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RESEARCH ARTICLE

GC-MS ANALYSIS AND BIOLOGICAL ACTIVITY OF FIXED OIL FROM SUDANESE *CAJANUS CAJAN* (L.) (LEGUMINOSEAE) SEEDS.

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Abstract

The present study was designed to identify the constituents of the fixed oil from seeds of Sudanese *Cajanus cajan* (L), and to evaluate its antimicrobial activity. GC- MS analysis of the fixed oil revealed the presence of 9,12–octadecadienoic acid (35.28%)**,** hexadecanoic acid (19.74%), 9-octadecenoic acid (19.14%) , methyl stearate(6.71%) 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.

Introduction:-

Leguminous plants belong to the family Fabaceae (Leguminosae). The fruits of these plants are called legumes . Legume seed and foliage have comparatively higher protein content than non legume materials, probably due to the additional nitrogen that legume received from nitrogen fixation symbiosis(David, 2014).

Cajanus cajan (L) known in Sudan (Aladassia) is one of the major grain legume crops grown in semi- arid tropics. It is valued both as food and fodder(Rama *et.al*.2013) . Pigeon pea (*Cajanus cajan* L.) probably evolved in South Asia and appeared about 2000 B.C . in West Africa which is considered as a second major center of origin(Van Den Beldt, 1988) . The slave trade took it to the Western Indies, where it is used as bird feed and this led to the name " Pigeon Pea" in 1692. Pigeon Pea (*Cajanus cajan* L.) is a leguminous plant (Van Der Maesen,1986) .

Pigeon Pea is a rich source of protein, carbohydrates and certain minerals. The protein content of commonly grown pigeon pea cultivars range between 17.9-24.3 g/100ml g for whole grain samples(Salunkhe *et.al.*,1985) . The pigeon pea plant as whole has been found useful. It is used for food and fuel . It is most widely eaten in the form of seeds ; it contains protein with amino acids profile similar to that of soybean (Singh *et.al*.,1990).Pigeon pea seeds contain about 57.3-58.7% carbohydrate, 1.2 – 8.1 crude fiber and 0.6 – 3.8% lipids(Singh , 1977**).**

Cajanus cajan is an erect, branched, hairy shrub, 1-2 meters high. Leaves are oblong-lanceolate to oblanceolate with three leaflets. Flowers are yellow, in sparse peduncled racemes, about 1.5-cm long.

It has been cultivated in ancient Egypt, Africa and Asia since prehistoric times, and was later introduced to America. Now it acclimatizes in several tropical countries. The major producer is India contributing about 90% of world production. Its altitude range is 1250 m in Hawaii, 0-3000 m in India and Columbia. It is essentially a plant of the semi-dry lowlands but has wide adaptability (Duke ,2004).

Pod is hairy, 4-7 cm long, 1 cm wide, containing two to seven seeds. The leaves are used for rearing silkworms while green pods are used as a vegetable.Also leaves and tops are used as fodder(Ambasta ,2004). Recently this

species has also been explored for the treatment of an array of human diseases including: an ischemic necrosis of the caput femoris, bed-sore and wound healing. Phytochemical investigations have revealed the presence of globulins, cajanin and concajanin(Ambasta ,2004) . *Cajanus cajan* is used traditionally as a sedative(Ahsan and Islam,2009**a**) and for treating , sores, skin irritations, hepatitis, measles, jaundice and dysentery . It is also employed for expelling bladder stones and stabilizing menstrual period(Yuan-gang ,2010).

Alcoholic extracts of the aerial parts of *C. cajan* were evaluated for anthelmintic properties(Pal *et.al.*,2007; Pal *et.al.*2008). This property was supposed to be due to the presence of phenolics (flavanoids and tannins) which are reported to have good anthelmintic property(Singh *et.al*. ,2010).In a study, the glycemic profile of the aqueous extract of *C. cajan* leaves in streptozocin-induced Type 2 diabetic rats was evaluated. This extract showed significant increment in fasting blood glucose levels of normal rats. The study of leaves was taken into consideration on the basis of earlier reported hypoglycemic activity of *C. cajan* seeds. However, the results observed for leaves were found to be just opposite and therefore leaves may be useful in controlling hypoglycemia occasionally caused due to excess of insulin and other hypoglycemic drugs (Jaiswal *et.al*.,8008).In animal model study, the methanolic extract of *C. cajan* was investigated for hepatoprotective activity against carbon tetrachloride – induced liver damage. It was found that the extract exhibited a moderate protective effect by lowering the serum levels of alanine aminotransferase (ALT) or serum glutamate pyruvate transaminase (SGPT), aspartate aminotransferase (AST) or serum glutamate oxaloacetate transaminase (SGOT), and cholesterol to a significant extent(Ahsan and Islam,2009**b**). Aqueous methanol fraction of the leaf extract also prevented alcohol- induced rat liver damage.

Cajanol, an isoflavanone from *C. cajan* roots is an important phytoalexin. The anticancer activity of cajanol towards MCF-7 human breast cancer cells was investigated . Cajanol inhibited the growth of MCF-7 cells in a time- and dose-dependent manner. Cajanol arrested the cell cycle in the G2/M phase and induced apoptosis via a reactive oxygen species (ROS) -mediated mitochondria-dependent pathway(Luo *et.al.* ,2010)

Materials and Methods.

Plant material:-

Seeds of *Cajanus cajan* were purchased from the local market-Khartoum.The plant was kindly 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**.**

Test organisms:-

Cajanus cajan oil was screened for antibacterial and antifungal activities using the standard microorganisms shown in Table(1).

Table 1: Test organisms.

Ser. 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*	fungi
6	*Candida albicans*	fungi

Methods:-

Extraction of oil from seeds of *Cajanus cajan*

Powdered seeds of *Cajanus cajan* (200g) 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 shaked 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 shaked 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:-

Cajanus cajan 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 µm, thickness)was used.Helium (purity; 99.99 %) was used as carrier gas.Oven temperature program is given in Table 2, while other chromatographic conditions are depicted in Table 3.

Table 2: Oven temperature program.

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

Table 3: Chromatographic conditions.

Column oven temperature	150.0^{o}C
Injection temperature	300.0^{o}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°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 10^{8}-10^{9}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.

Preparation of fungal suspensions:-

Fungal cultures were maintained on 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. 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°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:-

The GC-MS analysis of *Cajanus cajan* fixed oil:-

GC-MS analysis of *Cajanus cajan* 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 48 components(Table 4).The typical total ion chromatograms(TIC) is depicted in Fig.1.

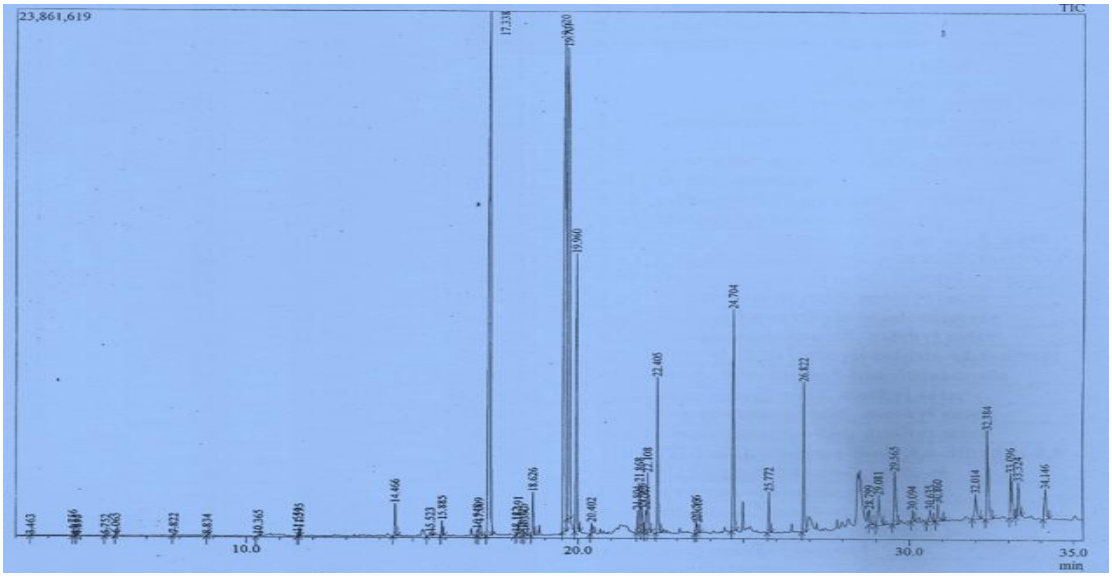

Fig. 1: Total ion chromatograms-*Cajanus cajan* oil.

Table 4: Contituents of *Cajanus cajan* oil.

Peak Report TIC

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 este
22	18.626	4264521	0.81	Heptadecanoic acid, methyl ester
23	19.620	114698256	21.83	9,12-Octadecadienoic acid (Z,Z)-, meth
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, methy
29	21.936	2285322	0.43	9,12,15-Octadecatrienoic acid, methyl
30	22.047	2746069	0.52	Oxiraneoctanoic acid, 3-octyl-, methyl
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-dimet
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
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.,24
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	

The most important constituents are discussed below:

9-Octadecenoic acid methyl ester(19.14%):-

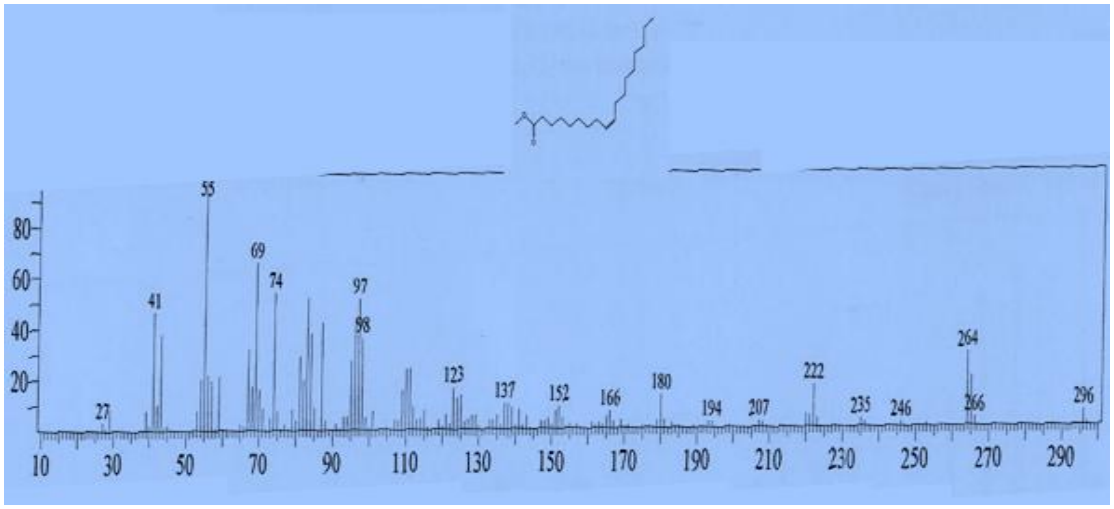

Fig. 2: Mass spectrum of 9-octadecenoic acid methyl ester.

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. 19.707 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z266 is due to loss of a methoxyl function.

9,12-Octadecadienoic acid methyl ester (21.83%):-

The EI mass spectrum of 9,12-octadecadienoic acid methyl ester is shown in Fig.3 .The peak at m/z294, which appeared at R.T. 19.620 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.

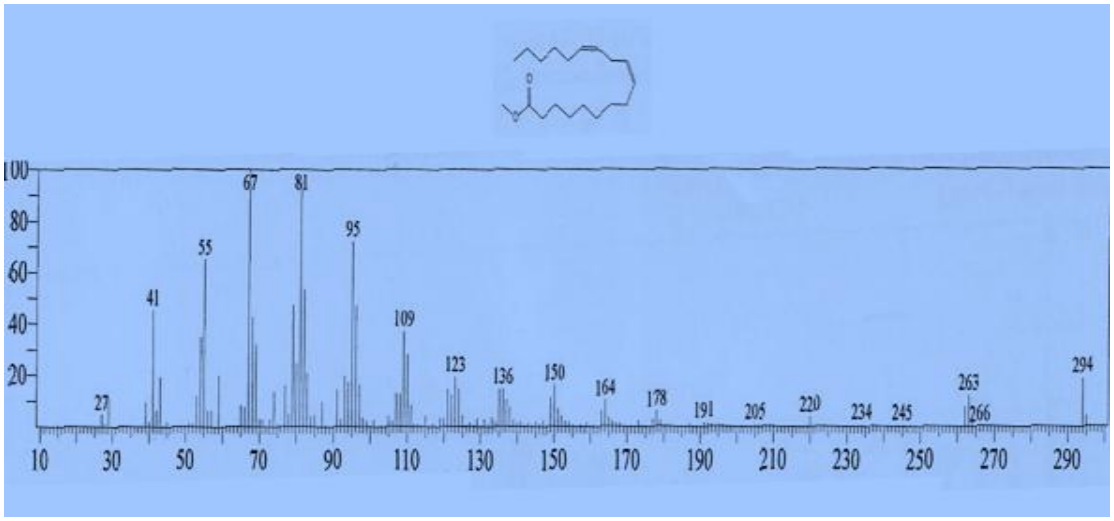

Fig. 3: Mass spectrum of 9,12-octadecadienoic acid methyl ester.

Hexadecanoic acid methyl ester(19.74%):-

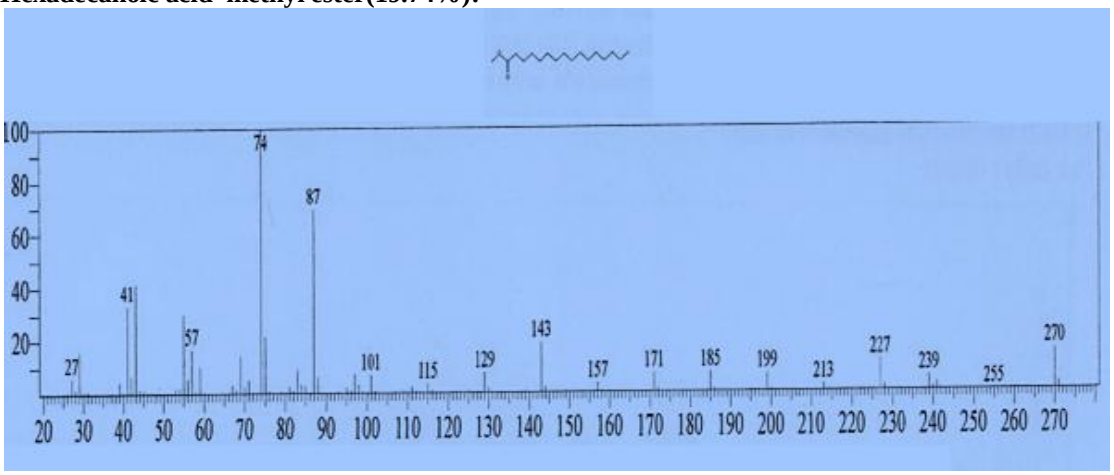

Fig. 4: Mass spectrum of hexadecanoic acid methyl ester.

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. 17.338 in total ion chromatogram, corresponds to $M^+[C_{17}H_{34}O_2]^+$ .The peak at m/z239 is due to loss of a methoxyl function.

Methyl stearate(6.71%):-

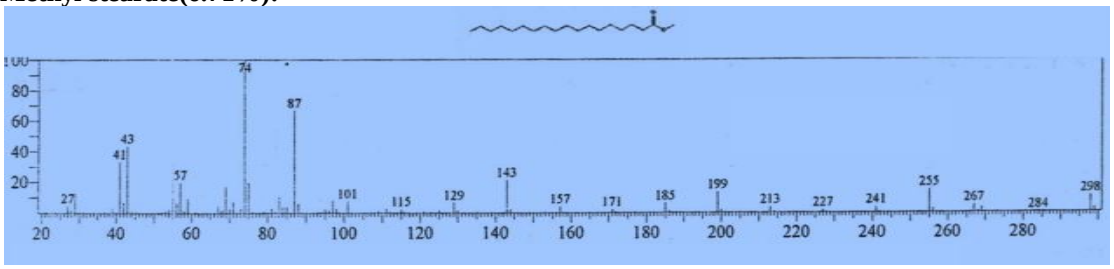

Fig. 5: Mass spectrum of methyl stearate.

The EI mass spectrum of methyl stearate is shown in Fig. 5.The peak at m/z 298, which appeared at R.T. 19.960 in total ion chromatogram, corresponds to $M^+[C_{19}H_{38}O_2]^+$ .The peak at m/z267 corresponds to loss of a methoxyl function.

Tetracosanoic acid methyl ester(3.45%):-

The EI mass spectrum of Tetracosanoic acid methyl ester is shown in Fig. 6.The peak at m/z382, which appeared at R.T. 26.822 in total ion chromatogram, corresponds to $M^+[C_{25}H_{50}O_2]^+$ .The peak at m/z351 corresponds to loss of a methoxyl function.

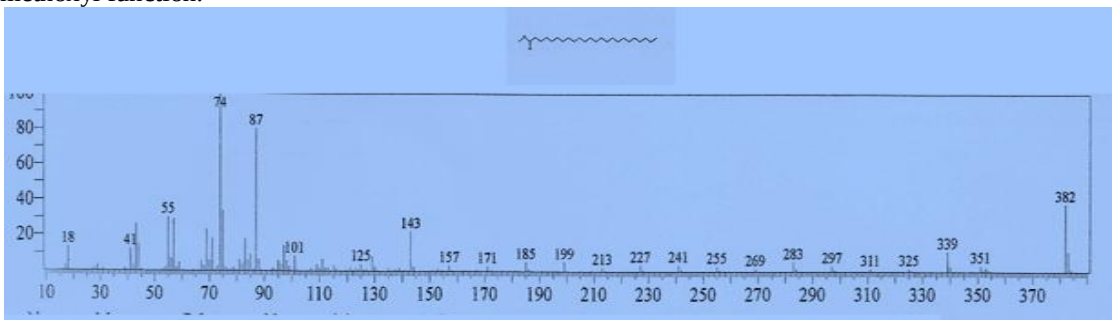

Fig. 6: Mass spectrum of Tetracosanoic acid methyl ester

Antibacterial activity:-

The oil was screened for antimicrobial activity against six standard organisms. The average of the diameters of the growth of inhibition zones are depicted in Table (5) .The results were interpreted in terms of the commonly used terms (<9mm: inative;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 *Cajanus cajan* oil :M.D.I.Z (mm).

	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca	An
oil	100	14	15	16	13	15	16

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 agents 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 more active against the fungus *Aspergillus niger* and the bacterial strain *Staphylococcus aureus* .

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