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RESEARCH ARTICLE

GC-MS ANALYSIS AND ANTIMICROBIAL ACTIVITY OF FIXED OIL FROM SUDANESE
PEGANUM HARMALA L. (ZYGOPHYLLACEAE).

*Abdel Karim. M.¹,Zohor,M.² and Sufian,A.³

1. Sudan University of Science and Technology, Faculty of Science, Dept. of Chemistry.
2. Institute of Industrial Consultancy and Research
3. Taibah University, Faculty of Science, Dept. of Chemistry

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***Corresponding Author**

Abdel Karim. M.

Abstract

This study was planned to identify and quantify the lipid composition of Sudanese *Peganum harmala* (L.) seeds and to evaluate the potential antimicrobial activity of the extracted fixed oil . GC- MS analysis of the fixed oil revealed the presence of : 9,12- octadecadienoic acid (49.76%),9- octadecenoic acid (19.30%) , hexadecanoic acid(10.86%) , methyl stearate(6.37%) beside other minor constituents. In cup plate agar diffusion assay, the oil was screened for antimicrobial activity against six standard human pathogens and promising results were obtained.

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Introduction:-

Peganum harmala L. (Zygophyllaceae) is native to eastern Mediterranean region. Due to resemblance to plants of the rue family, It is also known as Wild Rue or Syrian Rue(Mikaili ,2012). *Peganum harmala* is a perennial plant which can grow to about 0.8 m tall, but normally it is about 0.3 m tall. It blossoms between June and August in the northern hemisphere . The roots of the plant can reach a depth of up to 6.1 m, if the soil is very dry. The flowers are white and are about 2.5–3.8 cm in diameter. The round seed capsules measure about 1–1.5 cm in diameter(Frison *et. al.*,2008; El Gendy *et.al.*,2009; Wanntorp *et.al.*,2012; Sheahan *et.al.*,2000).

Peganum harmala is used traditionally in the treatment of a wide array of human disorders.β-Carboline alkaloids were identified in different parts(seeds,roots,barks) of *Peganum harmala*. Various pharmacological surveys demonstrated that harmala alkaloids namely; harmaline, harmine, harmalol and harmol are biologically active compounds.

The plant is employed in ethno-medicine to treat hypertension and cardiac disease(Tahraoui *et.al.*,2007; Fortunato *et.al.*,2009). Extracts of seeds exert vasorelaxant effects(Hamsa and Kuttan,2010) and alkaloids of *Peganum harmala* were shown to have anti-platelet aggregation effects(Saeed *et.al.*,1993).Furthermore,these alkaloids were shown to be psychoactive in mammalian body(Airaksinen and Kari , 1981).Various studies demonstrated a wide range of effects produced by *Peganum harmala* extracts on the central nervous system including ;analgesic(Airaksinen and Kari , 1981; Monsef and Ghobadi ,2004),hallucination, excitation (Nasehi *et.al.*,2010) and antidepressant(Fortunato *et.al.* ,2009; Farzin *et.al.*,2006) effects.Harmal alkaloids were shown to be involved in pathogenesis of Parkinson`s disease(Splettstoesser *et.al.*,2005; Storch *et.al.*,2004).

Various studies indicated antiparasidal (Storch *et.al.*,2008; Akhtar *et.al.*, 2000), antifungal (Akhtar ,2010;Saadabi,2006) ,anti-bacterial (Akhtar ,2010; Prashanth and John,1999) and insecticidal (Rharrabe *et.al.*, 2007; Jbilou *et.al.*,2008) effects for Harmal alkaloids.

Significant *in vitro* and *in vivo* antileishmanial activity was exhibited by *Peganum harmala* seeds extract (Rahimi-Moghaddam *et al.*, 2011). Also it was reported that methanolic extracts of *Peganum harmala* reduced the number of living pups and produced a dose-dependent decrease in litter size of model animals (Shapira *et al.*, 1989). Frequent abortion is observed in animals that digest this plant in dry seasons (Mahmoudian *et al.*, 2002).

Recently there has been renewed interest in the use of medicinal plants by local communities to treat a broad spectrum of human disorders. In parallel there has been an increased scientific interest in the bioconstituents (steroids, alkaloids, flavonoids...etc) of these plants. A knowledge of bio-active components would evidently site a rationale for traditional uses of medicinal plants and enrich the global database of phytochemicals. The constituents and the antimicrobial potential of *Peganum harmala* oil were addressed by many authors. However, such constituents are influenced to some extent by geographical distribution. To the best of our knowledge there are no reports addressing the constituents and antimicrobial potency of the Sudanese material of *Peganum harmala* fixed oil. So we planned this study to evaluate the antimicrobial activity of fixed oil from *Peganum harmala* native to Sudan and to probe the constituents present in this oil.

Materials and Methods:-

Plant material:-

Seeds of *Peganum harmala* were collected from a forest reserve at "Hawata" – eastern Sudan. The plant was identified by direct comparison with reference herbarium sample.

Instruments:-

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

Test organisms:-

Peganum harmala 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	<i>Bacillus subtilis</i>	G+ve
2	<i>Staphylococcus aureus</i>	G+ve
3	<i>Pseudomonas aeruginosa</i>	G-ve
4	<i>Escherichia coli</i>	G-ve
5	<i>Aspergillus niger</i>	fungi
6	<i>Candida albicans</i>	fungi

Methods:-

Extraction of oil from *Peganum harmala* seeds:-

Powdered shade-dried seeds of *Peganum harmala* (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°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. (5 μ l) of the hexane extract were mixed with (5ml) diethyl ether. The solution was filtered and the filtrate (1 μ l) was injected in the GC-MS vial.

GC-MS analysis:-

Peganum harmala fixed oil was analyzed by gas chromatography – mass spectrometry. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 μ m, thickness)was used. Helium (purity; 99.99 %) was used as carrier gas. Oven temperature program is given in Table 2 and other chromatographic conditions are depicted in Table 3 .

Table 2:- Oven temperature program.

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

Table 3:- Chromatographic conditions.

Column oven temperature	150.0 $^{\circ}$ C
Injection temperature	300.0 $^{\circ}$ 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:-**Preparation of 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 $^{\circ}$ 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 10⁸-10⁹colony forming units per ml. The suspension was stored in the refrigerator at 4 $^{\circ}$ 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 dry 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 $^{\circ}$ C for 24 hours.

Preparation of fungal suspensions:-

Fungal cultures were maintained on dextrose agar incubated at 25 $^{\circ}$ 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 $^{\circ}$ 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. Separate Petri dishes were designed for standard antibacterial chemotherapeutics (ampicillin and gentamycin).

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 $^{\circ}$ 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 *Peganum harmala* fixed oil:-

GC-MS analysis of *Peganum harmala* 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 oil:-

The GC-MS spectrum of the studied oil revealed the presence of 43 components (Table 4). The typical total ion chromatograms (TIC) is depicted in Fig. 1.

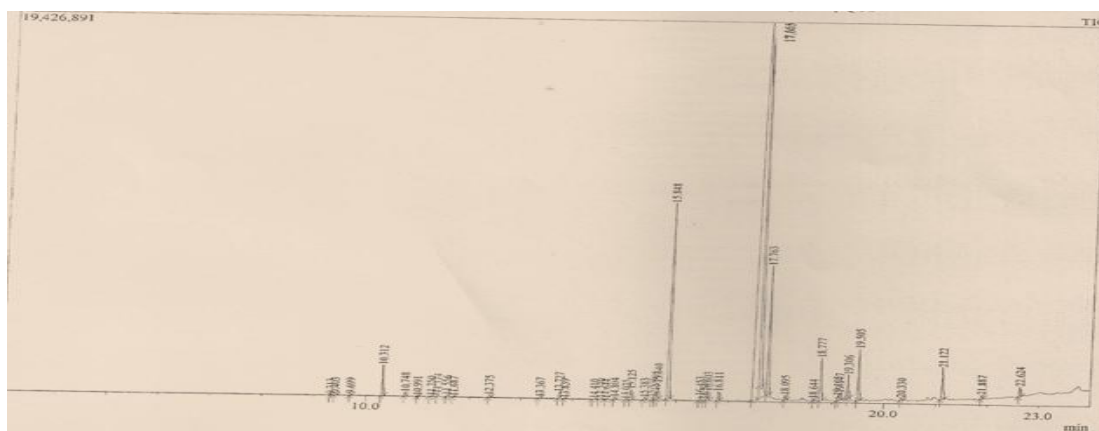


Fig. 1:- Chromatograms of *Peganum harmala* oil.

Table 4:- Constituents of *Peganum harmala* oil.

Peak Report TIC				
Peak#	R.Time	Area	Area%	Name
1	9.313	38106	0.02	.alpha.-Cubebene
2	9.403	161408	0.08	Eugenol
3	9.699	117267	0.06	.alfa.-Copaene
4	10.312	2534770	1.28	Caryophyllene
5	10.748	307066	0.16	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramet
6	10.991	33949	0.02	gamma,-Muurolene
7	11.250	58704	0.03	.alpha.-Farnesene
8	11.374	237716	0.12	Butylated Hydroxytoluene
9	11.556	126716	0.06	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-d
10	11.687	37042	0.02	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-d
11	12.375	172655	0.09	1H-Cycloprop[e]azulen-4-ol, decahydro-1,
12	13.367	98368	0.05	Santalol, cis,.alpha.-
13	13.727	493507	0.25	Methyl tetradecanoate
14	13.839	35339	0.02	.beta.-Santalol
15	14.410	47077	0.02	Methyl 13-methyltetradecanoate
16	14.540	93168	0.05	5-Octadecenoic acid, methyl ester
17	14.644	57896	0.03	6-Octadecenoic acid, methyl ester
18	14.804	163133	0.08	Pentadecanoic acid, methyl ester
19	15.025	18141	0.01	2-Pentadecanone, 6,10,14-trimethyl-
20	15.125	769918	0.39	Musk ambrette (artificial)
21	15.383	228703	0.12	n-Nonadecanol-1
22	15.536	197684	0.10	7,10-Hexadecadienoic acid, methyl ester
23	15.595	777759	0.39	Methyl hexadec-9-enoate
24	15.640	1221537	0.62	9-Hexadecenoic acid, methyl ester, (Z)-
25	15.848	21455053	10.86	Hexadecanoic acid, methyl ester
26	16.453	337846	0.17	Hexadecanoic acid, 15-methyl-, methyl est
27	16.540	91310	0.05	Hexadecanoic acid, 14-methyl-, methyl est
28	16.603	768084	0.39	cis-10-Heptadecenoic acid, methyl ester
29	16.811	743750	0.38	Heptadecanoic acid, methyl ester
30	17.565	98353486	49.76	9,12-Octadecadienoic acid (Z,Z)-, methyl e
31	17.605	38154905	19.30	9-Octadecenoic acid (Z)-, methyl ester
32	17.763	12582612	6.37	Methyl stearate
33	18.095	391479	0.20	Cyclopropaneoctanoic acid, 2-octyl-, meth
34	18.644	144498	0.07	Nonadecanoic acid, methyl ester
35	18.777	3907267	1.98	Methyl 9.cis.,11.trans.t,13.trans.-octadecat
36	19.105	437657	0.22	Methyl 5,13-docosadienoate
37	19.147	605638	0.31	gamma,-Linolenic acid, methyl ester
38	19.306	2002847	1.01	11-Eicosenoic acid, methyl ester
39	19.505	4871911	2.46	Methyl 18-methylnonadecanoate
40	20.330	219451	0.11	Heneicosanoic acid, methyl ester
41	21.122	3239274	1.64	Methyl 20-methyl-heneicosanoate
42	21.887	351954	0.18	Tricosanoic acid, methyl ester
43	22.624	964636	0.49	Tetracosanoic acid, methyl ester
		197651287	100.00	

The following important constituents were detected:

9-Octadecenoic acid methyl ester(19.30%):-

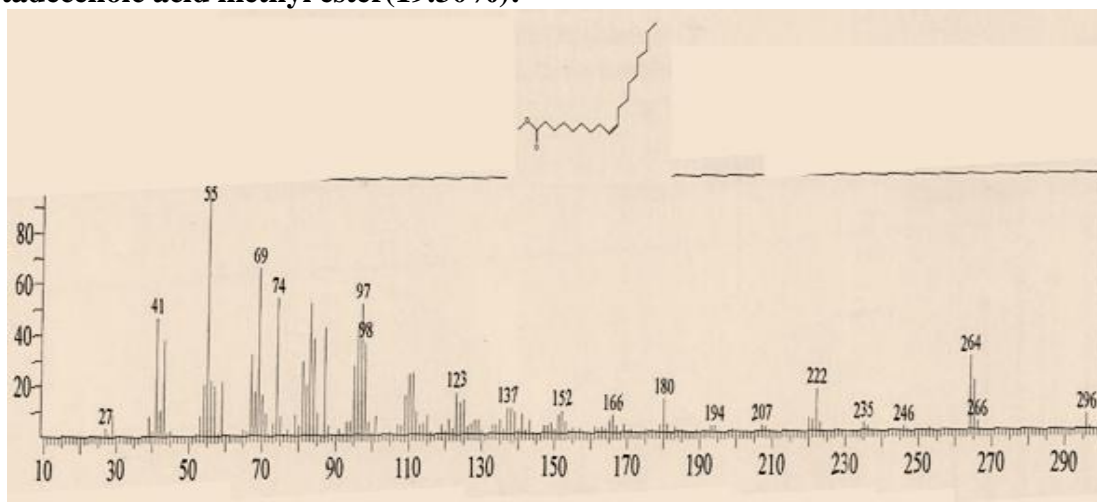


Fig. 2:- Mass spectrum of 9-octadecenoic acid methyl ester.

The EI mass spectrum of 9-octadecenoic acid methyl ester is shown in Fig. 2. The peak at m/z 296, which appeared at R.T. 17.605 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$. The peak at m/z 266 is due to loss of a methoxyl function.

As part of animal fats and vegetables, 9-octadecenoic acid (oleic acid) is included in the normal human diet. It is used as emollient (Currasco, 2002). It is also a major component of soap. The acid is used in small amounts as excipient in pharmaceutical industries and as soldering flux in stained glass work. Oleic acid is a common monounsaturated fat in human diet. This acid may be responsible for the hypotensive potential of olive oil (Terese *et al.*, 2008). The consumption of oleate in olive oil has been associated with decreased risk of breast cancer (Martin-Moreno *et al.*, 1994).

9,12-Octadecadienoic acid methyl ester (49.76%):-

The EI mass spectrum of 9,12-octadecadienoic acid methyl ester is shown in Fig. 3. The peak at m/z 294, which appeared at R.T. 17.565 in total ion chromatogram, corresponds to $M^+[C_{19}H_{34}O_2]^+$. The peak at m/z 263 corresponds to loss of a methoxyl function.

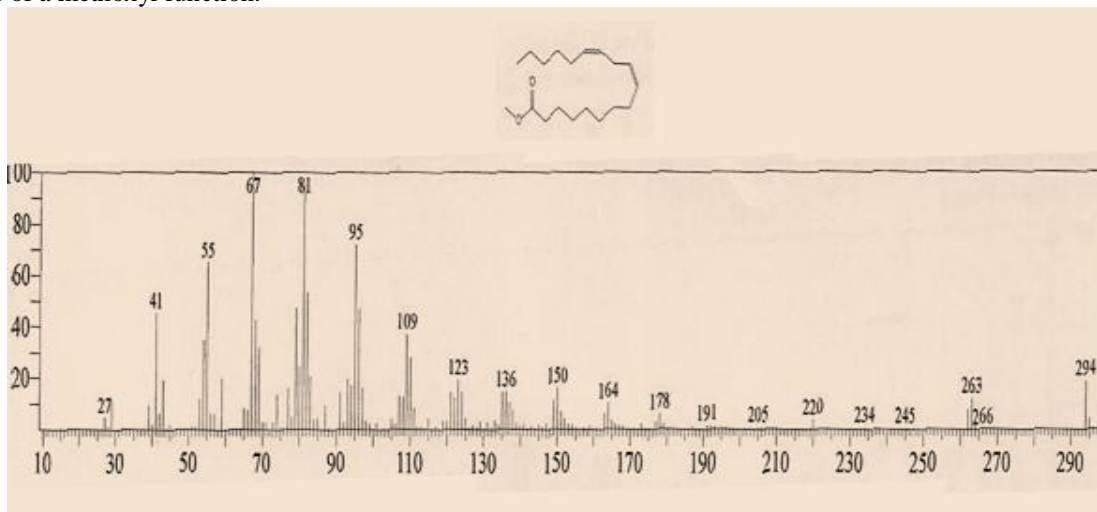


Fig. 3:- Mass spectrum of 9,12-octadecadienoic acid methyl ester

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 (Burr *et.al.*, 1930). Linoleic acid is used in the biosynthesis of arachidonic acid. It exists in lipids of cell membrane. Rich sources are: nuts and fatty seeds. 9,12-Octadecadienoic is converted by some enzymes like lipoxygenases into mono-hydroxy products which are enzymatically oxidized to keto metabolites. Such metabolites are implicated in human physiology and pathology. Linoleate deficiency caused mild skin scaling, hair loss and poor wound healing in model animals (Cunnane and Anderson, 1997; Ruthig and Mecklung-Gill, 1999).

Hexadecanoic acid methyl ester (10.86%):-

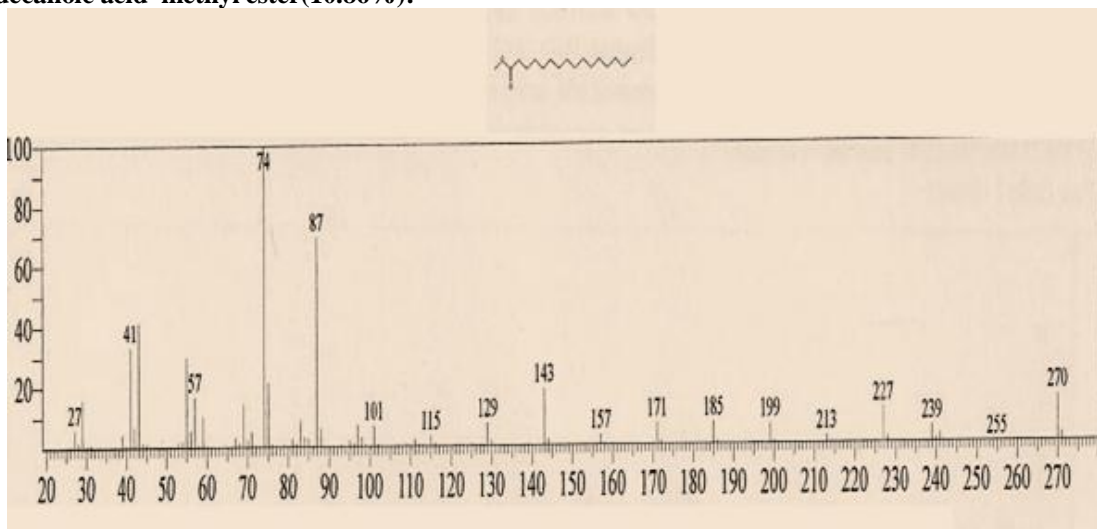


Fig. 4:- Mass spectrum of hexadecanoic acid methyl ester.

The EI mass spectrum of hexadecanoic acid methyl ester is shown in Fig. 4. The peak at m/z 270, which appeared at R.T. 15.848 in total ion chromatogram, corresponds to $M^+[C_{17}H_{34}O_2]^+$. The peak at m/z 239 is due to loss of a methoxyl function.

hexadecanoic acid (palmitic acid) is a saturated fatty acid. In plants and humans, it is the most common fatty acid. During the synthesis of fatty acids, palmitic acid is produced first (Gunstone *et.al.*, 2007). It is the precursor of long-chain fatty acids. This acid is a major lipid component of human breast milk (Kingsbury *et.al.*, 1961; Jensen *et.al.*, 1978). Palmitic acid is used in production of soaps and cosmetics, it is also widely used in food industry.

Methyl stearate (6.37%):-

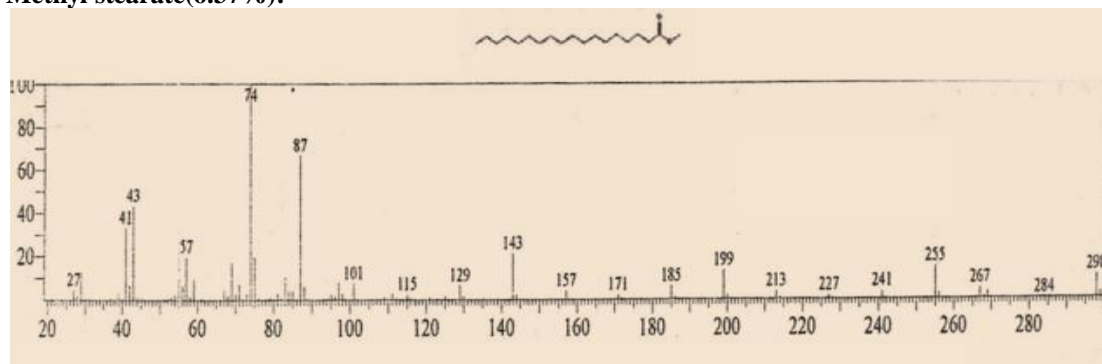


Fig. 5:- Mass spectrum of methyl stearate.

The EI mass spectrum of methyl stearate is shown in Fig. 5. The peak at m/z 298, which appeared at R.T. 17.763 in total ion chromatogram, corresponds to $M^+[C_{19}H_{38}O_2]^+$. The peak at m/z 267 corresponds to loss of a methoxyl function.

Antibacterial activity:-

In cup plate agar diffusion assay, the oil was evaluated for antimicrobial activity. The averages of the diameters of the growth inhibition zones are shown in Table (5). The results were interpreted in terms of the commonly used terms ; <9mm: inactive;9-12mm:partially active;13-18mm: active;>18mm:very active). Tables (6) and (7) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table 5:- Antibacterial activity of *Peganum harmala* oil : M.D.I.Z,(mm)

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca	An
oil	100	15	9	17	17	16	18

Table 6:- 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 7:- Antifungal activity of standard chemotherapeutic agent against standard fungi.

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 all test organisms, but it was partially active against *Pseudomonas aeruginosa*. Significant activity was observed against the Gram positive bacteria: *Staphylococcus aureus*, *Bacillus subtilis* and the fungus *Aspergillus niger*. Thus the oil is a plausible candidate for further optimization.

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