.25mm diameter; 0.25 μm, thickness) was used.

Test organisms

The antimicrobial activity was estimated by the cup plate agar diffusion assay using the standard microorganisms: *Bacillus subtilis* (G+ve), *Staphylococcus aureus* (G+ve), *Pseudomonas aeroginosa* (G-ve), *Escherichia coli* (G-ve) and the fungal species *Candida albicans*.

Methods

Extraction of oil

Powdered seeds of *Haplophyllum tuberculatum* (300g) were macerated with n-hexane for 48h.The solvent was removed under reduced pressure giving the oil

GC-MS analysis

Haplophyllum tuberculatum oil was analyzed by GC-MS using a Shimadzo GC-MS-QP2010 Ultra instrument. Chromatographic conditions are shown below.

Table 1: Oven temperature program

Rate (min.$^{-1}$)	Temperature (^{o}C)	Hold Time
-	150.0	1.00
4.00	300.0	0.00

Table 2: Chromatographic conditions

Column oven temperature	150.0 ^{o}C
Injection temperature	300.0^{o}C
Injection mode	Split
Flow control mode	Linear velocity
Pressure	139.3 KPa
Total flow	50.0 ml/ min
Column flow	1.54 ml/sec
Linear velocity	47.2 cm/sec
Purge flow	3.0 ml/min
Spilt ratio	- 1.0

Antimicrobial Assay

The cup-plate agar diffusion bioassay was adopted , with some minor modifications, to assess the antimicrobial activity of the studied oil . (2ml) of the standardized microbial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45°C in a water bath. (20 ml) aliquots the nutrient agar were distributed into sterile Petri dishes. The agar was left to settle and in each of these plates a cup (6 mm in diameter) was cut using sterile cork borer (No 4). The agar discs were removed and cups were filled with test samples and allowed to diffuse at room temperature for 2h.The plates were then incubated at 37^{o}C for 24h. Inhibition zones were recorded as averages of two replicates.

Results and Discussion

Haplophyllum tuberculatum seed oil was studied by GC-MS. The analysis showed 22 constituents (Table 3). The total ions chromatograms is depicted in Figure 1.

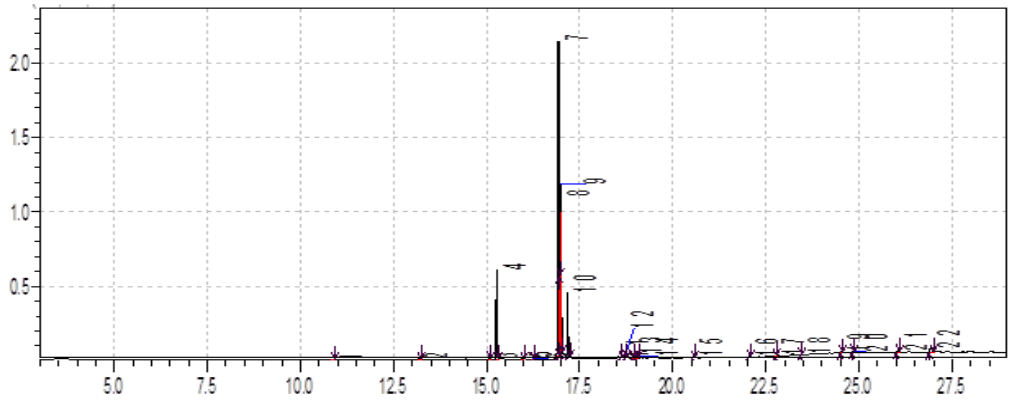

Figure 1 : Total ions chromatograms

Table 3: Constituents of *Haplophyllum tuberculatum* seed oil

No.	Name	Ret. Time	Area %
1.	Dodecanoic acid, methyl ester	10.871	0.12
2.	Methyl tetradecanoate	13.179	0.22
3.	9-Hexadecenoic acid, methyl ester, (Z)-	15.071	0.09
4.	Hexadecanoic acid, methyl ester	15.263	11.70
5.	Hexadecanoic acid, 14-methyl-, methyl ester	15.969	0.31
6.	Heptadecanoic acid, methyl ester	16.239	0.14
7.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	16.927	49.60
8.	9-Octadecenoic acid (Z)-, methyl ester	16.970	7.89
9.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	16.993	17.91
10.	Methyl stearate	17.174	8.07
11.	Methyl 8,11,14,17-eicosatetraenoate	18.587	0.11
12.	cis-11-Eicosenoic acid, methyl ester	18.721	0.24
13.	Eicosanoic acid, methyl ester	18.922	0.52
14.	2(1H)-Quinolinone, 3-(3-methyl-2-butenyl)-4-[(3-methyl-2-butenyl)oxy]-	19.005	0.32
15.	Docosanoic acid, methyl ester	20.541	0.27
16.	Tetracosanoic acid, methyl ester	22.041	0.24
17.	Squalene	22.766	0.13
18.	Hexacosanoic acid, methyl ester	23.442	0.09
19.	Tetratetracontane	24.522	0.22
20.	Urs-12-ene	24.833	0.17
21.	Ergost-5-en-3-ol, (3.beta.)-	26.033	0.45
22.	.gamma.-Sitosterol	26.923	1.19

Major constituents of the oil are:

i) 9,12-octadecadienoic acid methyl ester (49.60%); 9,12,15-octadecatrienoic acid methyl ester (17.91%); hexadecanoic acid methyl ester (11.70%) and methyl stereate (8.07%).

9,12-Octadecadienoic acid-(ZZ)-, methyl ester (49.60 %)

The EI mass spectrum of 9,12-octadecadienoic acid methyl ester is shown in Figure 2. The peak at m/z 294 (R.T. 16.927) coincides with $M^+[C_{19}H_{34}O_2]^+$, while the peak at m/z 263 is due to loss of a methoxyl. 9,12-Octadecadienoic (linoleic acid) belongs to one of the two families of essential fatty acids. Such acids can not be synthesized by human bodies and are available through diet [25]. Linoleic acid which exists in lipids of cell membrane is used in the biosynthesis of arachidonic acid .

9,12,15-octadecatrienoic acid methyl ester (17.91 %)

Mass spectrum of 9,12,15-octadecatrienoic acid, methyl ester is depicted in Figure 3. The peak at m/z 292, which appeared at R.T. 16.993 corresponds to M + $[C_{19}H_{32}O_2]^+$, while the peak at m\z 261 is attributed to loss of methoxyl.

Hexadecanoic acid , methyl ester (11.70 %)

Figure 4 shows the mass spectrum of hexadecanoic acid methyl ester. The molecular ion: $M^+[C_{17}H_{34}O_2]^+$ appeared at m/z 270 at R.T. 15.263 in total ion chromatogram. The fragment at m/z239 is due to loss of a methoxyl function. Hexadecanoic acid (palmitic acid) is a C_{16} saturated fatty acid. It is the most common fatty acid in plants and animals. This acid is the precursor of long chain fatty acids. Pamitic acid is a lipid constituent of human breast milk [26-27].

Methyl stearate (8.07 %)

Figure 5 shows the mass spectrum of methyl stereate. The signal at m/z 298 (R.T. 17.174) corresponds $M^+[C_{19}H_{38}O_2]^+$, while the peak at m/z 267 accounts for loss of a methoxyl.

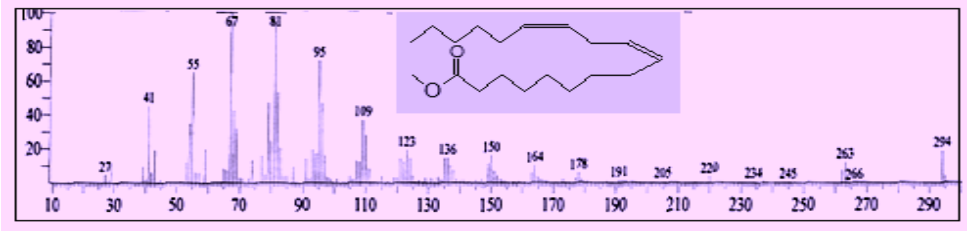

Figure 2: Mass spectrum of 9,12-octadecadienoic acid

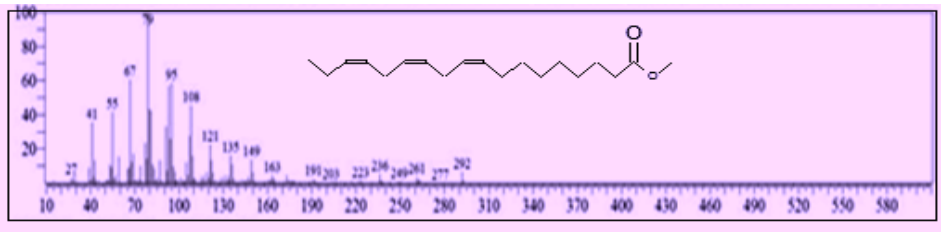

Figure 3: Mass spectrum of 9,12,15-octadecatrienoic acid

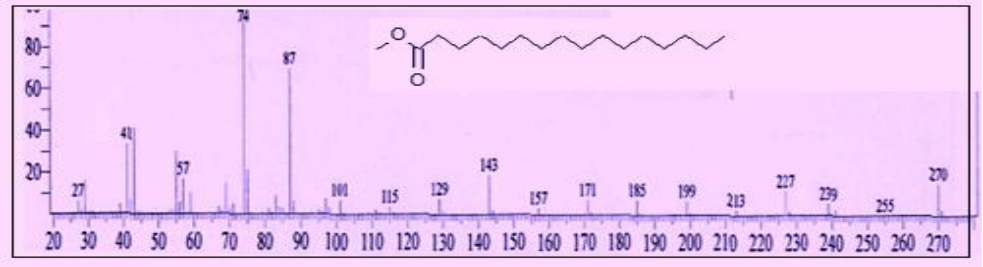

Figure 4: Mass spectrum of hexadecanoic acid methyl ester

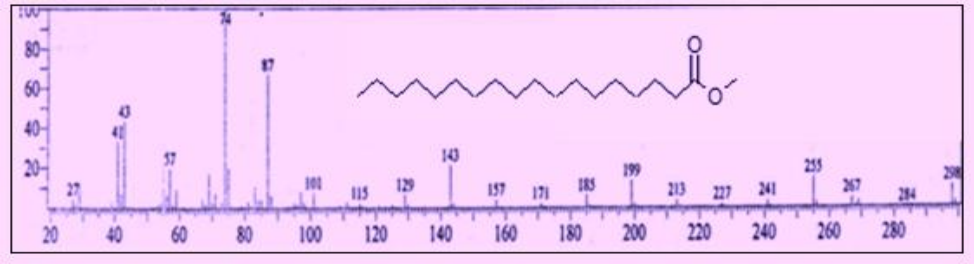

Figure 5: Mass spectrum of methyl stearate

Antimicrobial Activity

The antimicrobial activity of the oil was examined against Gram positive bacteria *Bacillus subtilis,* and *staphylococcus aureus,* Gram negative bacteria *Escherichia coli, Pseud*o*monas aeruginose* and fungus *candida albicans*. The obtained results are compared with reference drugs (ampicilin, gentamicin and clotrimazole). As shown in Table 4, the oil exhibited good activity against *Bacillus subtilis.*

Table 4: Inhibition zones (mm/mg sample)

Type	Sa	Bs	Ec	Ps	Ca
Oil (100mg/ml)	12	15	--	12	--
Ampicilin (40mg/ml)	30	15	--	--	--
Gentamicin (40mg/ml)	19	25	22	21	--
Clotrimazole (30mg/ml)	--	--	--	--	38

<9mm : Inactive; 9-12mm : partially active; 13-18mm: active ; >18mm very active
Sa.: *Staphylococcus aureus*; Bs.: *Bacillus subtilis*; Ec.: *Escherichia coli*; Pa.: *Pseudomonas aeruginosa*;
Ca.: *Candida albicans*

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