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GC-MS Analysis and Antimicrobial Activity of Sudanese *Brassica nigra* L. (Brassicaceae) Fixed Oil

Abdel Karim. M.[1(*)] , Weam,A.[2] M. Yousif, Inas, O.[3]

1. Sudan University of Science and Technology, Faculty of Science, Dept. of Chemistry
2. Omdurman Islamic University , Faculty of Pharmacy, Dept. of Pharmaceutical Chemistry
3. University of Bahri, College of Applied and Industrial Sciences

Abstract

The present study was designed to investigate the chemical constituents of *Brassica nigra* seed oil and to evaluate its potential antimicrobial activity. 31 components were detected by GC-MS analysis. Major constituents are: 13-docosenoic acid methyl ester(33.53%),9,12-Z,Z-octadecadienoic acid methyl ester(16.20%),11-eicosenoic acid methyl ester(12.12%),9,12,15-octadecatrienoic acid, methyl ester (10.75%),9-Z--octadecenoic acid methyl ester(5.90%), hexadecanoic acid methyl ester(4.26%) ,. Butylated hydroxytoluene, a potent antioxidant, was detected as a minor constituent(0.12%). The antibacterial activity of the oil was evaluated via cup plate agar diffusion assay against six standard human pathogens(Gram positive: *Staphylococcus aureus* and *Bacillus subtilis*; Gram negative : *Escherichia coli* and *Pseudomonasa aeruginosa* and the fungi *Candida albicans* and *Aspergillus niger*) . The oil showed different antimicrobial responses against test organisms. The oil showed activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Aspergillus niger*.However,it gave partial activity against *Pseudomonas aeruginosa* and *Candida albicans*.It seems that the oil is a lead for further optimization.

Keywords: *Brassica nigra*, , Fixed oil, GC-MS, Antimicrobial Activity

Introduction

In developing countries , medicinal plants play an important rule in treating a wide array of human disorders. A considerable number of modern drugs have been isolated or derived from plant material(Butler,2004; Johnes et. al.,2006 ; Abdel Wahab,2009). More than 80% of the world

population now depend on medicinal plants which contribute to the primary healthcare of different

communities(Anonymous,2002; Amri et. al.,2012 ; Muthu et. al.,2006). This is mainly due to the side effects of several synthetic drugs and the unaffordable cost of modern drugs . Medicinal plants include bioactive constituents(steroids, alkaloids,flavonoids..etc) which are very helpful in treating various ailments(Vellisek et. al.,1995).

Black mustard (*Brassica nigra* **L.**) is an annual plant in the Brassicaceae family.It grows up to 2 meters in height. It has branching , angular stem with large alternate leaves blooming during summer.Black mustard is now widely cultivated as a primary source of the mustard seeds.

Brassica nigra is widely used in ethnomedicine to treat several non-communicable diseases,chronic diseases and other degenerative disorders(Velosek et. al.,1995).Seeds of this plant contain : fixed oil, proteins, isothiocyanate glycoside (sinigrin) , albumins, mucilage , gum,lecithin,inosite and coloring materials(Evans,2007 ; Kirtikar and Basu,2005 ; Kakate et.al.2007 ; Nadkarni,1994).Leaves are reported to contain many minerals (Ca, K, Na..) beside carotenes, thiamine, riboflavin , niacin and ascorbic acid(Matai,1973).

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Some flavonoids were recently isolated from *Brassica nigra* including some kaempferol derivatives(Kamal and Ahmed,2013). The plant is a source of α-tocopherol which is known to protect cells against oxidative damage triggered by free radicals(Yusif et.al. 2007). Aqueous extract of *Brassica nigra* is claimed to inhibit lipid peroxidation on human erythrocyte membranes(Sujatha,1995).

The cytotoxicity of *Brassica nigra* extract was demonstrated by Coggiola(2005).Different fractions of *Brassica nigra* were evaluated against 5 cancer cell lines(including cervical carcinoma, colon carcinoma and breast carcinoma cells) and significant growth inhibition was documented(Sujatha,1995).Furthermore ,essential oil from *Brassica nigra* has been shown to reduce tumour cell proliferation in model animals probably through antiangiogensis and apoptotic mechanisms(Kumar,2009; Eskin,2007).

Since *Brassica nigra* is a key species in Sudanese ethnomedicine , it was aimed to investigate the constituents of the oil of the Sudanese material of *Brassica nigra* and to evaluate the extracted oil for antimicrobial potential.

Materials and Methods

Materials

Plant material

The seeds of *Brassica nigra* were collected from Nyala, west Sudan in October 2015. The plant was authenticated by direct comparison with a herbarium sample.

Instruments

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

Brassica nigra oil was screened for antibacterial and antifungal activities using the standard microorganisms shown in table(1).

Table 1: Test organisms

Ser. No	Micro organism	Type
1	*Bacillus subtilis*	G+ve
2	*Staphylococcus aureus*	G+ve
3	*Pseudomonas aeroginosa*	G-ve
4	*Escherichia coli*	G-ve
5	*Aspergillusniger*	fungi
6	*Candida albicans*	fungi

Methods

Biological activity

Antimicrobial assay

Brassica nigra seed oil was screened for antimicrobial activity against six standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger*) using the cup plate agar method with some minor modifications.

Preparation of bacterial suspensions

One ml. aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37^{o} 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 suspension containing about 10^{8}. 10^{4} colony forming units per ml. The suspension was stored in refrigerator at 4^{o}C until used. The average number of viable organism

per ml of the saline 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 volume (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 to dry, and then incubated at 37^{o} C for 24 hours.

Preparation of fungal suspensions

Fungal cultures were maintained on potato dextrose agar incubated at 25^{o}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 modification, to assess the antibacterial activity of the oil.

Two ml of the standardized bacterial stock suspention were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45^{o} C in water bath.

(20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes and the agar was left to settle. Each of these plates was divided into two halves . Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for a sample.

The agar discs were removed and cups were filled with(0.1) ml of sample 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 ^{o} C for 24 hours.After incubation the diameters of the resultant growth inhibition zones were measured in duplicates and averaged.

Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar dextrose agar was used. Samples were used here by the same concentrations used above.

Extraction of oil from seeds of *Brassica niger*

Powdered seeds of *Brassica niger* (300g) were exhaustively extracted with n-hexane (soxhlet).The solvent was removed under reduced pressure and the oil was kept in the fridge at 4^{o}C for further manipulation.

Esterification of oil

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

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added, then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes .The hexane layer was then separated.(5µl) of the hexane extract were mixed with 5ml diethyl ether . The solution was filtered and the filtrate(1µl) was injected in the GC-MS vial.

GC-MS analysis

The oil of *Brassica nigra* seeds was analysed by 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 (purity; 99.99 %) was used as carrier gas.Oven temperature

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program is given in Table 2, while other chromatographic conditions are depicted in Table 3.

Table 2: Oven temperature program

Rate	Temperature(°C)	Hold Time (min.[1])
-	150.0	1.00
4.00	300.0	0.00

Table 3: Chromatographic conditions

Column oven temperature	150.0°C
Injection temperature	300.0°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

Results and Discussion

Antimicrobial activity

*Brassica nigr*a oil was evaluated for antimicrobial activity via the cup plate agar diffusion assay. The average of the diameters of the growth inhibition zones are shown in Table (4) .The results were interpreted in terms of the commonly used 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 : Antimicrobial activity of *Brassica nigra* seed oil :M.D.I.Z (mm)

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca	An
Brasssica nigra oil	100	12	10	15	14	11	13

Table 5 : Antibacterial activity of standard chemotherapeutic agents :M.D.I.Z (mm)

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

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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 activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Aspergillus niger*.However,it gave partial activity against *Pseudomonas aeruginosa* and *Candida albicans*.

The GC-MS analysis of *Brassica nigra* oil

GC-MS analysis of *Brassica nigra* oil was conducted and the identification of the constituents was initially accomplished by comparison with the MS library (NIST) and further confirmed by interpreting the observed fragmentation pattern. Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

Constituents of the oil

The GC-MS spectrum of the studied oil revealed the presence of 31 components(Table 7).The typical total ion chromatograms(TIC) is shown in Fig.1.

Table 7: Constituents of *Brassica.nigra* oil

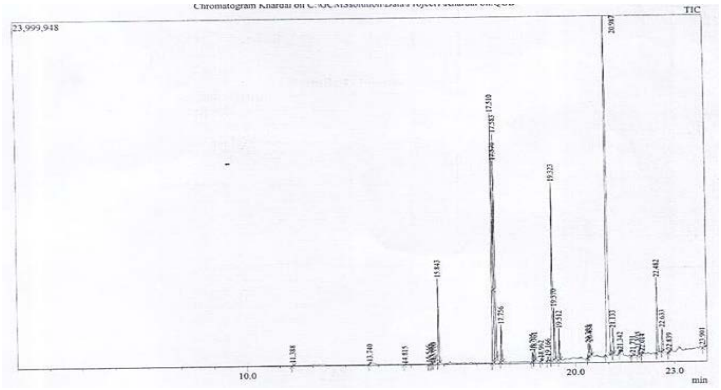

Fig.1:Cromatograms of *Brassica nigra* oil

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The following major constituents were detected in the chromatograms:

13-Docosenoic acid methyl ester(33.53%)

The EI mass spectrum 13-docosenoic acid methyl ester is shown in Fig. 2.The peak at m/z 352, which appeared at R.T. 20.987 in total ion chromatogram, corresponds to $M^+[C_{23}H_{44}O_2]^+$ .The peak at m/z320 corresponds to loss of a methoxyl function.

Ser. No.	RT	%age	Name
1	11.388	0.12	Butylated Hydroxy Toluene
2	13.740	0.11	Methyl Tetradecanoate
3	14.815	0.02	Pentadecanoic acid , methyl ester
4	15.546	0.05	7,10-Hexadecadienoic acid, Methyl ester
5	15.613	0.13	Methyl 8,11,14-Heptadecatrienoate
6	15.650	0.09	9-Hexadecenoic acid, Methyl ester(Z,Z)
7	15.700	0.16	Adipic acid Isohexyl , 2-methoxyethyl ester
8	15.843	4.26	Hexadecenoic acid , Methyl ester
9	17.510	16.20	9,12-Octadecadienoic acid(Z,Z) , Methyl ester
10	17.570	5.90	9-Octadecenoic acid(Z), methyl ester
11	17.583	10.75	9,12,15-Octadecatrienoic acid, Methyl ester
12	17.756	1.80	Methyl Stereate
13	18.705	0.54	Adipic acid , 2-Methoxyethyl octyl ester
14	18.761	0.51	Hexanedioc acid, Dioctyl ester
15	18.962	0.15	Octadecanoic acid, ,2,3-dihydroxypropyl ester
16	19.166	0.21	Gamma Linolenic acid , Methyl ester
17	19.323	12.12	11-Ecosenoic acid, Methyl ester
18	19.370	2.63	Cis-11-Ecosenoic acid , Methyl ester
19	19.512	1.54	Methyl , 18- Methylnonadecanoate
20	20.385	0.82	Adipic acid, decyl , 2-methoxyethyl ester
21	20.438	0.72	Adipic acid ,dodecyl 2-methoxyethyl ester
22	20.987	33.53	13-Docosenoic acid methyl ester
23	21.133	1.26	Methyl 20- methylheneicosanoate
24	21.342	0.10	1,3-Dioxolane 4-methyl-2-(1-pentyl)-
25	21.731	0.17	Cis-13-Eicosenoic acid , Methyl ester
26	21.895	0.33	Oxiraneoctanoic acid , 3-octyl , Methyl ester
27	22.014	0.15	Octadecanoic acid 9,10-epoxy, isopropyl ester
28	22.482	4.15	15-Tetracosenoic acid , Methyl ester,(z)-
29	22.633	1.20	Tetracosanoic acid , Methyl ester
30	22.839	0.11	9-Octadecenoic acid, 1,2,3-propanetriyl ester
31	23.901	0.18	Cyclpropaneoctanoic acid , 2-hexyl Methyl ester

9,12-Z,Z-Octadecadienoic acid methyl ester(16.20%)

The EI mass spectrum of 9,12-Z,Z-octadecadienoic acid methyl ester shown in Fig.3.The peak at m/z 294, which appeared at R.T. 17.51 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.

11-Eicosenoic acid methyl ester(12.12%)

The EI mass spectrum of 11-eicosenoic acid methyl ester is shown in Fig.4.The peak at m/z 324, which appeared at R.T. 19.32 in total ion chromatogram, corresponds to $M^+[C_{21}H_{40}O_2]^+$ .The peak at m/z292 corresponds to loss of a methoxyl function.

9,12,15-Octadecatrienoic acid, methyl ester (10.75%)

The EI mass spectrum of 9,12,15-octadecatrienoic acid, methyl ester is shown in Fig.5.The peak at m/z 292, which appeared at R.T. 17.58 in total ion chromatogram, corresponds to $M^+[C_{19}H_{32}O_2]^+$ .The peak at m/z277 corresponds to loss of a methyl group while the signal at m/z261 is due to loss of a methoxyl function.

9-Z--Octadecenoic acid methyl ester(5.90%)

The EI mass spectrum of 9-Z-octadecenoic acid methyl ester is shown in Fig.6.The peak at m/z 296, which appeared at R.T. 17.57 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z266 corresponds to loss of a methoxyl function.

Hexadecanoic acid methyl ester(4.26%)

The EI mass spectrum of hexadecanoic acid methyl ester is shown in Fig.7.The peak at m/z 270, which appeared at R.T. 15.84 in total ion chromatogram,

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corresponds to $M^+[C_{17}H_{34}O_2]^+$ .The peak at m/z239 corresponds to loss of a methoxyl function.

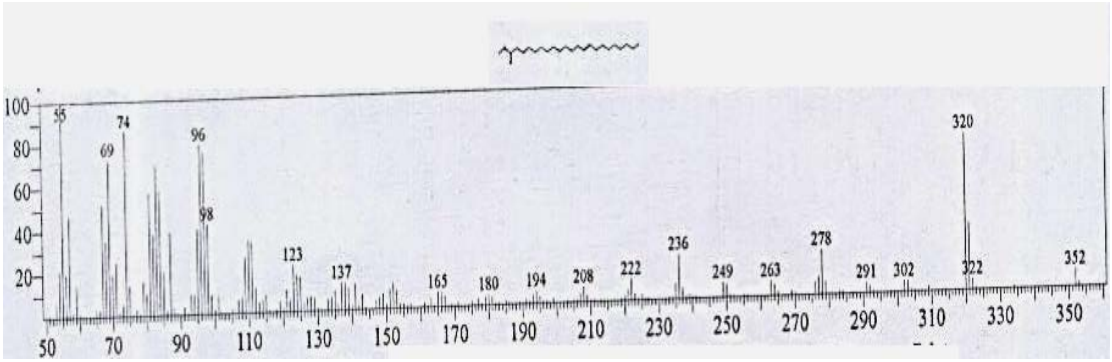

Fig. 2: Mass spectrum of 13-docosenoic acid methyl ester

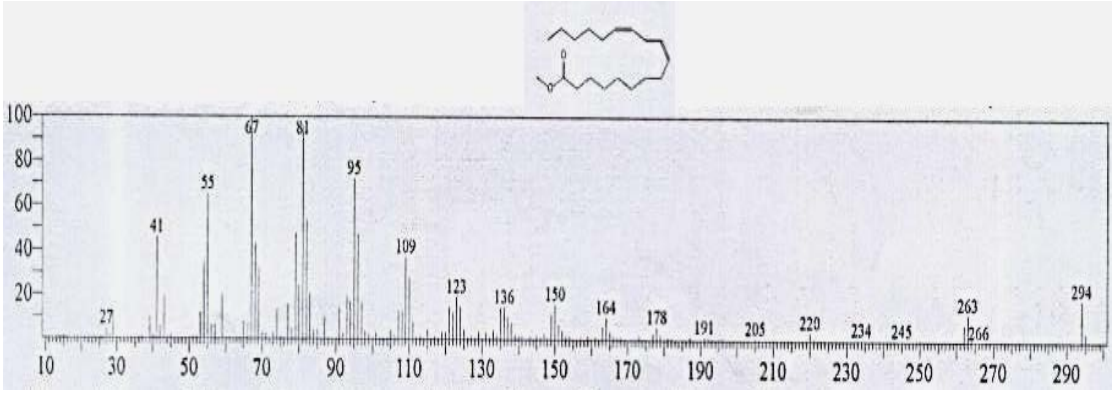

Fig. 3: Mass spectrum of 9,12-Z-Z-octadecenoic acid methyl ester

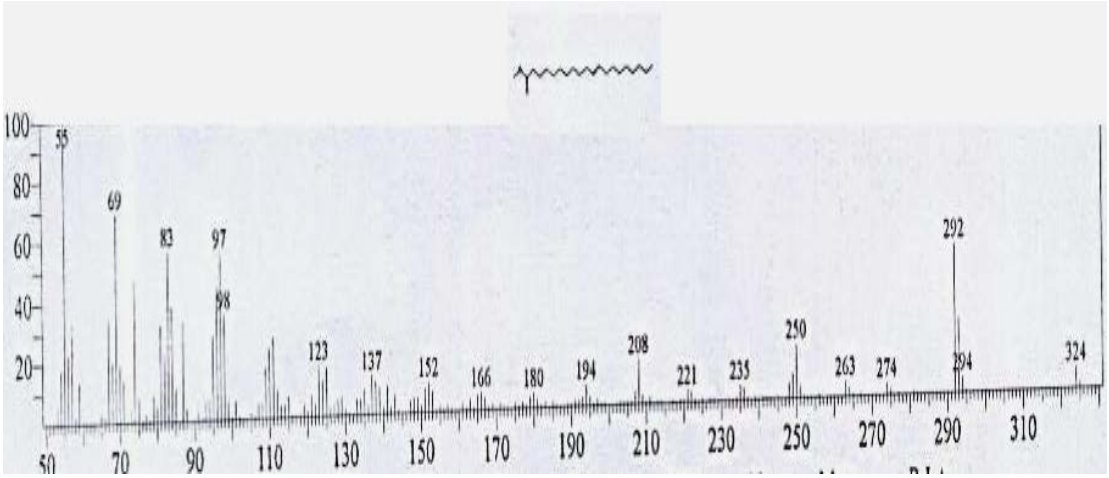

Fig. 4: Mass spectrum of 11-eicosenoic acid methyl ester

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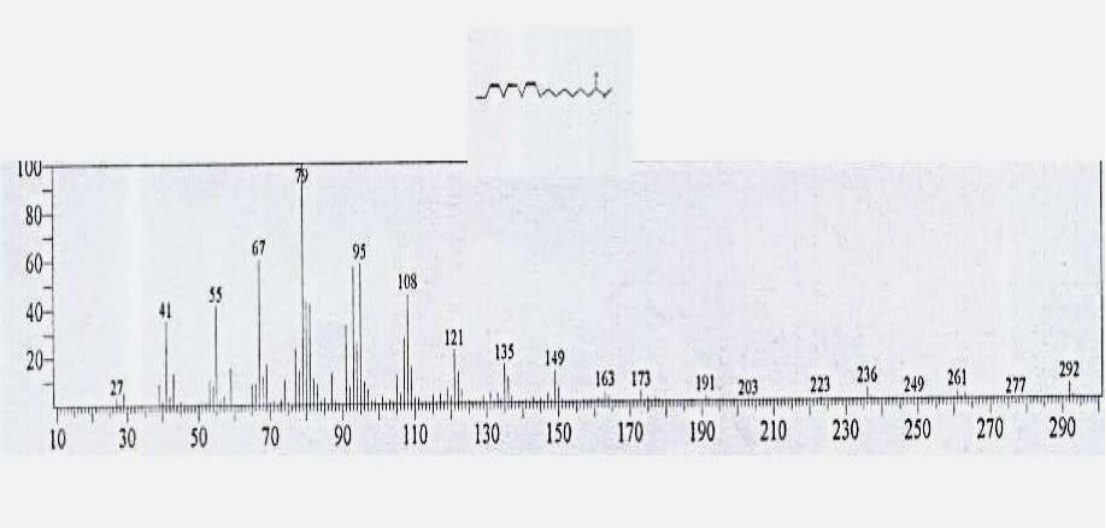

Fig.5: Mass spectrum of 9,12,15-octadecatrienoic acid, methyl ester

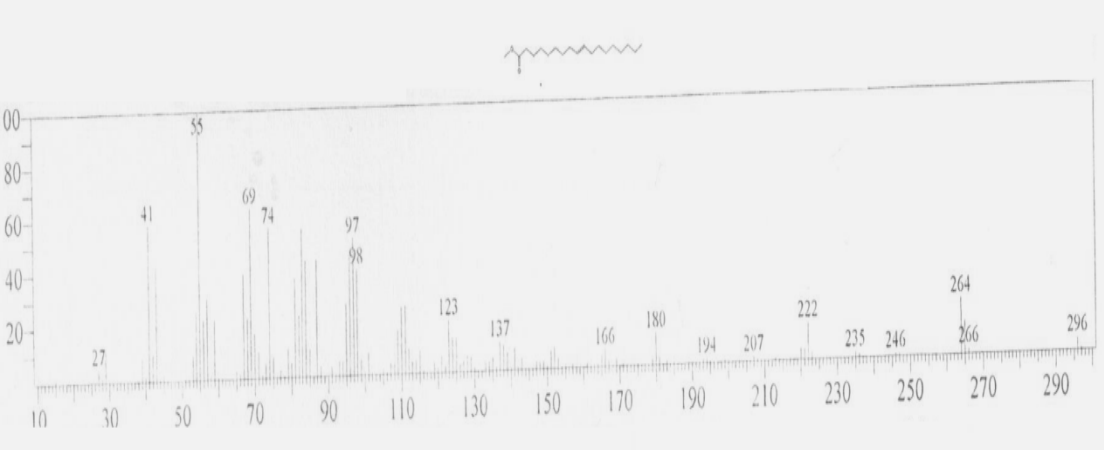

Fig. 6: Mass spectrum of 9-Z-octadecenoic acid methyl ester

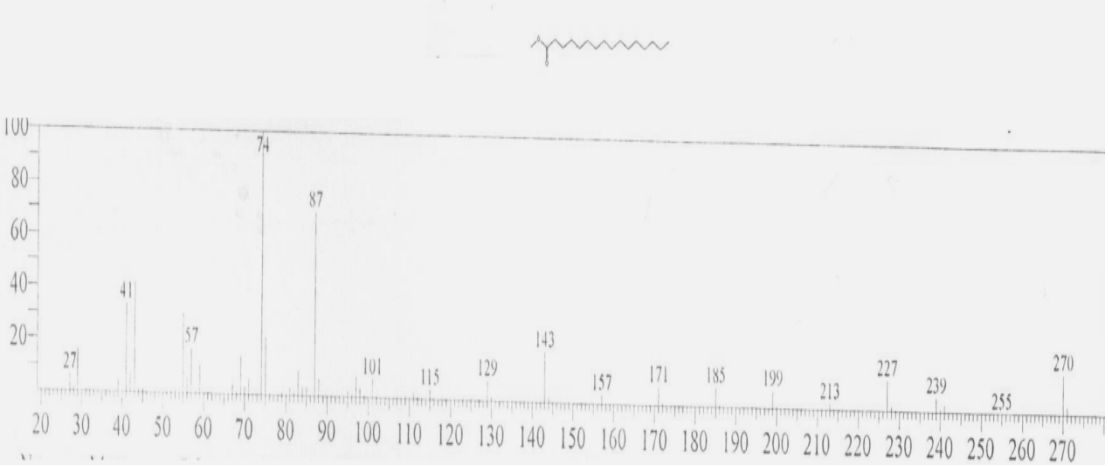

Fig. 7: Mass spectrum of hexadecanoic acid methyl ester

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