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RESEARCH ARTICLE

GC-MS Analysis and Antimicrobial Activity of Fixed Oil from Saudi Negella sativa (Ranunculaceae)

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ABSTRACT

Constituents of Negella sativa seed oil were identified by GC-MS analysis. Major constituents are: methyl 12-hydroxy-9-octadecenoate(17.37%) , 9,12,15-octadecatrienoic acid (14.98%) , 9-octadecenoic acid methyl ester(12.84%), β-sitosterol (11.95%),9,12-octadecadienoic acid methyl ester(10.15%), 11-eicosenoic acid(9.67%), hexadecanoic acid(8.63%) , 13-docosenoic acid(5.20%) . The oil was extracted by two different methods (soxhlet and maceration) and each sample was screened separately for antimicrobial activity against a panel of pathogens.

INTRODUCTION

Negella sativa (Ranunculaceae) is a small herb about 45cm in height. The plant is cultivated in many countries for its economic value. This plant is widely used in ethnomedicine to treat a wide array of human disorders.The popularity of this herb in Islamic communities is probably due to ideological belief that the herb is a remedy for multidisease. It is used as astringent, stimulant, diuretic, emmenagogue and anthelmintic. It is also used for fever, dyspepsia, piles and skin diseases[1-3]. Seed oil is used as a local anesthetic.The alcoholic fraction constitutes about 20%(w/w) ; aqueous extract 15%(w/w); fixed oil 25-32% (w/w) ; volatile oil 0.42%(w/w)[4,5].Seeds contain , among others, nigellone [6] ; negellidine[7] negellimine ; steroids; terpenoids; tannins; oleic acid; linoleic acid; saponins; protein; reducing sugar and bitter principle [5,8,9] .

Studies on pancreatic cancer cells testifies that thymoquinone- a major constituent of seed oil- is synergic with gemcitabine and oxaliplatin [10]. . Thymoquinone was also found cytotoxic for several types of human cancer cells[11] .Also it was reported that thymoquinone inhibited benzopyrene – induced carcinogenesis in model animals[12]. Some thymoquinone conjugates were found to be active against some resistant tumor cells[13]. A combined dose of selenium and thymoquinone resulted in decreased cell count, decreased alkaline phosphotase level and decreased glutathione level on the proliferation of osteoblast cells(MG 63) [14]. Thymoquinone was found to inhibit tumor incidence and tumor burden significantly in 20-methylcholanthrene-induced fibrosarcoma [15] . It was demonstrated that it protects rats against NAME-induced hypertension and renal damage probably by its antioxidant potential[16] .

The essential oil of *Negella sativa* and ethyl acetate fraction were cytotoxic against P815 cell line[14] . A decoction comprising *Negella sativa* seeds, *Hemidesmus indicus* root bark and *Smilax glabra* rhizomes is said to inhibit DEN-mediated expression of GST-P. It also inhibited histopathological changes leading to tumor development in model animals[17,18] . *In vitro* studies testified that the ethanol extract of seeds inhibited cancer cells and endothelial cell progression [19,20] , while *in vivo* studies demonstrated that topical application of seed extract inhibited skin carcinogensis[21].The aqueous and ethanol extracts inactivated MCF-7 breast cancer [22] .

Aqueous exract of seeds inhibited electrogenic intestinal absorption of glucose *in vitro*. After chronic oral administration , seed extract improved glucose tolerance and body weight in model animals[23] . Such findings validate the traditional use of *Negella sativa* seeds against diabetes. The ethanol extract of seeds significantly reduced elevated blood glucose, plasma insulin and improved antioxidant enzymes like superoxide dismutase. Furhemore , it reduces glutathione and glutathione peroxidase in liver and kidney[24,25] .

MATERIAL AND METHODS

Materials

Plant material

Negella sativa seeds were purchased from the local market-Reyad-Saudi Arabia and authenticated by Botanist- Dr. Mohamed Abhery- Biotechnology

Laboratory, Faculty of Science, Taiba University, Saudi Arabia .

Extraction and esterification of oil

Powdered *Negella sativa* seeds (400g) were extracted with n-hexane (soxhlet).The solvent was removed *in vacuo* to afford the oil . The extracted oil was esterified by a methanolic solution of sodium hydroxide and methanolic sulphuric acid. After the usual workup, the ester solution (1µl) was directly injected in the GC-MS vial

GC-MS analysis

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. Oven temperature program :

Rate : -- ; **Temperature** ^{o}C: 150.0; **Hold time** (Min^{-1}) : 1.00

Rate : 4.00 ; **Temperature** ^{o}C : 300.0 ; **Hold time** (Min^{-1}) : 0.00

A Tabulation of chromatographic conditions is shown below:

Column oven temperature	150.0^{o}C
Injection temperature	300.0^{o}C
Injection mode	Split
Flow control mode velocity	Linear
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

Aliquots(1ml) of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours. Bacterial growth was harvested and washed off with sterile normal saline, and then suspended in normal saline(100ml) giving about 10^{8}-10^{9}colony forming units per ml. The suspension was kept at 4°C until used. Average number of viable organism per ml was assessed by surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline. Volumes (0.02 ml) of the appropriate dilutions were transferred into the surface of dry nutrient agar plates. Plates were left at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

Fungal cultures were prepared on potato dextrose agar and incubation was continued for four days at 25^{o} C. Fungal growth was harvested , washed with sterile normal saline and stored in the refrigerator until used.

Testing for antibacterial activity

Sterile molten nutrient agar(200ml) was heated at 45°C in a water bath. Bacterial stock suspension (2ml) was added to the agar and mixed. Aliquots(20ml) of the incubated nutrient agar were distributed into sterile Petri dishes. The Petri dishes were divided into two halves(each half was assigned for a test solution).Two cups in each half (10 mm in diameter) were cut by a cork borer (No 4). Positive controls - standard antimicrobial chemotherapeutics-were assigned separate Petri dishes .

Alternate cups were filled with 0.1 ml samples and allowed to diffuse at room temperature for two hours and then incubated at 37°C for 24 hours.

Following incubation, the diameters of the growth inhibition zones were measured in triplicates and averaged.

RESULTS AND DISCUSSION

Identification of oil constituents

Sixteen components were detected by GC-MS(Table1).Typical total ion chromatogram(TIC) is shown in Fig.1.

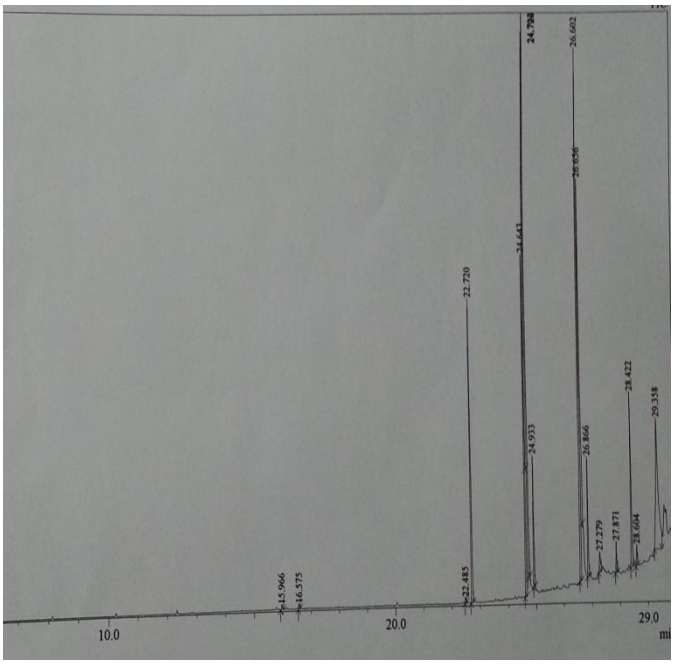

Fig.1: Total ion chromatograms of hexane extract

Peak#	R.Time	Area	Area%	Name
1	15.966	181695	0.16	1s,4R,7R,11R-1,3,4,7-Tetramethyltricyclo[
2	16.575	177698	0.16	Butylated Hydroxytoluene
3	22.485	161356	0.14	9-Hexadecenoic acid, methyl ester, (Z)-
4	22.720	9747365	8.63	Hexadecanoic acid, methyl ester
5	24.643	11461904	10.15	9,12-Octadecadienoic acid (Z,Z)-, methyl e
6	24.702	14494959	12.84	9-Octadecenoic acid (Z)-, methyl ester
7	24.726	16915744	14.98	9,12,15-Octadecatrienoic acid, methyl este
8	24.933	3749464	3.32	Methyl stearate
9	26.602	19619456	17.37	Methyl 12-hydroxy-9-octadecenoate
10	26.656	10916390	9.67	11-Eicosenoic acid, methyl ester
11	26.866	3575666	3.17	Eicosanoic acid, methyl ester
12	27.279	951745	0.84	9-Octadecenamide, (Z)-
13	27.871	921182	0.82	Phenol, 2,2'-methylenebis[6-(1,1-dimethyl
14	28.422	5871103	5.20	13-Docosenoic acid, methyl ester
15	28.604	678176	0.60	Docosanoic acid, methyl ester
16	29.358	13497165	11.95	.beta.-Sitosterol
		112921068	100.00	

Table 1: Consituents of *Negella sativa* seed oil

Major components are discussed below:

Methyl 12-hydroxy-9-octacecenoate(17.37%)

In Fig. 2 , the peak at m/z294 (R.T.26.602) in total ion chromatogram corresponds $M^+[C_{21}H_{42}O_2]^+$ .The peak at m/z263 accounts for loss of a methoxyl .

9,12,15-Octadecatrienoic acid (14.98%)

The mass spectrum of 9,12,15-octadecatrienoic acid is displayed in Fig. 3.The peak at m/z 292, which appeared at R.T.24.726 in total ion chromatogram, corresponds $M^+[C_{19}H_{32}O_2]^+$ .The loss of a methyl function is testified by a peak at m/z277 .

9-Octadecenoic acid methyl ester(12.84%)

The peak at m/z 296(Fig.4) , which appeared at R.T.24.702 , corresponds M^+ $[C_{19}H_{36}O_2]^+$, while the signal at m/z 264 is attributed to loss of a methoxyl function.

β-Sitosterol(11.95%)

The EI mass spectrum of β-sitosterol is shown in Fig.5.The peak at m/z414 , which appeared at R.T. 29.358 in total ion chromatogram, corresponds M^+ $[C_{29}H_{50}O]^+$ .The loss of a methyl group is demonstrated by a peak at m/z 299 .

9,12-Octadecadienoic acid methyl ester(10.15%)

The EI mass spectrum of 9,12-octadecadienoic showed m/z294 for M^+ $[C_{19}H_{34}O_2]^+$ .The signal at m/z 263 is due to loss of a methoxyl(Fig.6)**.**

11-Eicosenoic acid(9.67%)

The peak at m/z324 which appeared (Fig.7) at R.T.26.656 corresponds $M^+[C_{21}H_{40}O_2]^+$, while

the signal at m/z292 accounts for loss of a methoxyl group.

Hexadecanoic acid(8.63%)

The EI mass spectrum of hexadecanoic acid is shown in Fig. 8.The peak at m/z 270 (R.T. 22.720) in total ion chromatogram, corresponds M^+ $[C_{17}H_{34}O_2]^+$ .The signal at m/z 239 is attributed to loss of a methoxyl .

13-Docosenoic acid(5.20%)

The peak which appeared at m/z352 (R.T.28.422) corresponds $M^+[C_{23}H_{44}O_2]^+$, while the signal at m/z320 corresponds to loss of a methoxyl.

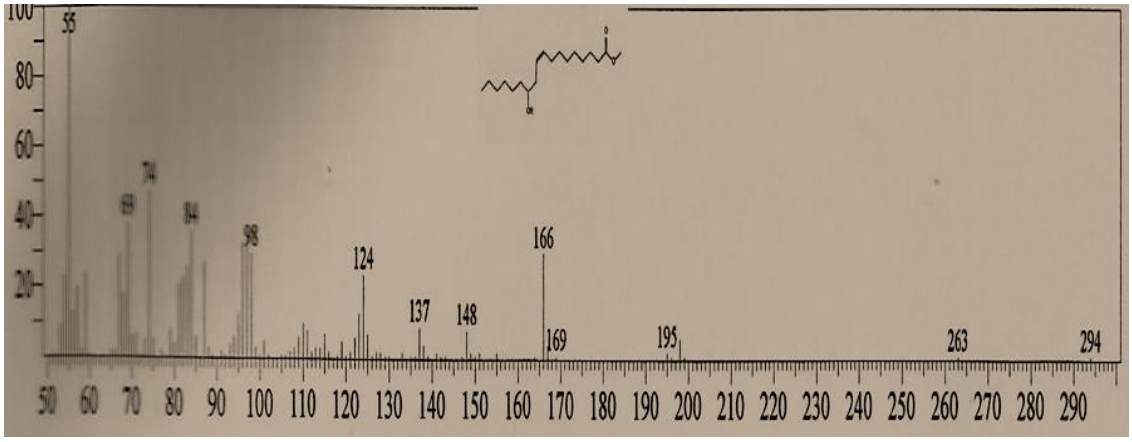

Fig.2: Mass spectrum of methyl 12-hydroxy-9-octadecenoate

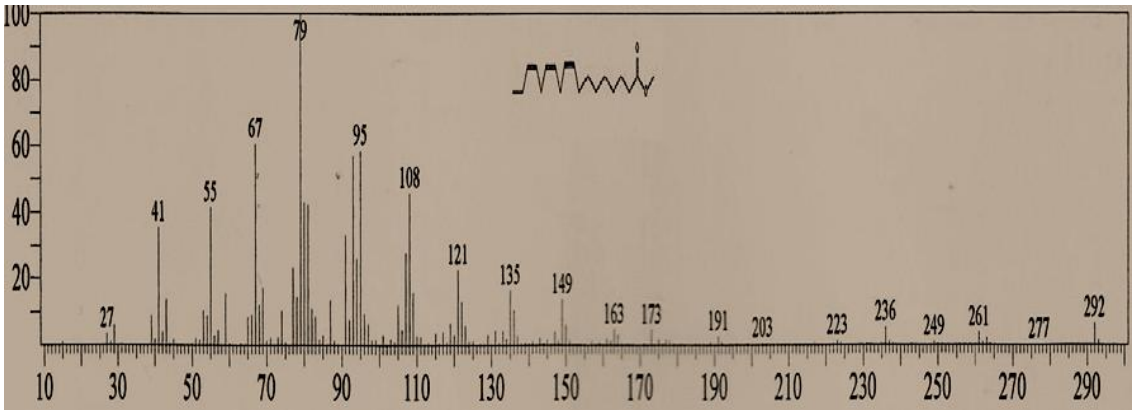

Fig.3: Mass spectrum of 9,12,15-octadecatrienoic acid methyl ester

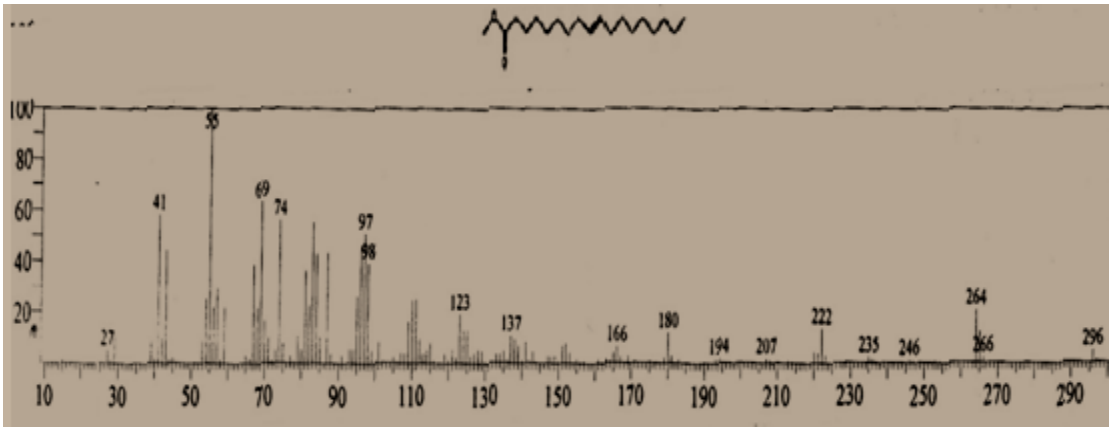

Fig.4: Mass spectrum of 9-octadecanoic acid methyl ester

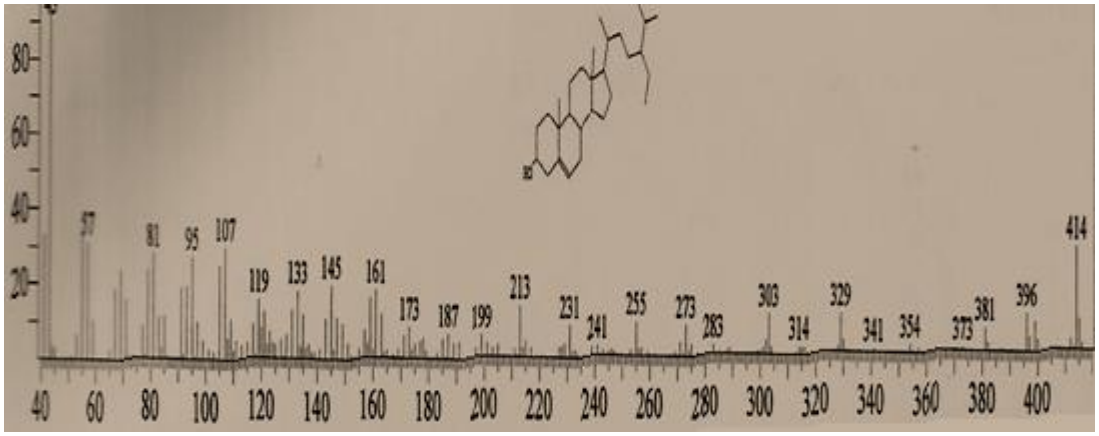

Fig.5: Mass spectrum of β-sitosterol

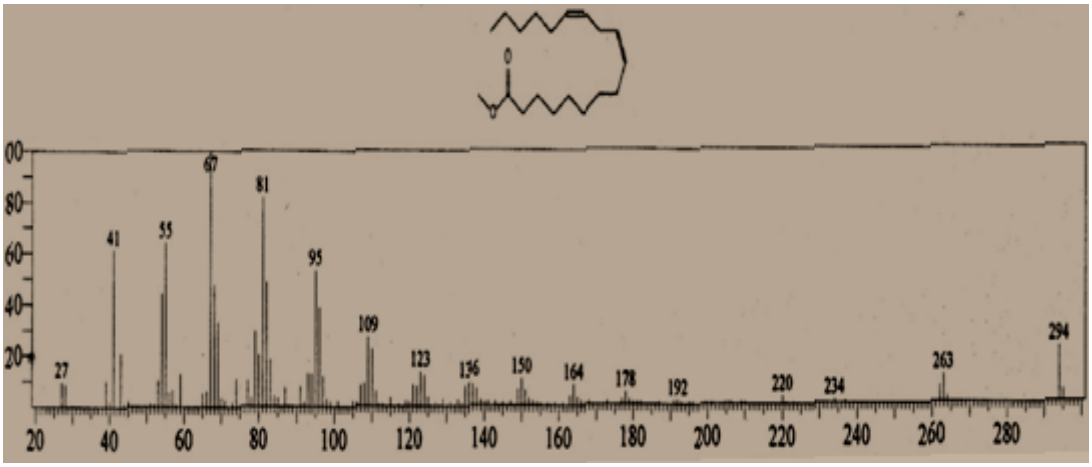

Fig.6: Mass spectrum of 9,12-octadecadienoic acid methyl ester

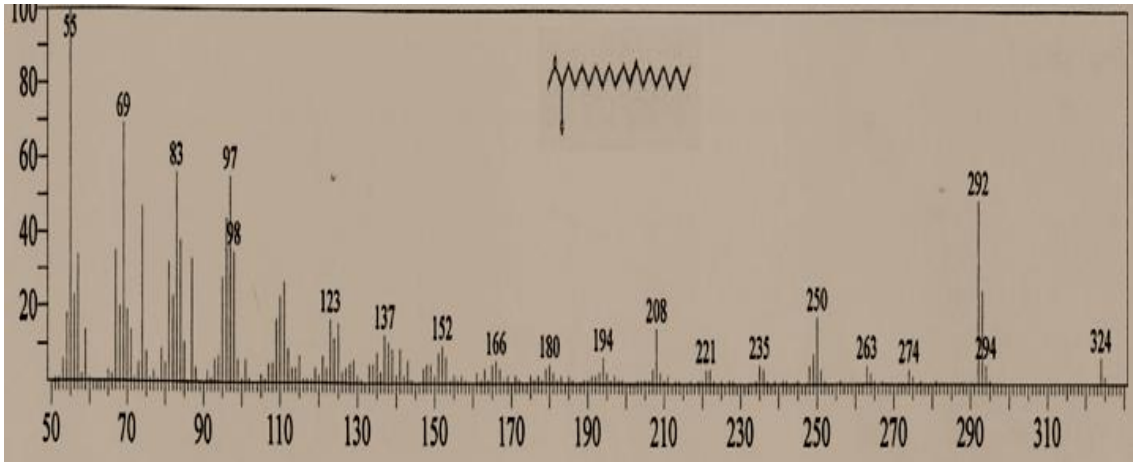

Fig.7: Mass spectrum of 11-eicosenoic acid

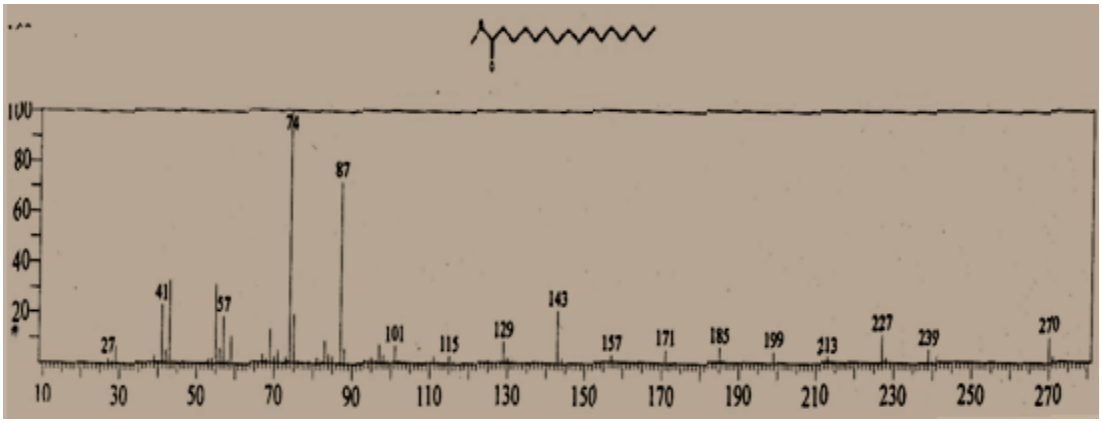

Fig.8: Mass spectrum of hexadecanoic acid methyl ester

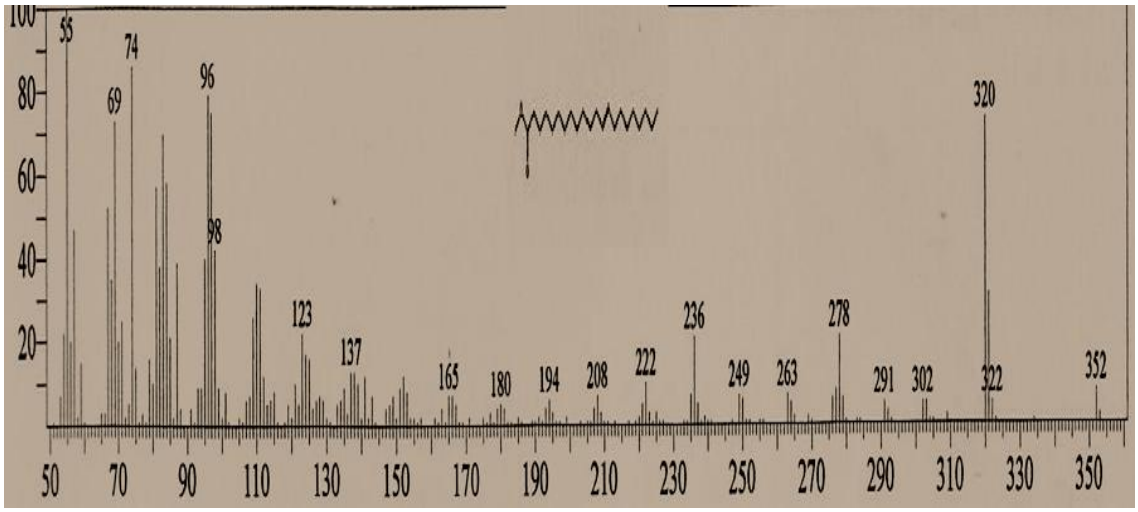

Fig.9: Mass spectrum of 13-docosenoic acid

Antimicrobial assay

Negella sativa seed oil was extracted by two different methods (A; soxhlet and B ; maceration) to evaluate the effect of heating(soxhlet) on the antimicrobial potency of the extracted oil. The oil extracted by soxhlet was partially active against *Escherichia coli* at 5µg/ml , active at concentrations of 10 and very active at 50 µg/ml while the macerated sample was merely partially active at 50µg/ml (Table 2). A partial activity was observed for *Klebsiella pneumonia* when using the soxhlet sample at 5 , 10 and 50 µg/ml ,while the macerated sample was inactive at 5 µg/ml. For *Acinetobacter baumannii*, the macerated sample showed partial activity at 10 and 50 µg/ml while the soxhlet sample was devoid of activity at all test concentrations. Noteworthy that the macerated sample gave excellent activity against the fungus *Candida albicans* at 50µg/ml , while the soxhlet sample was devoid of activity at all tested concentrations. It seems that the potency of such extracts depends largely on the type of test organism and the concentration of the sample. Similar trends

were observed for the minimum inhibition concentration(Table 3).

Table 2 : Minimum inhibition zone (mm)

Gram	Strain	A			B			Amp	Kan	Strip	Nys
		5	10	50	5	10	50	10	10	10	10
-ve	Escherichia coli	11±0.2	14±0.2	16±0.7	7±0.0	8±0.0	9±0.4	15±0.4	19±0.3	25±0.4	0
-ve	Klebsiella pneumoniae	9±0.3	10±0.3	12±0.6	7±0.0	9±0.0	10±0.4	10±0.4	19±0.0	26±0.4	0
-ve	Acinetobacter baumannii	-	7±0.0	8±0.0	8±0.0	10±0.4	11±0.3	12±0.3	19±0.3	12±0.3	0
-ve	Pseudomonas aureginosa	-	-	-	-	-	-	12±0.3	14±0.3	20±0.5	0
+ve	Staphylococcus aureus	-	-	-	-	-	-	15±0.5	17±0.5	15±0.5	0
+ve	Bacillus subtilis	-	-	-	-	-	10±1.0	13±0.4	19±0.4	30±0.4	0
C	Candida albicans	-	-	-	9±0.4	14±0.5	17±0.8	N	16±0.5	N	12±0.5
F		-	-	-	10±0.4	10±0.6	10±0.8	N	15±0.5	N	15±0.5

-ve: gram negative, +ve: gram positive, C: colony forming, F: filamentous, -: no activity. Aspergillus flavus

Table 3: Minimum inhibition concentration(mg/ml)

Gram	Strain	A			B			Amp.	Kan.	Strep.	Nys.
		Concn.(mg/ml)									
		100	250	500	100	250	500	10	10	10	10
-ve	Escherichia coli	R	I	S	R	R	R	S	S	S	N
-ve	Klebsiella pneumonia	R	R	I	R	R	R	S	S	S	N
-ve	Acinetobacter baumannii	R	R	R	R	R	I	S	S	S	N
-ve	Pseudomonas aureginosa	R	R	R	R	R	R	S	S	S	N
+ve	Staphylococcus aureus	R	R	R	R	R	R	S	S	S	N
+ve	Bacillus subtilis	R	R	R	R	R	R	S	S	S	N
C	Candida albicans	R	R	R	R	S	S	N	S	N	S
F	Aspergillus flavus	R	R	R	R	R	I	N	S	N	S

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