International Journal of Research in Pharmacy and Pharmaceutical Sciences
ISSN: 2455-698X; Impact Factor: RJIF 5.22
www.pharmacyjournal.in
Volume 2; Issue 4; July 2017; Page No. 83-87

GC-MS analysis and antimicrobial activity of Sudanese *Ziziphus spina-christi* (Rhamnaceae) fixed oil

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Abstract
The present study was carried out to investigate the chemical constituents of *Ziziphus spina-christi* seed oil and to assess its potential antimicrobial activity. GC-MS analysis revealed 21 constituents. Major constituents are: 9-octadecenoic acid methyl ester (30.81%), 9, 12-octadecadienoic acid methyl ester (21.28%), hexadecanoic acid methyl ester (12.23%) and Methyl stearate (10.29%).
Ziziphus spina-christi seed oil was evaluated for antimicrobial activity via cup plate agar diffusion bioassay against six standard bacterial strains: (Gram positive: *Staphylococcus aureus* and *Bacillus subtilis*; Gram negative: *Escherichia coli* and *Pseudomonasa aeruginos*a and the fungi *Candida albicans* and *Aspergillus niger*). The oil gave excellent activity against the bacterial strains *Escherichia coli* and *Bacillus subtilis* at 100mg/ml. It also showed excellent activity against *Bacillus subtilis* at 50mg/ml. Good activity was observed for *Escherichia coli* at the concentrations: 50, 25 and 12.5mg/ml.

Keywords: *ziziphus spina-christi*, fixed oil, gc-ms, antimicrobial activity

Introduction
The Rhamnaceae family comprise about 60 genera and more than 850 species. The genus *Ziziphus* in the Rhamnaceae family includes about 100 species of deciduous or evergreen trees and shrubs [1]. *Ziziphus spina-christi* (Rhamnaceae) is an evergreen tree native to northern and tropical Africa and southern and western Asia [2]. Since ancient times *Ziziphus spina-christi* has been among the key plants used in Sudanese ethnomedicine. The edible ripe fruits significantly contribute to the improvement of human health [3]. Phytochemical screening of *Ziziphus spina-christi* leaves revealed the presence of bioactive constituents including: triterpenes, alkaloids, flavonoids and saponins [4].
The extremely nutritious fruits are usually consumed fresh. The flowers are important source for honey in Yemen and Eritrea [5] and Sudan. Roots are used to cure skin diseases [6], while seeds are sedative and anti-emetic. The fruits are used as poultice for ulcers. The plant is claimed to treat pulmonary ailments and fevers and to promote the healing of fresh wounds [1]. Fruits are also used by local healers to treat liver complaints, urinary disorders, obesity, diabetes, skin infections, bronchitis, anemia, diarrhea,and insomnia. leaves are helpful in liver troubles, asthma and fever [7-9].
Extracts of *Z. spina-christi* have been shown to possess anti-conceptive properties in model animals beside protective effect against aflatoxicosis [10].
The plant is claimed to exert a calming effect on the central nervous system and leaf extract improved glucose utilization in diabetic models by increasing insulin secretion [11]. The insulinotropic and subsequent hypoglycemic effects of leaves may be attributed to a sulfonylurea-like activity [11]. The ethanol and petroleum ether fractions were tested for antioxidant potential against stable 1, 1-dipheny l-2-picrylhydrazyl (DPPH) radicals and significant antioxidant activity was demonstrated.
Aqueous extract of *Z. spina-christi* stem bark demonstrated significant antibacterial activity against a panel of human pathogens [12].

Materials and Methods
Plant material
The seeds of *Ziziphus spina-christi* were collected from Nyala, western Sudan.The plant was authenticated by Institute of Aromatic and Medicinal Plants- Khartoum, Sudan.

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

Test organisms
Ziziphus spina-christi oil was screened for antimicrobial activity against the following standard microorganisms:

Table 1

S. No	Microorganism	Type
1	Bacillus subtilis	G+ve
2	Staphylococcus aureus	G+ve
3	Pseudomonas aeroginosa	G-ve
4	Escherichia coli	G-ve
5	Aspergillus niger	fungus
6	Candida albicans	fungus

Methods
Extraction of oil from *Ziziphus spina-christi*
Powdered, shade-dried seeds of *Ziziphus spina-christi* (300g) were macerated with n-hexane at room temperature for 48h.The solvent was removed under reduced pressure to afford the oil. For GC-MS analysis, the oil was esterified via a methanolic solution of sodium hydroxide (prepared by

dissolving (2g) of sodium hydroxide in 100ml methanol) and a methanolic solution sulphuric acid (prepared by mixing 1ml of concentrated sulphuric acid with 99ml methanol).

GC-MS analysis

Ziziphus spina-christi oil was analyzed by gas chromatography – mass spectrometry. Oven temperature program is given in Table 1, while other chromatographic conditions are depicted in Table 2.

Table 2: Oven temperature program

Rate	Temperature(C)	Hold time (mim.$^{-1}$)
-	60.0	0.00
10.00	300.0	0.00

Table 3: Chromatographic conditions

Column	1300.0 °C
oven temperature	280.0 °C
Injection temperature	Split
Injection mode	Linear velocity
Flow control mode	93.1KPa
Pressure	50.0ml/ min
Total flow	1.50ml/sec
Column flow	44.7cm/sec
Linear velocity.	3.0ml/min.
Purge flow	- 1.0
Spilt ratio	

Antimicrobial assay

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 108-109 colony forming units per ml. The suspension was stored in the refrigerator at 4°C until used. The average number of viable organism 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 cultures were maintained on potato dextrose agar incubated at 25°C for four days. The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

Testing for antibacterial activity

The cup-plate agar diffusion method was adopted, with some minor modifications, to assess the antibacterial activity. (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 solutions. The agar discs were removed, alternate cups were filled with (0.1 ml) samples of each test solution using adjustable volume microtiter 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 solutions and the standard chemotherapeutics. After incubation, the diameters of the resultant growth inhibition zones were measured in triplicates and averaged.

Results and Discussion

GC-MS analysis of *Ziziphus spina-christi* oil

GC-MS analysis of *Ziziphus spina-christi* oil was conducted via a Shimadzu GC-MS instrument. Identification of the constituents was accomplished by comparison with the MS library (NIST) and further confirmed by observed fragmentation pattern. A match of 90-95% was observed.

Constituents of oil

The GC-MS spectrum of the studied oil revealed the presence of 21 components (Table 3).The typical total ion chromatograms (TIC) is depicted in Fig.1.

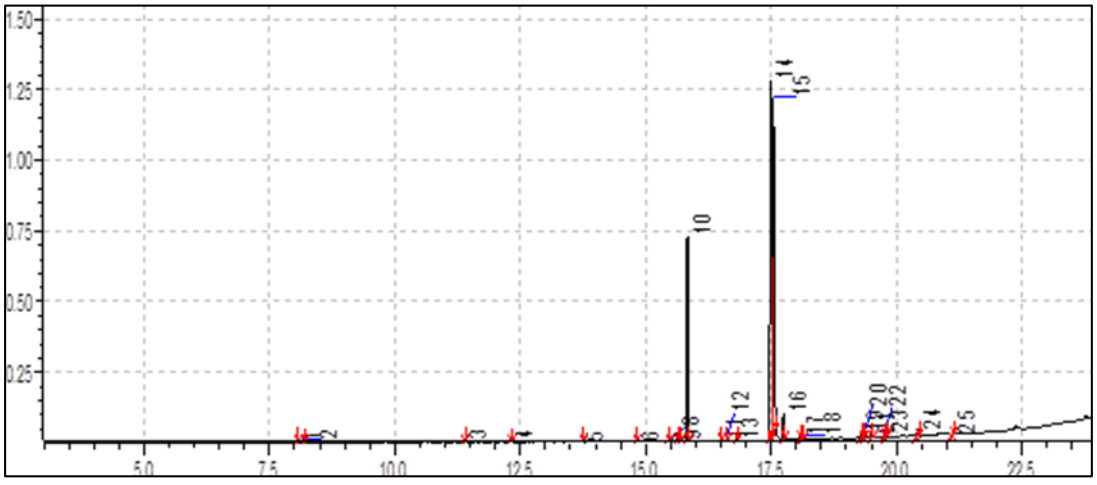

Fig 1: Total ion chromatograms

Table 3: Contituents of *Ziziphus spina-christi* oil

Peak#	R.Time	Area	Area%	Name
1	13.750	389478	0.13	Methyl tetradecanoate
2	14.827	95222	0.03	Pentadecanoic acid, methyl ester
3	15.620	138529	0.04	9-Hexadecenoic acid, methyl ester, (Z)-
4	15.666	272276	0.09	cis-10-Nonadecenoic acid, methyl ester
5	15.866	38082284	12.23	Hexadecanoic acid, methyl ester
6	16.838	494480	0.16	Heptadecanoic acid, methyl ester
7	17.538	66251981	21.28	9,12-Octadecadienoic acid (Z,Z)-,methyl ester
8	17.608	95922908	30.81	9-Octadecenoic acid (Z)-, methyl ester
9	17.787	32027283	10.29	Methyl stearate
10	18.448	953485	0.31	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tet
11	19.343	22413415	7.20	11-Eicosenoic acid, methyl ester
12	19.541	18246370	5.86	Eicosanoic acid, methyl ester
13	20.368	1197743	0.38	Heneicosanoic acid, methyl ester
14	20.484	975729	0.31	Phenol, 2,2-methylenebis{6-(1,1-dimethyl
15	20.986	1044958	0.34	13-Docosenoic acid, methyl ester
16	21.163	16677616	5.36	Docosanoic acid, methyl ester
17	21.928	718283	0.23	Tricosanoic acid, methyl ester
18	22.126	1020789	0.33	Cholesterol
19	22.669	6577540	2.11	Tetracosanoic acid, methyl ester
20	23.407	2459176	0.79	Squalene
21	23.797	5384130	1.73	.gamma,-Ergostenol
		311343675	100.00	

Some important constituents are discussed below:

9-Octadecenoic acid methyl ester (30.81%)

Fig. 2 shows the EI mass spectrum of 9-octadecenoic acid methyl ester.The peak at m/z 296, which appeared at R.T. 17.608 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$, while the peak at m/z266 accounts for loss of a methoxyl function**.**

9, 12-Octadecadienoic acid methyl ester (21.28%)

Fig. 3 shows the EI mass spectrum of 9,12-octadecadienoic acid methyl ester.The peak at m/z294, which appeared at R.T. 17.538 in total ion chromatogram, corresponds to $M^+[C_{19}H_{34}O_2]^+$. The peak at m/z263 corresponds to loss of a methoxyl function.

Hexadecanoic acid methyl ester (12.23%)

Mass spectrum of hexadecanoic acid methyl ester is depicted in Fig. 4.The peak at m/z 270, which appeared at R.T. 15.866 corresponds to $M^+[C_{17}H_{34}O_2]^+$ while the peak at m/z239 is attributed to loss of a methoxyl group.

Methyl stearate (10.29%)

Mass spectrum of methyl stearate is shown in Fig. 5.The peak at m/z 298, which appeared at R.T. 17.787 corresponds to $M^+[C_{19}H_{38}O_2]^+$.

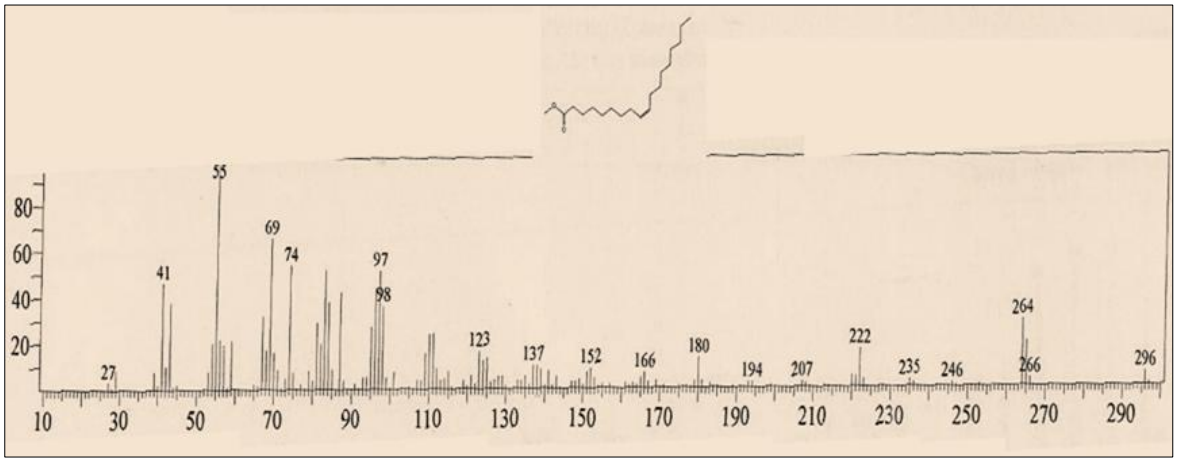

Fig 2: Mass spectrum of 9-octadecenoic acid methyl ester

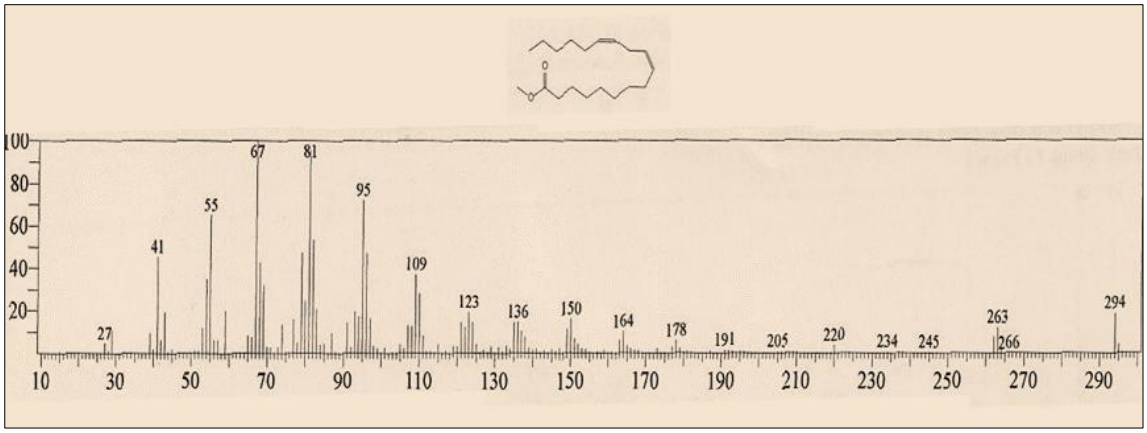

Fig 3: Mass spectrum of 9, 12-octadecadienoic acid methyl ester

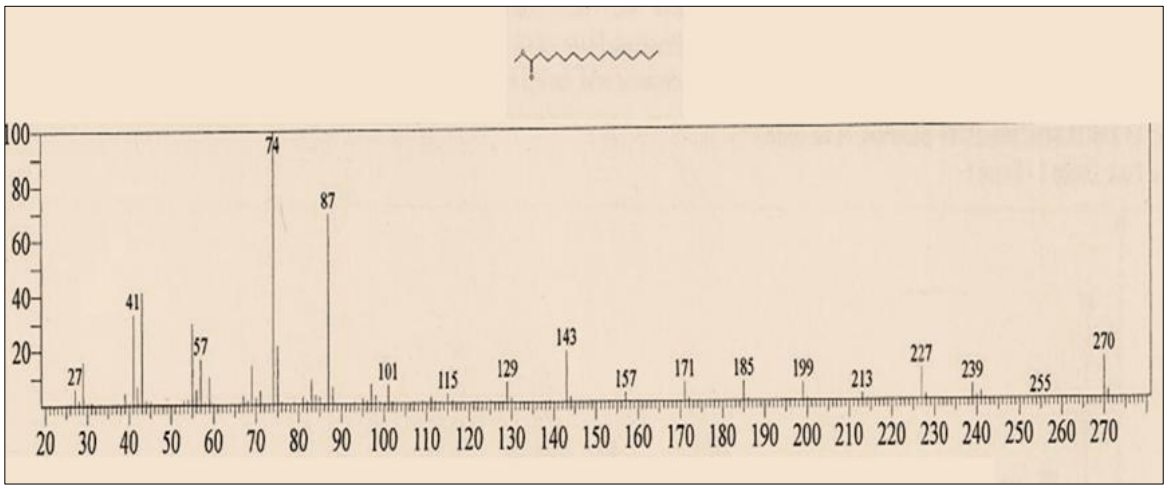

Fig 4: Mass spectrum of hexadecanoic acid methyl ester

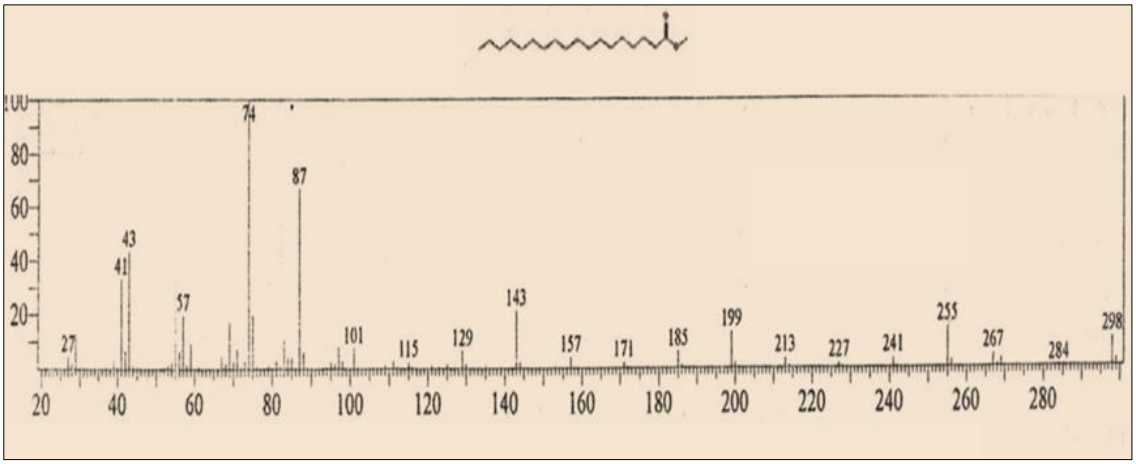

Fig 5: Mass spectrum of methyl stearate

Antibacterial activity

In cup plate agar diffusion assay, the oil was screened for antimicrobial activity against six standard human pathogens. The average of the diameters of the growth of inhibition zones are depicted in Table (4).The results were interpreted in conventional terms: (<9mm: inative; 9-12mm: partially active;13-18mm: active;>18mm:very active). Tables (5) and (6) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table 4: Antibacterial activity of *Ziziphus spina-christi* oil

Type	Conc.(mg/ml)	Sa	Bs	Ec	Ps	Ca
Oil	100	15	18	18	16	12
	50	13	18	14	-	-
	25	13	14	14	-	-
	12.5	12	-	14	-	-
	6.25	10	-	12	-	-

Table 5: Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	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

Sa.: *Staphylococcus aureus*
Ec.: *Escherichia coli*
Pa.: *Pseudomonas aeruginosa*
An.: *Aspergillus niger*
Ca.: *Candida albicans*
Bs.: *Bacillus subtilis*
The oil showed excellent activity against *Escherichia coli* and *Bacillus subtilis* at 100mg/ml. It also showed excellent activity against *Bacillus subtilis* at 50mg/ml.Good activity was observed against *Escherichia coli* in the concentration range: 50-12.5mg/ml.

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