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RESEARCH ARTICLE

GC-MS Analysis and Antimicrobial Activity of Sudanese *Vigna unguiculata* (L.) Walp. Oil.

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Abstract

The present study was designed to investigate the chemical constituents of *Vigna unguiculata* (L) Walp. oil and to evaluate its potential antimicrobial activity.. Forty eight components were detected by GC-MS analysis. Major constituents are: 9,12-octadecadienoic acid(49.76%),9-octadecenoic acid(19.30%) and hexadecanoic acid(10.86%) .Some sterols were detected as minor components including : 3-β-9,19-cyclolanost-24-en-3-ol(1.11%) and fucosterol(1.08%).The antibacterial activity of the oil was evaluated by cup plate agar diffusion assay against six standard human pathogens(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 showed activity against all test organisms. Significant activity was observed against the Gram positive bacteria: *Staphylococcus aureus* and *Bacillus subtilis* and the fungus *Aspergillus niger* .

Introduction:-

Vigna uguiculata (L) Walp (cowpea) - known in Sudan as "Luba Helo" belongs to the Fabaceae family .It grows extensively in lowlands and mid – altitude regions of Africa (particularly in the dry savanna) sometimes as sole crop but more often intercropped with cereals such as sorghum or millet(Agbogidi and Egho,2012). Cowpea is of major importance for millions of relatively poor people in less developed countries of tropics (FAO 2002). All part of the plant are nutritious providing proteins and vitamins. Immature pods and peas are used as vegetables while several snack dishes are prepared from the grains(Duke , 1981).Among the legumes, cowpea is the most extensively grown, distributed and traded food crop in Africa and Asia (Phillips and Walters,1991). This is because the plant is of considerable nutritional value to man and livestock. They form a major stable in the diet in Africa and Asian continents (Awe ,2008). The seeds make up the largest contributor of the overall protein intake of several rural and urban families hence cowpea is regarded as the poor man's source of protein. Their amino acids complements those of cereals (Fashakin and Ojo,1988) .Their mineral contents : calcium and iron are higher than that of meat, fish and egg and the iron content equates that of milk; the vitamins- thiamin , riboflavin and niacin exist in levels comparable with that found in lean meat and fish (Platt ,1962). Many researchers have shown that daily consumption of 100-135g of dry beans reduces serum cholesterol level by 20%, thereby, reducing the risk of

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coronary heart diseases by 40% (Anderson ,1985). Besides its health related benefits ,beans are inexpensive , considerably cheaper than rice or any other dietary fiber type (Aynelere ,2012; Singh and Rachei,1985; Allen and Allen,1981).

Materials and methods:-

Plant material:-

Vigna unguiculata (L) Walp. seeds were purchased from a local market at Omdurman city, Sudan, and was identified at the herbarium of the Aromatic and Medicinal Plants Research Institute.

Extraction of oil:-

(500g) of the seeds were milled into fine powder. Powdered seeds were extracted with n-hexane using Soxhlet extractor for two days. The volume of hexane was reduced under reduced pressure. The oil of *vigna unguiculata* was obtained by evaporating the reduced hexane by air drying in a steady current. The oil was kept in a refrigerator for further manupilation.

Refractive index determination:-

Few drops of *vigna unguiculata* oil were applied in the refract meter slide .The readings of the scale were recorded.

Acid value determination:-

(25ml) of diethyl ether were mixed with (80%) ethyl alcohol.(1 ml) of phenolphthalein (1%) was added and the solution was carefully neutralized with (0.1 N) alkali. (2g) of the oil was dissolved in the neutral solvent and was titrated against (0.1N) sodium hydroxide solution until a pink color which persisted for 15 seconds was obtained . Consumed volume of sodium hydroxide was recorded.

Antimicrobial assay:-

The oil of *vigna unguiculat* was screened for its antimicrobial activity against six standard human pathogens (*Bacilus subtilis* (B.S), *Staphylococcus aureus* (Sa), *Escherichia colli* (Ec), *Pseudomonas aeruginosa* (Pa) , *Aspergillus niger* (An) and *Candida albacans* (Ca).

Preparation of bacterial suspensions:-

One ml aliquot 24 hours broth culture of the test organisms were aseptically distributed onto hutment 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 producing a suspension containing about 10^{8} - 10^{4} colony forming units per ml. The suspension was stored in the refrigerator at 4^{o} C until used. The average of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilution of the stock suspension were made in sterile saline in tubes and one drop volumes (0-20ml) of the appropriate dilution were transferred by adjustable volume micropipette onto the surface of dried agar plates.

The plates were allowed to stand for two hours at room temperature for drop to dry, and then incubated at 37^{o} C for 24 hours.

Preparation of fungal suspensions:-

Fungal cultures were maintained on dextrose agar and 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:-

To determine the antimicrobial activity of the oil , the cup- plate agar diffusion method was adopted with some minor modification. (2ml) of the standard bacteria stock suspension were mixed with (200ml) of sterile molten nutrient agar which was maintained at 45^{o} C.

(20ml) aliquot of incubated agar were distributed into sterile Petri dishes. The agar was left to settle and each plate was cut using sterile cork-borer (No.4) and agar discs were removed. Alternates cups were filled with (0.1ml) of test sample using adjustable pipette and allowed to diffuse at room temperature . The Petri dishes were then incubated in the upright position at 37^{o} C for 18 hours. After incubation, the diameter of the resultant growth inhibition zones were measured.

Testing for antifungal activity:-

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar dextrose agar was used.

Results and Discussion:-

GC-MS analysis of *vigina unguiculata* fixed oil:-

Lipid Consituents of *vigna unguiculata* oil were identified and quantified by GC-MS. Identification of the components was accomplished by comparison with the MS library (NIST) .Furthermore, the observed fragmentation pattern was interpreted. Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

Constituents of oil:-

The GC-MS analysis of *vigna unguiculata* oil revealed the presence of 48 components(Table 1).The typical total ion chromatograms(TIC) is depicted in Fig.1.

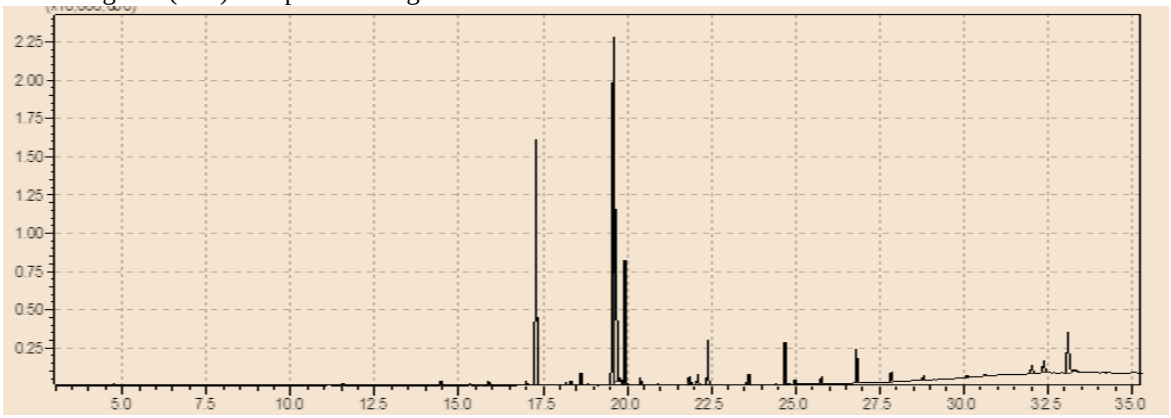


Fig. 1:- Typical Total Ion Chromatograms of *vigna unguiculata* oil

Table 1 :- Constituents of *vigna unguiculata* oil

Peak#	R.Time	Area	Area%	Name
1	3.463	45181	0.01	Hexanoic acid, methyl ester
2	4.756	378117	0.07	1-Hexanol, 2-ethyl-
3	4.835	28965	0.01	D-Limonene
4	4.895	38131	0.01	Eucalyptol
5	5.752	67374	0.01	Benzoic acid, methyl ester
6	6.065	130506	0.02	Octanoic acid, methyl ester
7	7.822	148640	0.03	Propanal, 2-methyl-3-phenyl-
8	8.834	107545	0.02	Decanoic acid, methyl ester
9	10.365	168379	0.03	Nonanoic acid, 9-oxo-, methyl ester
10	11.551	288422	0.05	Butylated Hydroxytoluene
11	11.595	574204	0.11	Dodecanoic acid, methyl ester
12	14.466	2678501	0.51	Methyl tetradecanoate
13	15.523	365995	0.07	5-Octadecenoic acid, methyl ester
14	15.885	1339792	0.25	6-Octadecanoic acid,methyl ester
15	16.948	724529	0.14	7-Hexadecenoic acid, methyl ester, (Z)-
16	17.009	1425287	0.27	9-Hexadecenoic acid, methyl ester, (Z)-
17	17.338	103704422	19.74	Hexadecanoic acid, methyl ester
18	18.132	255757	0.05	Heptadecanoic acid, methyl ester
19	18.191	1092231	0.21	Hexadecanoic acid, ethyl ester
20	18.326	619157	0.12	Methyl 5,12-octadecadienoate
21	18.390	352836	0.07	cis-10-Heptadecenoic acid, methyl ester
22	18.626	4264521	0.81	Heptadecanoic acid, methyl ester
23	19.620	114698256	21.83	9,12-Octadecadienoic acid (Z,Z)-, methyl e
24	19.707	100575541	19.14	9-Octadecenoic acid (Z)-, methyl ester
25	19.960	35281712	6.71	Methyl stearate
26	20.402	1040252	0.20	n-Propyl 9,12-octadecadienoate
27	21.804	2389371	0.45	Methyl 5,13-docosadienoate
28	21.868	4351750	0.83	6,9,12,15-Docosatetraenoic acid, methyl est
29	21.936	2285322	0.43	9,12,15-Octadecatrienoic acid, methyl este
30	22.047	2746069	0.52	Oxiraneoctanoic acid, 3-octyl-, methyl este
31	22.108	7199025	1.37	11-Eicosenoic acid, methyl ester
32	22.405	17963125	3.42	Methyl 18-methylnonadecanoate
33	23.566	1348350	0.26	Heneicosanoic acid, methyl ester
34	23.615	951870	0.18	Phenol, 2,2'-methylenebis[6-(1,1-dimethyle
35	24.704	28773655	5.48	Methyl 20-methyl-heneicosanoate
36	25.772	4439533	0.84	Tricosanoic acid, methyl ester
37	26.822	18338029	3.49	Tetracosanoic acid, methyl ester
38	28.799	1746727	0.33	Hexacosanoic acid, methyl ester
39	29.081	6234786	1.19	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-din
40	29.565	10051199	1.91	Stigmast-8(14)-en-3.beta.-ol
41	30.094	1214910	0.23	.gamma.-Tocopherol
42	30.635	2208114	0.42	Stigmast-5-en-3-ol, oleate
43	30.860	3014340	0.57	Cholest-5-en-3-ol, (3.alpha.)-
44	32.014	5335788	1.02	.gamma.-Ergostenol
45	32.384	14984963	2.85	Stigmasterol
46	33.096	7968014	1.52	Stigmast-7-en-3-ol, (3.beta.,5.alpha.,24S)-
47	33.324	5655388	1.08	Fucosterol
48	34.146	5854661	1.11	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-
		525449242	100.00	

Some important constituents are discussed below:

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

Fig. 2 shows the EI mass spectrum of 9-octadecenoic acid methyl ester.The peak at m/z 296, which appeared at R.T. 17.602 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

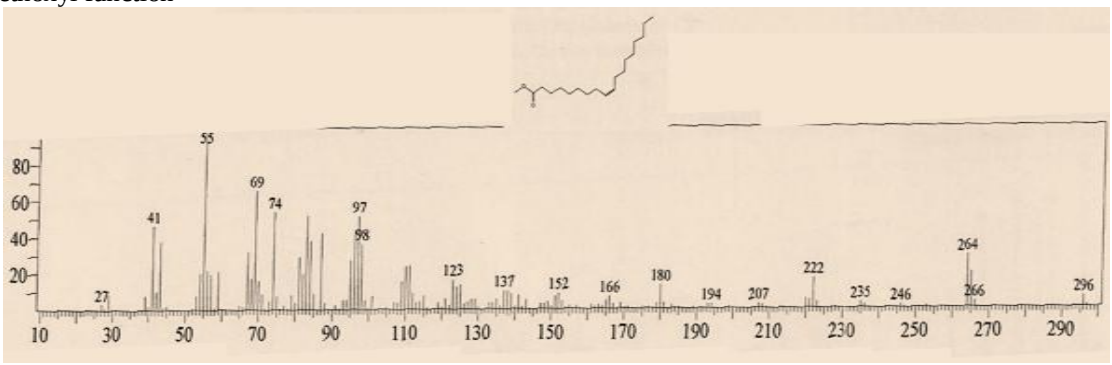


Fig. 2:- Mass spectrum of 9-octadecenoic acid methyl ester

9-octadecenoic acid(oleic acid) is a common monounsaturated fat in human diet. It may be responsible for the hypotensive potential of olive oil(Terese et.al.,2008). Oleic acid finds some applications in soap industry and it is used in small amounts as excipient in pharmaceutical industries. It is also used as soldening flux in stained glass work. Oleic acid is employed as emollient(Currasco,2002). 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%):-

Fig. 3 shows the EI mass spectrum of 9,12-octadecadienoic acid methyl ester.The peak at m/z294, which appeared at R.T. 17.565 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.

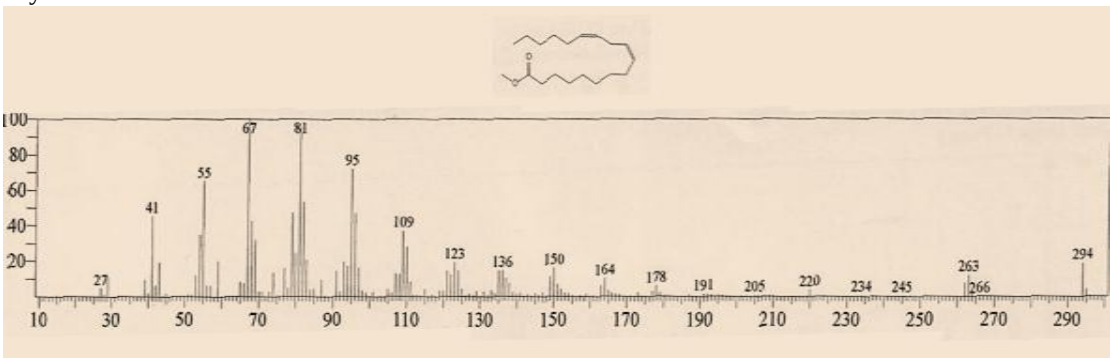


Fig. 3:- Mass spectrum of 9,12-octadecadienoic acid methyl ester

9,12-Octadecadienoic **(**linoleic) acid can not be synthesized by human bodies and is available through diet(Burr *et.al.*,1930).It belongs to one of the two families of essential fatty acids. It exists in lipids of cell membrane and is used in the biosynthesis of arachidonic acid . Oleic acid is converted enzymatically into mono-hydroxy products which are subsequently oxidized by some enzymes to keto metabolites.These metabolites are implicated in human physiology and pathology. Deficiency of linolate caused hair loss and poor wound healing in model animals(Cunnane and Anderson,1997; Ruthig and Mecklung-Gill,1999).

Hexadecanoic acid methyl ester(10.86%):-

Mass spectrum of hexadecanoic acid methyl ester is depicted in Fig. 4.The peak at m/z 270, which appeared at R.T. 15.848 corresponds to $M^+[C_{17}H_{34}O_2]^+$ while the peak at m/z239 is attributed to loss of a methoxyl function.

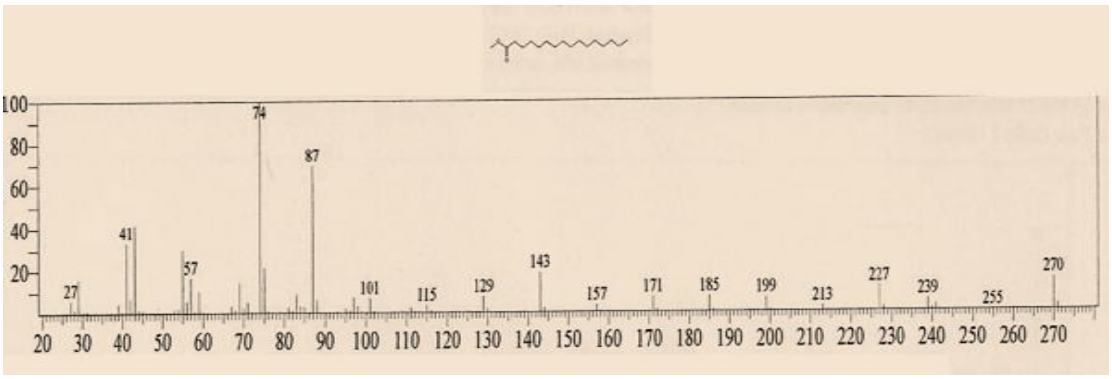


Fig. 4:- Mass spectrum of hexadecanoic acid methyl ester

Palmitic acid (hexadecanoic acid) is a saturated fatty acid .It is wide-spread in plants and humans . This acid is produced first during the synthesis of fatty acids (Gunstone et.al.,2007) and is considered as precursor of long-chain fatty acids. Palmitic acid is a major lipid component of human breast milk(Kingsbury et.al.,1961; Jensen et.al.,1978). The acid finds applications in soap and cosmetics industries. It is also used in food industry .

Methyl stearate(6.37%):-

Mass spectrum of methyl stearate is shown in Fig. 5.The peak at m/z 298, which appeared at R.T. 17.763 corresponds to $M^+[C_{19}H_{38}O_2]^+$ .The peak at m/z267 corresponds to loss of a methoxyl function.

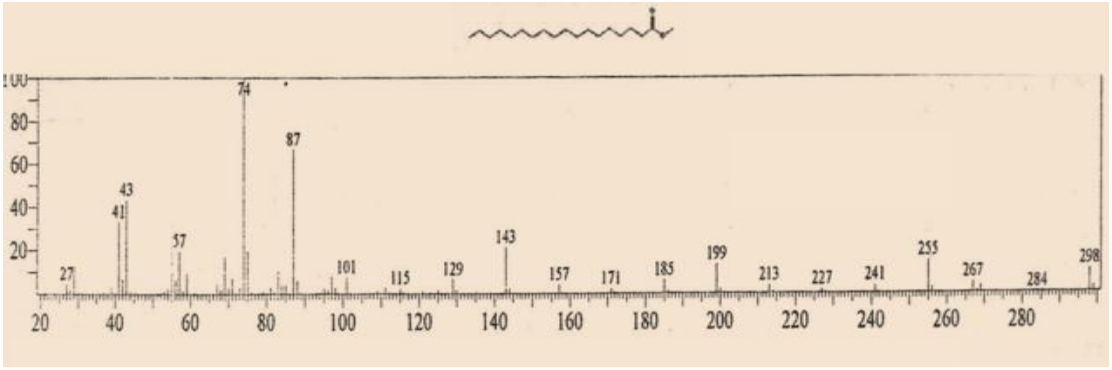


Fig. 5:- Mass spectrum of methyl stearate

3-β-9,19-Cyclolanost-24-en-3-ol(1.11%):-

The EI mass spectrum of 3-β-9,19-cyclolanost-24-en-3-ol is shown in Fig. 6.The peak at m/z 426, which appeared at R.T. 34.14 in total ion chromatogram, corresponds to $M^+[C_{30}H_{50}O]^+$.

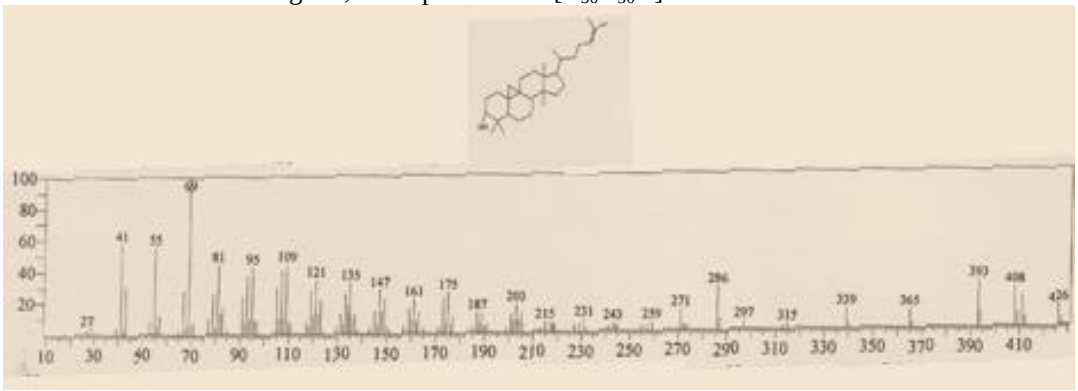


Fig. 6:- Mass spectrum of 3-β-9,19-Cyclolanost-24-en-3-ol

Cycloartenol (3-β-9,19-cyclolanost-24-en-3-ol) is a phyto- terpenoid. It is the starting material for the synthesis of almost all plant steroids(Schaller,2003). In contrast, fungi and animals steroids are produced from lanosterol

Fucosterol(1.08%):-
The EI mass spectrum of fucosterol is shown in Fig. 7.The peak at m/z 412, which appeared at R.T. 33.324 in total ion chromatogram, corresponds to $M^+[C_{29}H_{48}O]^+$.

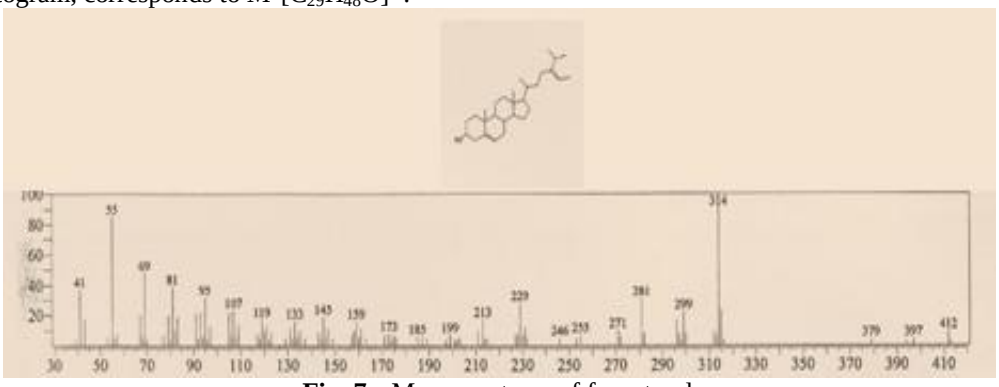


Fig. 7:- Mass spectrum of fucosterol

In animal model studies ,Sanghym *et.al*.(2003) claimed antioxidant acivity for fucosterol in addition to hepatoprotective properties. While Lee *et.al*.(4004) demonstrated *in vivo* antidiabetic activity of fucosterol

δ-Tocopherol(1.19%):-
The mass spectrum of **δ** -tocopherol is depicted in Fig. 7.The peak at m/z 402, which appeared at R.T. 33.324 in total ion chromatogram, corresponds to $M^+[C_{27}H_{46}O_2]^+$.

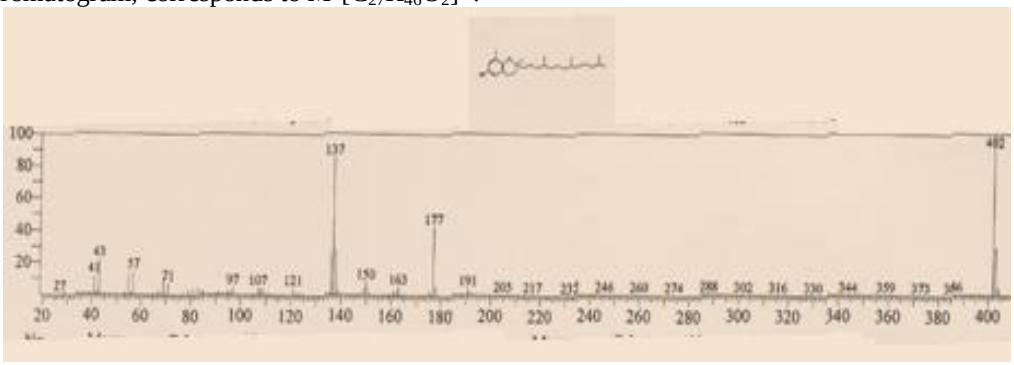
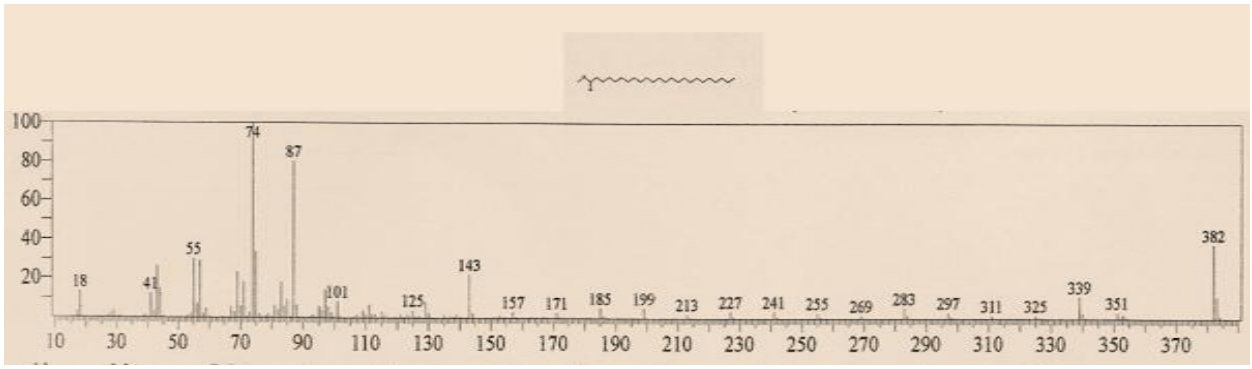


Fig. 7:- Mass spectrum of **δ** –tocopherol

Tocoferols and tocotrienols are essential nutrients for the body found in selected vegetable oils including rice bran and palm oil. Tocoferols and tocotrienols act as antioxidants protecting cell membranes, active enzymes and DNA from free radical damage(Cerecetto and López 2007). For δ-tocoferol ,Yoshinori *et.al.* claimed that a synergistic antioxidant effects was observed in the presence of epicatechin and epigallocatechin gallate.

Tetracosanoic acid(3.49%)
Mass spectrum of tetracosanoic acid is shown in Fig. 8.The peak at m/z 382, which appeared at R.T. 26.822 in total ion chromatogram, corresponds to $M^+[C_{25}H_{50}O_2]^+$,while the peak at m/z351 accounts for loss of a methoxyl function.



Tetracosanoic (lignoceric) acid, is a saturated fatty acid found in small amounts in most natural fats. It is also found in wood tar . Peanut oil contains small amounts of tetracosanoic acid.

Antibacterial activity:-

In cup plate agar diffusion assay, *vigna unguiculata* oil was evaluated for antimicrobial activity. The averages of the diameters of the growth inhibition zones are shown in Table (2) .The results were interpreted in commonly used terms ; <9mm: inative; 9-12mm:partially active; 13-18mm: active; >18mm:very active) .Tables (3) and (4) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table 2 :- Antibacterial activity of *vigna unguiculata* oil: M.D.I.Z(,mm)

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca	An
oil	100	14	15	18	18	16	18

Table 3 :- 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 4 :- 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. Significant activity was observed against the Gram positive bacteria: *Staphylococcus aureus, Bacillus subtilis* and the fungus *Aspergillus niger* .

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