Research Article

Constituents and Antimicrobial Activity of Sudanese *Azadirachta indica* (Meliaceae) Fixed Oil

Abdel Karim, M.[1*], AbdelAzim, I.[1] and Um-Alahasan, T.[2]

1- Sudan University of Science and Technology, Faculty of Science.

2- Nile Valley University, Faculty of Education

Abstract:

In this study Azadirachta indica fruit oil was studied by GC-MS. The oil was also assessed for antimicrobial activity. The GC-MS analysis revealed the presence of 29 constituents .The major constituents of the oil are: i) 9-octadecenoic acid methyl ester (37.20%) , ii) methyl stearate(20.42%) , iii) hexadecanoic acid methyl ester(19.13%) and iv) 9, 12-octadecadienoic acid methyl ester (12.60%).The antimicrobial activity of the oil was evaluated via the agar diffusion bioassay against five standard pathogenic bacteria (Gram positive: Staphylococcus aureus and Bacillus subtilis; Gram negative: Esherichia coli, Pseudomonasaaeruginosa) and the fungus Candida albicans. At a concentration of 100mg/ml the oil showed good activity against Escherichia coli. However, at the same concentration, it exhibited partial activity against Staphylococcus aureus and Bacillus subtilis. The oil failed to give any anticandidal activity.

Keywords: *Azadirachta Indica, Oil, GC-MS analysis, Antimicrobial Activity*.

I. INTRODUCTION

Neem (*Azadirachta indica*) tree which belongs to the family Meliaceae , is a large ever green tree growing up to 60ft high in the dry tropical forests of Africa, India , Burma and Pakistan. Neem twigs are used as tooth brushes by some communities. Flowers are used in pharmaceutical, food and cosmetic industries [(1)].

The seed kernels constitute 50-60% of the seed weight and 25% of the fruit, The fat content of the kernels ranges from 33 to 45%[(2)]. Neem oil is usually opaque, bitter and inedible but can be processed into non bitter edible oil with 42-50% oleic acid and 15% linoleic acid. Neem is a medicinal plant of potential attributes; it provides many useful compounds that are used as pesticides and could be applied to protect storage against insects[(3)]. Neem seed has high nutritional potential for livestock[(4)]. Tignic acid is the principle component responsible for the distinctive odour of neem seed[(5)] as well as sulphur- containing compounds like nimbin , nimbidin and nimbosterol[(6)]. The neem constituents- which belong to chemically diverse classes- have been divided into two major groups viz. i) isoprenoids, ii) non-isoprinoides. The later category comprises glycerides, polysaccharides, sulphurone compounds, flavonoids, amino acids, aliphatic compounds… etc[(1)].

Azadirachta indica is a very useful traditional medicinal plant in the African continent. Each part of the tree has some medicinal properties which can be used to treat several diseases [(7,8)]. The branches are used as one of the most effective forms of dental care in traditional medicine [(9)]. Interestingly, the neem trees are an excellent alternative for modern tooth care products. Leaves are also used as natural treatment for acne sufferers [(10)]. Treatment of infected eyes can be carried out by the use of neem leaves. A similar infusion can also be used in the treatment of sore throats [(10)]. All parts of neem trees including leaves, seeds, roots, bark and the flowers are used to cure different ailment, such as stomach ulcer, jaundice and to overcome a variety of infectious and parasitic disease, ranging from leprosy, chicken pox to malaria [(11)]. Infusions and teas made from leaves are used to alleviate malaria attacks, intestinal complaint, dental headache, loss of appetite and heartburn. In addition to that it was also used as a diuretic and for diabetes as well as to treat numerous skin diseases. [(12)] The use of aqueous extracts from seeds to treat head lice is widely known. Neem oil showed good antiseptic properties. It is applied in the treatment of eczema, as well as to relieve intestinal worm infections [(13)]. Apart from that, neem-based products are traditionally used for pest control in agriculture and gardening [(14,15)].

II. Materials and Methods

Materials

Plant material

Fruits of Azadirachta indica, were collected from Khartoum, Sudan. The plant was authenticated by the Medicinal and Aromatic Plants Research Institute, Khartoum (Sudan).

Instruments

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

Test organisms

The studied oil was screened for antimicrobial activity using the standard microorganisms: Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeroginosa, Escherichia coli and Candida albicans.

Methods

Extraction of oil

Powdered air-dried plant material (300g) was exhaustively macerated with n-hexane. The solventt was removed under reduced pressure to afford the oil.

GC-MS analysis

The target oil was analyzed by the hyphenated technique gas chromatography- mass spectrometry. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m, length; 0.25mm diameter; 0.25 μm, thickness) was used. Helium (99% pure) was used as carrier gas. Oven temperature program and other chromatographic conditions are presented below:

Table 1: Oven temperature program

Rate	Temperature(^{o}C)	Hold Time (min.$^{-1}$)
-	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	139.3KPa
Total flow	50.0ml/ min
Column flow	1.54ml/sec.
Linear velocity	47.2cm/sec
Purge flow	3.0ml/min.
Spilt ratio	- 1.0

Antimicrobial assay

Bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to

produce a suspension containing about 10^8-10^9colony forming units per ml. The suspension was stored in the refrigerator at 4°C until used. The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique. Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volumes (0.02 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

Fungal suspensions

Fungal cultures were maintained on sabouraud dextrose agar incubated at 25°C for 72h. The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

Antibacterial test

The cup-plate agar diffusion method was adopted with some minor modifications, to assess the antibacterial activity of the oil. 2ml of the standardized bacterial 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 of the incubated nutrient agar were distributed into sterile Petri dishes, the agar was left to settle and in each of these plates which were divided into two halves, two cups in each half (10 mm in diameter) were cut using sterile cork borer (No 4), each one of the halves was designed for one of the test samples. Separate Petri dishes were designed for standard antibacterial chemotherapeutic agents (ampicillin and gentamicin).

The agar discs were removed, alternate cup was filled with 0.1 ml samples of the test solution using adjustable volume micrometer pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours.

The above procedure was repeated for different concentrations of the test sample and the standard antibacterial chemotherapeutics. After incubation, the diameters of the resultant growth inhibition zones were measured in duplicates and averaged as indicator of activity.

III. RESULTS AND DISCUSSION

GC-MS analysis

GC-MS analysis of *Azadirachta indica* oil was conducted and the identification of the constituents was accomplished by retention times and MS fragmentation pattern. A 90-95% match was observed when comparing the mass spectra with the database on MS library.

Constituents of oil

The GC-MS spectrum of the studied oil revealed the presence of 29 constituents (Table 3). The typical total ion chromatograms (TIC) is depicted in Fig.(1)

The major constituents of the oil are:

i) 9-Octadecenoic acid methyl ester (37.20%)

ii) Methyl stearate(20.42%)

iii) Hexadecanoic acid methyl ester (19.13%)

iv) 9, 12-Octadecadienoic acid methyl ester (12.60%)

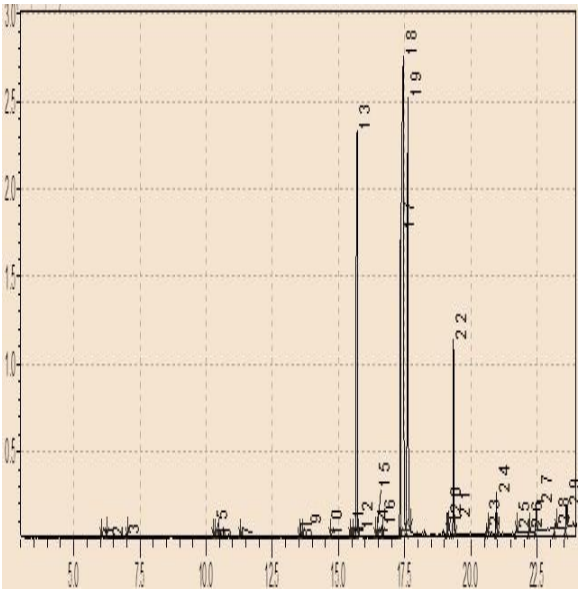

Fig. 1: Total ions chromatograms

Table 3: **Constituents of *Azadirachta indica* oil**

No.	Name	R.T	Area%
1.	Cyclopropane, 1,2-dimethyl-1-pentyl-	6.020	0.05
2.	(S)-(+)-6-Methyl-1-octanol	6.215	0.05
3.	L-.alpha.-Terpineol	6.978	0.11
4.	trans-.alpha.-Bergamotene	10.250	0.01
5.	.alpha.-ylangene	10.316	0.00
6.	1,5,9,11-Tridecatetraene, 12-methyl-, (E,E)-	10.435	0.01
7.	Dodecanoic acid, methyl ester	11.253	0.04
8.	6,10-Dodecadienoic acid, 3,7,11trimethyl-, methyl ester, (E)-(S)-	13.488	0.02
9.	Methyl tetradecanoate	13.563	0.37
10.	Pentadecanoic acid, methyl ester	14.639	0.05
11.	7-Hexadecenoic acid, methyl ester, (Z)-	15.429	0.05
12.	9-Hexadecenoic acid, methyl ester, (Z)-	15.470	0.22
13.	Hexadecanoic acid, methyl ester	15.698	19.13
14.	Hexadecanoic acid, 14-methyl-, methyl ester	16.371	0.03
15.	cis-10-Heptadecenoic acid, methyl ester	16.433	0.06
16.	Heptadecanoic acid, methyl ester	16.641	0.28
17.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.341	12.60
18.	9-Octadecenoic acid (Z)-, methyl ester	17.439	37.23
19.	Methyl stearate	17.621	20.42
20.	Oxiraneoctanoic acid, 3-octyl-, methyl ester, cis-	19.099	0.61
21.	cis-11-Eicosenoic acid, methyl ester	19.133	0.25
22.	Eicosanoic acid, methyl ester	19.334	5.25
23.	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	20.591	0.19
24.	Docosanoic acid, methyl ester	20.950	1.07
25.	Tricosanoic acid, methyl ester	21.715	0.12
26.	Hexatriacontane	22.192	0.11
27.	Tetracosanoic acid, methyl ester	22.454	0.80
28.	Squalene	23.194	0.28
29.	Tetratriacontane	23.599	0.59

Major constituents are discussed below:

i) 9-Octadecenoic acid methyl ester (37.20%)

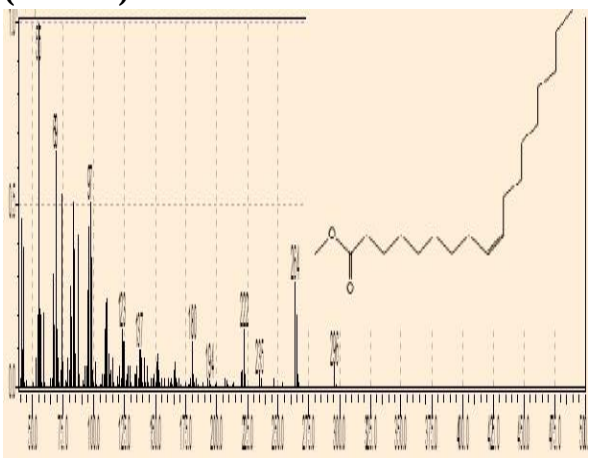

Fig (2): Mass spectrum of 9-octadecenoic acid methyl ester

The mass spectrum of 9-octadecenoic acid methyl esteris shown in Fig.(2).

The peak at m/z 296, which appeared at R.T. 17.439 in total ion chromatogram, corresponds the molecular ion: $M^+[C_{19}H_{36}O_2]^+$. The signal at m/z 266 is due to loss of a methoxyl .

ii) Methyl stearate (20.42%)

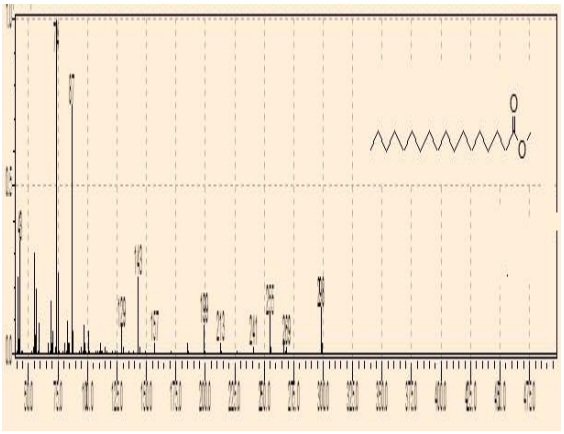

Fig (3): Mass spectrum of methyl stearate

The EI mass spectrum of methyl stearate is displayed in Fig.(3).The peak at m/z 298 (R.T. 17.621) is due to $M^+[C_{19}H_{38}O_2]^+$, while the signal at m/z267 corresponds to loss of a methoxyl group.

iii) Hexadecanoic acid methyl ester (19.13%)

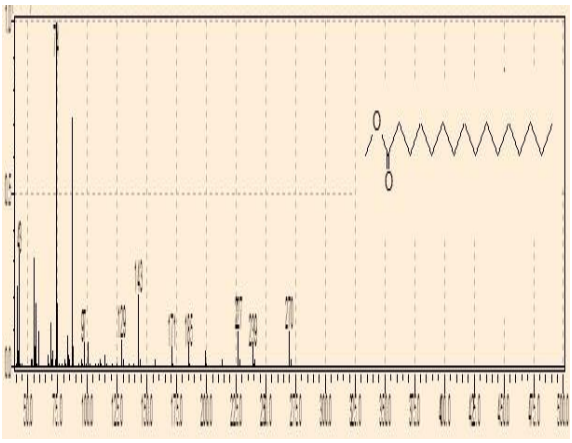

Fig (4): Mass spectrum of hexadecanoic acid methyl ester

Fig (4) shows the mass spectrum of hexadecanoic acid methyl ester. The peak m/z 270(R.T. 15.698) was detected in the spectrum. It corresponds $M^+[C_{17}H_{34}O_2]^+$ .The peak at m/z239 is due to loss of a methoxylfunction.

iv) 9,12-Octadecadienoic acid methyl ester (12.60%).

The mass spectrum of 9,12octadecadienoic acid methyl ester is depicted in Fig (5). The signal which was observed at m/z294(R.T. 17.341) is due to $M^+[C_{19}H_{34}O_2]^+$, while the signal at m/z263 corresponds to loss of a methoxyl.

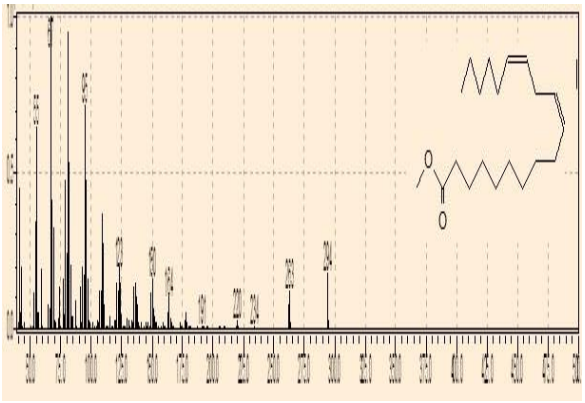

Fig (5): Mass spectrum of 9,12octadecadienoic acid methyl ester

Antimicrobial activity

Azadirachta indica oil was screened for antimicrobial activity against five standard human pathogens. The average of the diameters of the growth of inhibition zones are depicted in Table (4). The results were interpreted in the following manner: (>9mm: inactive; 9-12mm: partially active; 1318mm: active; < 18mm: very active). Tables (5) and (6) represent the antimicrobial activity of standard antibacterial and antifungal drugs respectively.

At a concentration of 100mg/ml the oil showed good activity against *Escherichia coli*. However, at the same concentration, it exhibited partial activity against *Staphylococcus aureus* and *Bacillus subtilis*. The oil failed to give any anticandidal activity.

Table (4): Diameters of inhibition zones of the *Azadirachta indica* oil

Oil	Diameters of inhibition zones				
	Gram positive		Gram negative		
Concn. mg/ml	Bs.	Sa.	Ec.	Pa.	Ca.
100	10	10	14	--	--

Table (5): Antibacterial activity of standard chemotherapeutic agents

Drug	Conc. mg/ml	Bs.	Sa.	Ec.	Ps.
Ampicillin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table (6): Antifungal activity of standard chemotherapeutic agent

Drug	Conc. mg/ml	An.	Ca.
Clotrimazole	30	22	38
	15	17	31
	7.5	16	29

IV. REFERENCES

1. Eckey. E., "Vegetable fats and oils" Reinhold publishing, P. 559 (1954).

2. Rahim. M., Biological activity of Azadiract inenrichedneem kernel extracts against Rhyzoperthadominiea (f) (coleopteran: Bostrichidae) in stored wheat, Journal of stored product Research, 34:123128(1998).

3. Udayashekhara.P., "Chemieal composition and biological evaluation of debitterised and defatted neem (Azadirachta indica) seed kernel cake, Journal of American oil Chemists Society, 64:1348-1352(1987).

4. Iwu.M., "Handbook African Medicinal Plants"CRC Press London, P.124-128(1993).

5. KarKar.C., Utilization of neem (A.indica) and its by-products: Report of the modified neem cake manorial project 1969-1976. Directorate of non-edible oils soap industry khadi and village Industries Commossion, Bombay, India (1976).

6. (W.H.O) W.H., General guidelines for methodologies on research and evaluation of traditional medicine (2001).

7. Velusamy.P, Das.J, pachaiappon.R, Pandian.K.,Greener approach for synthesis of antibacterial silver nanoparticles using aqueous , solution of neem gum (Azadirachta Indica L) , Ind Crops Prod. , 66:103-109(2015).

8. Prashant.G, Chandu.G, Chandu.G, Shafiulla.M., The effect of mango and neem extract on four organisms causing dental caries: streptococcus mutans. streptococcus salivavius, streptococcus mitis, and Streptococcus sanguis: An in vitro study, Indian J Dent Res., 18(4):148-151(2007).

9. Bhowmik. D, Yadav.J, Tripathi.K, Kumar.K., Herbal remedies of Azadirachta Indica and its medicinal application, J Chem Pharm Res.,2(1): 62-72(2010).

10. Bis was.K, Chattopadhyay.I, Banerjee.R.,Biological activities and medicinal properties of neem(Azadirachta Indica),Curr Sci.,82(11):1336-45(2002).

11. Sujarwo.W, Keim.P, Caneva.G, Nicoletti.M.,Ethnobotanical uses of neem (Azadirachta IndicaA. juss, Meliaceae) leaves in Bali (Indonesia) and the Indian subeontinent in relation with historical background and photochemical properties, Ethnopharmacol.,189:18693(2010).

12. Forster.P, Moser.G., "Status report on global neem usage: Deutsche Gesellschaft fur Technische Zusammenarbeit (G T Z)". Eschborn, Germany, P:122(2000).

13. Habluetzel.A, Esposito,F., Azadirachta Indica as apublic health tool for the control of malaria and other vector-borne diseases , Indian JMed Res., 130(2): 112-114(2009).

14. Vietmeger,N., Neem: a tree for solving global problems, Report of an ad hoe panel of the Board on science and Technology for International development (1992).

15. FAO. "Traditional Food Plants: A Resource Book for Promoting the Exploration Consumption of Food Plant in Arid, Semi-arid and Subhumid lands of Eastern Africa. Rome: FAO Food and Nutritionpape: 42 (1988).