Chapter two

Materials and methods

2-1materials:-

2-1-1 sample:

Mango fruits are purchased from khartoum north local market.

2-1-2Chemicals

n-hexane

ethanol (96%)

methanol

light petroleum ether

diethyl ether
sodium hydroxide(0.1M)
sulfuric acid (1%)
hydrochloric acid (0.1)

Distilled water

Chloroform

Glacial acetic acid

Potassium Iodide

Starch

Phenolaphthaleine

Sodium thiosulphate

Di chloromethane

Iodine mono chloride solution

magnesium sulphate

super saturated sodium chloride solution.

Buffer solution

acetone

potassium Iodide

2-1-3Equipment:

- sensitive balance.
- soxhlet extractor.
- rotatory evaporation.
- thermometer

- Refractometer.
- Pycnometer.
- Viscometer
- PH meter
- FTIR- 8400S

Flourier transform infrared spectro photo meter

Model FTIR-8400 made in Japan

- GC-MS. QP2010 ULTRa

Gas chromatograph mass spectrometer

Shimadzu made in Japan

- Water bath
- Oven
- PH meter ELLCD LI120 Ph meter.

2-2methods:-

2-2-1Preparation of the sample:

The seeds were washed and air dried and the kernels were removed manually from seeds. The kernels were chopped and dried .
The dried material was ground in a hammer mill (Apex Wiley provided with a 100 mesh sieve) into a powdery form and kept in a closed dark glass bottle and stored at 4 C until utilization.

2-2-2Extraction of oil:

292.0g of mango seed kernel were extracted with n-hexane using soxhlet extractor apparatus for about eight hours. extract was then filtered through filter paper and the solvent was evaporated under reduced pressure using rotary evaporator apparatus.oil obtained was subjected to different physical and chemical tests.

2-3Physical tests of mango oil:

2-3-1Determination of density:

The density of oil sample was determined using pycnometer and sensitive balance.

The pycnometer was cleaned with ethanol and dried in an oven.

The dry pycnometer was filled with water and the water level was adjusted to proper point on pycnometer; the pycnometer was wiped dry with towel and weighed. After that the pycnometer was emptied and dried in an oven, then the dry pycnometer was filled with the oil sample in such a manner to prevent entrapment of air bubbles.

Carefully any oil that has come out of the capillary was wiped off, after that it was weighed. Both weights were carried out in the same temperature.

The method used for determination of density was adapted from

A.O.A.C.(2000), (method number 920-212).

2-3-2Determination of refractive index

The refractive index of oil sample was determined using refractometer.

The sample chamber containing the lens was opened and cleaned with acetone then plugged to source of light.

The equipment was calibrate with a drop of water, after which a drop of the oil sample was added into the sample chamber and closed the adjustment knob was turned crossed the cross bar then reading were taken. The reading was taken at 40C.

The method used for determination of refractive index was adapted from

A.O.A.C(2000), (method number 921-08).

2-3-3Determination of viscosity

Capillary tube viscometer test method

The method of determining viscosity (kinematic viscosity) utilized the capillary tube viscometer.

The oil sample was placed into a glass capillary U-tube and the sample was drawn through the tube using suction until it reached the start position indicated on the tube under gravity.

The narrow capillary section of the tube controls the oils flow rate.

Once the oil meniscus touched the lower line.

The method used for determination of viscosity was adapted from ASTMD445-06.

2-4 Chemical tests of mango oil:

2-4-1Determination of Acid value A_v:

Dissolve 1g of the substance to be examined, in 5ml of a mixture of equal volumes of ethanol(96%)and light petroleum ether previously neutralized with 0.1M potassium hydroxide, unless otherwise specified, using 0.1ml of phenolphthalein solution as indicator. Titrate with 0.1M potassium hydroxide until the pink color persists for at least 15s(n ml of titrate).
Calculate the acid value as followed:
Acid value =5.61*N/M
N:volume of alkaline
M:weight of sample

2-4-2Determination of Saponification value S_v:

Dissolve 4g potassium hydroxide in20ml of water and add sufficient ethanol(96%)to produce 1000ml. allow to stand overnight and pour off the clear liquid.
Weight 1g of the substance into a200-ml flask, add 25.0ml of the ethanolic solution of potassium hydroxide and boil under a

reflux condenser for 1 hour, rotating the content frequently . while the solution is still hot, titrate the excess of alkali with 0.5M hydrochloric acid using 1ml of phenolphthalein solution as indicator. Repeat the operation without the substance being examined.

Calculate the saponification value from the expression 28.05v/w where v is the difference, in ml, between the titrations and w is the weight, in g,of substance taken.

2-4-3Determination of peroxide value P_v:

Place 5g of the substance to be examined in a 250ml conical flask fitted with aground-glass stopper. Add 30ml of a mixture of 2volumes of chloroform and 3volumes of glacial acetic acid. Shake to dissolve the substance and add 0.5ml of saturated potassium iodide solution. Shake for exactly 1min then add 30mlof water titrate with 0.01M sodium thiosulphate , adding the titrant slowly with continuous vigorous shaking, until the yell0w color is almost discharged add5ml of starch solution and continue the titration ,shaking vigorously, until the color is discharged(A1ml of 0.01M sodium thio sulphate).carry out a blank test under the same conditions(A2ml of 0.01M sodiumthiosulphate). The volume of 0.01Msodium thio sulphate used in the blank titration must not exceed 0.1ml.calculate the peroxide value from the expression:

Peroxide value =10(A1-A2)/M

2-4-4Determination of Iodine value**:**

0.25g of oil was dissolved in 10ml of dichloromethane in a dry iodine flask, add 20ml of iodine mono chloride solution, insert the stopper, and allow standing in the dark at 15 to 25 for 30 minutes. Place 15 ml of dilute potassium iodide solution, carefully remove the stopper, rinse the stopper and the sides of the flask with 100ml of water, shake and titrate with 0.1M sodium

thiosulphate, using starch ,added towards the end of the titration, as indicator. At the same time carry out the operation in exactly the same manner, but without the substance being examined.

Calculate the iodine value from the expression 1.269v/w where v is the difference, in ml , between the titrations and w is the weight, in g ,of the substance taken.

2-4-5 Determination of ester value(E_v):

It is calculated from the saponification value S_v and the acid value A_v .

2-5 Test microorganisms:

2-5-1 bacterial microorganisms:

Bacillus subtilis	NCTC 8236 (gram + ve bacteria)
Staphylococcus aureus	ATCC 25923 (Gram+ve bacteria)
Klebsiella pneumonia	ATCC 53657(Gram – bacteria)
Pseudomonas aeruginosa	ATCC 27853
(Gram –ve bacteria)	
Escherichia coli	ATCC 25922(Gram –ve bacteria)

National collection of type culture (NCTC), Colindale, England.

American type culture collection (ATCC) Rockville, Maryland, USA.

2-5-2Fungal microorganism:

Candida albicans ATCC7596

2-5-2-1Methods:

2-5-2-2 Preparation of the test organisms:

2-5-2-3 Preparation of bacterial suspensions:

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours. The bacteria growth was harvested and washed off with 100ml sterile normal saline, to produce a suspension containing about 10^8-10^9 C.F.U/ml. the suspension was stored in the refrigerator at 4°C till used.

The aevarage number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique. Serial dilutions of the stock suspention were made in sterile normal saline solution and 0.02ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37°C FOR 24 hours. After incubation , the number of developed colonies in each drop was counted. The average number of colonies per drop(0.02ml)was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental condition were maintained constant so that suspensions with very close viable counts would be obtained.

2-5-2-4 Preparation of fungal suspension:

The fungal cultures were maintained on sabouraud dextrose agar, incubated at 25° C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspension in 100ml of sterile normal saline , and the suspension were stored in the refrigerator until used.

2-5-2-5 In vitro testing of extracts for antimicrobial activity:-

2-5-2-6 Testing for antibacterial activity:

The cup-plate agar diffusion method, was adopted with some minor modifications to assess the antibacterial activity of the prepared extracts.

One ml of the standardized bacterial stock suspension 10^8-10^9 C.F.U/ml were thoroughly mixed with 100ml of molten sterile nutrient agar which was maintained at 45°C. 20ml aliquots of the inoculated nutrient agar were distributed into sterile petri-dishes.

The agars was left to set and in each of these plates 4 cups(10mm in diameter)were cut using a sterile cork borer(no.4)and agar discs were removed.

Alternate cups were filled with 0.1ml sample of each of the oils dilutions in methanol using automatic microliter pipette, and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 18 hours.

Two replicates were carried out for each extract against each of the test organisms. After incubation the diameter of the resultant growth inhibition zones were measured, averaged and the mean values were tabulated.

2-5-2-7 Testing for antifungal activity:

The same method as for bacteria was adopted. Instead of nutrient agar , sabouraud dextrose agar was used. The inoculated medium was incubated at 25°C for two days for the candid albicans.

2-6 Determination of fatty acid composition by GC-MS:

2-6-1 Preparation of methyl esters of the fatty acid:

2ml of the mango seeds oil was taken and transfer to glass test tube , then 7ml of methanolic sodium hydroxide and 7ml of methanolic sulfuric acid was added, after that gentle shaking was made for three minutes then the test tube was placed in test tube rack for overnight, after that a suitable volume from super saturated sodium chloride was added and a suitable volume from normal hexane was added then the mixture was well shaken.

After three minutes two layer separated, the upper layer is the organic layer which contain the fatty acid and the lower layer is the aqueous layer.

From the upper organic layer 5µL WAS TAKEN and dilute it with 5ml diethyl ether then 1g sodium sulphate as drying agent was added. After that the sample was taken to GC-MS.

Methanolic sodium hydroxide prepared by dissolve 2g sodium hydroxide in 100ml methanol. Methanolic sulfuric acid prepared by taken 1ml from concentrated sulfuric acid in 99ml methanol.

2-6-2 **GC-MS analysis :**

GC-MS was carried out on sample of oil used GC-MS QP 2010Ultra instrument, operating with following parameter.

COLumn:DB-1(0.25mm* 0.25µM) length 30m, carrier gas: helium, column temp:60.0C̊, injection temp: 280.00C, injection mode: split, pressure: 93.1k pa, total flow: 50.0ml/min, column flow:1.50ml/min, linear velocity: 44.7cm/sec, purge flow:3.0ml/min, split ratio:-1.0, temperature program:(rate: 10.0,temperature:300,hold time: 0.00).

2-6-3 FTIR analysis:

Sample preparation for FTIR analysis:

One drop of mango seed oil was placed between two plates of sodium chloride (salt). The plates were transparent to the infrared light and did not introduce any lines onto the spectra. The drop formed a thin film between the plates, then the plates were taken to FTIR instrument.

CHAPTER THREE

RESULTS

3-resultS

3-1 Oil content:

Table (3.1) Yield percentage of extract of mango oil

Weight of sample	Weight of oil	Yield%
292 g	23.393 g	12.48

3-2 Physical properaties of mango oil

Table(3.2):

Density	0.8958
Viscosity	14.186 mpa.s
Refractive index	1.467
Melting point	35C
PH	5.6

3-3 chemical proparaties of mango oil:

Table(3.3)

Test	Value
Acid value	2.805
Saponification value	190
Peroxide value	5
Iodine value	49.173
Ester value	187.195

3-4 GC-MS analysis of fixed oil:

Table(3.4): ()retention time(R_t) and %area of the compounds identified in the fixed oil of mango seed oil by GC-MS.

Peak	R.T	Area	Area%	name
1	3.123	195128	1.21	Oxime, amethoxy-phenyl
2	4.029	1274380	7.92	Glycerin
3	4.766	45599	0.28	1-hexanol,2-ethyl
4	11.372	464091	2.88	Butylated hydroxyltoluene
5	15.828	1074898	6.68	Hexadecanoic acid
6	17.483	459054	2.85	9,12-octadecandienoicacid(z,z)
7	17.528	4944959	30.72	9,octadecanoic acid(z)
8	17.743	6216627	38.62	Methyl stearate
9	19.502	337107	2.09	Methyl18-methylnonadecanoate
10	20.413	142832	0.89	Phenol,2,2′-methylenebis[6-(1,1-dimethylethyl)-4-methyl
11	22.545	944277	5.87	9,19-cyclolanost-24-en-3-ol
		16098952	100.00	

3-5 Antimicrobial activity of mango oil:

Table(3.5)

Plant name	Antimicrobial activity				
Mango tree	Antibacterial activity				Antifungal activity
Extracts	Ec	Ps	Sa	BS	Ca
Mango oil	0	0	0	0	0

100mg/ml					

Es: Escherichiacoli

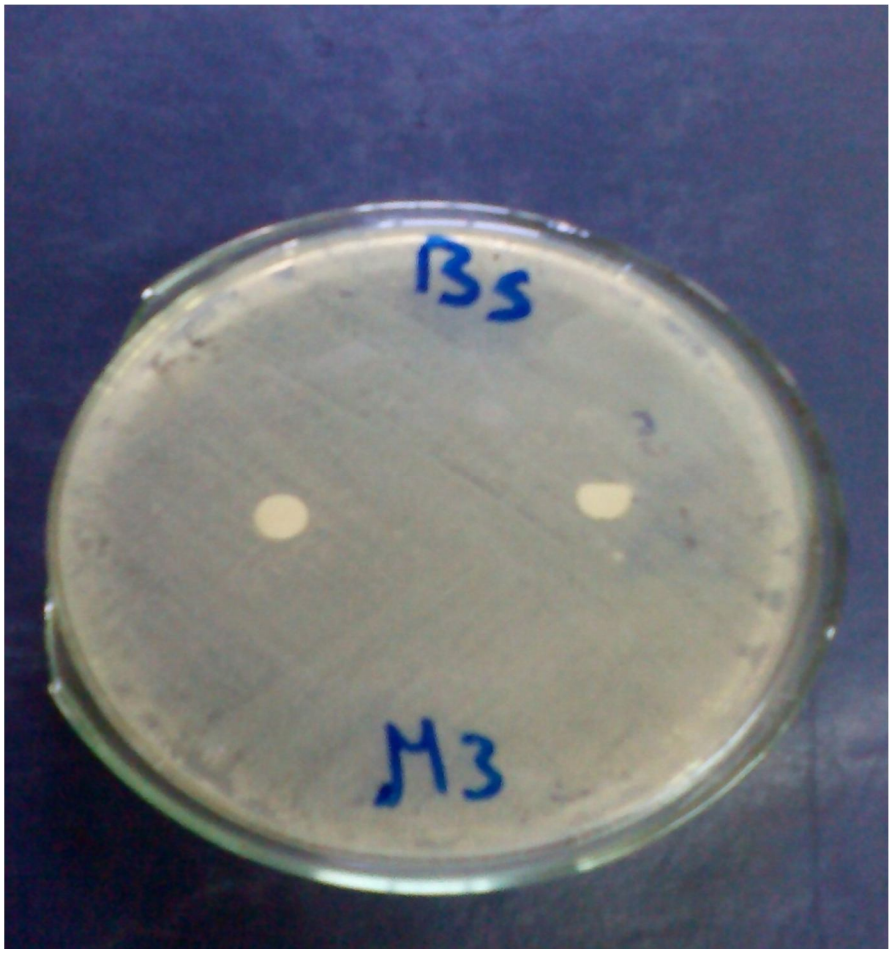
Ps: pseeudomonas aeruginosa

Sa: staphylococcus aureus

Bs: bacillus subtilis

Ca:candida

Figures:-



Fig(3-1) Antibacterial activity of mango oil-Bacillus subtilis

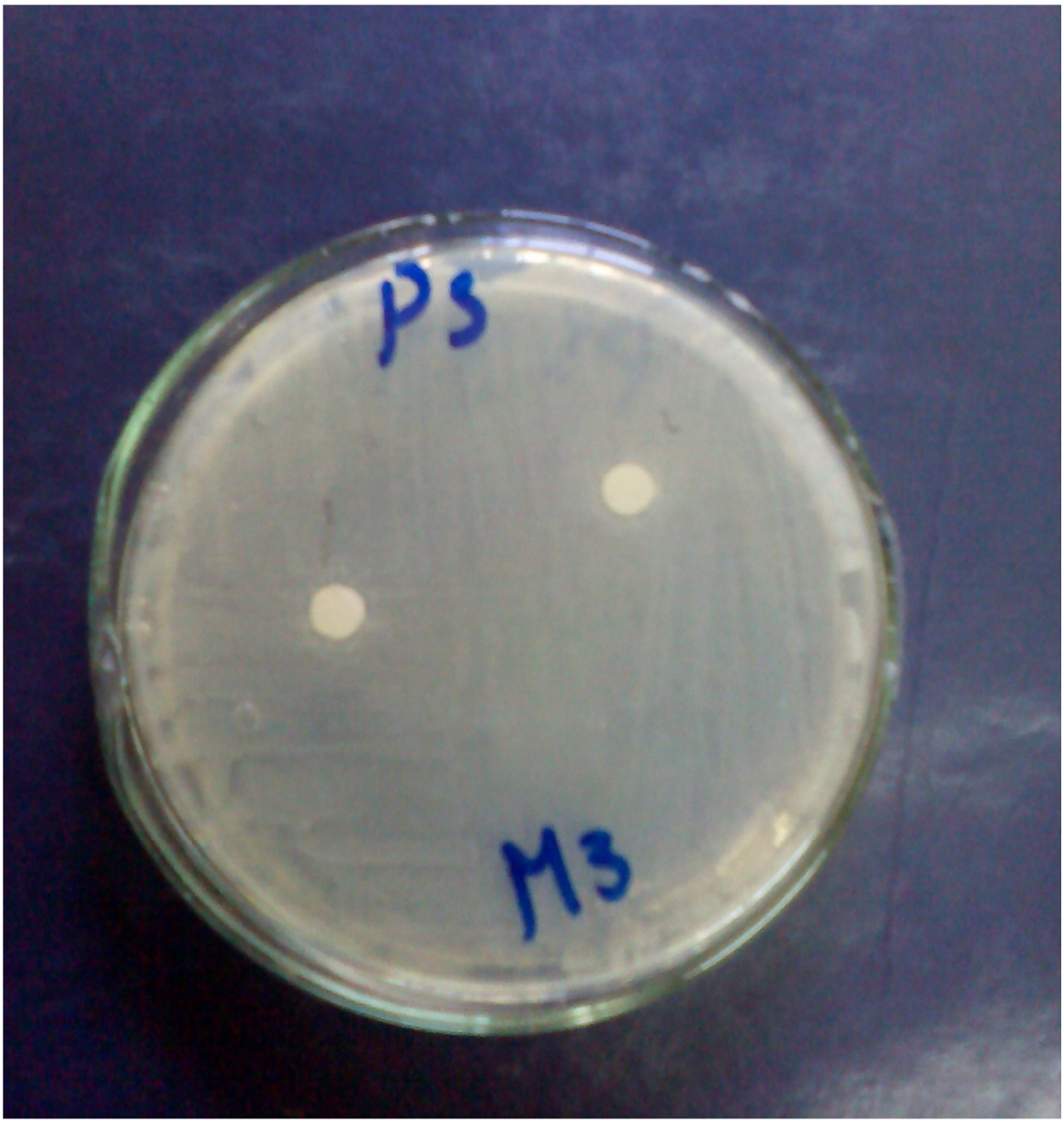
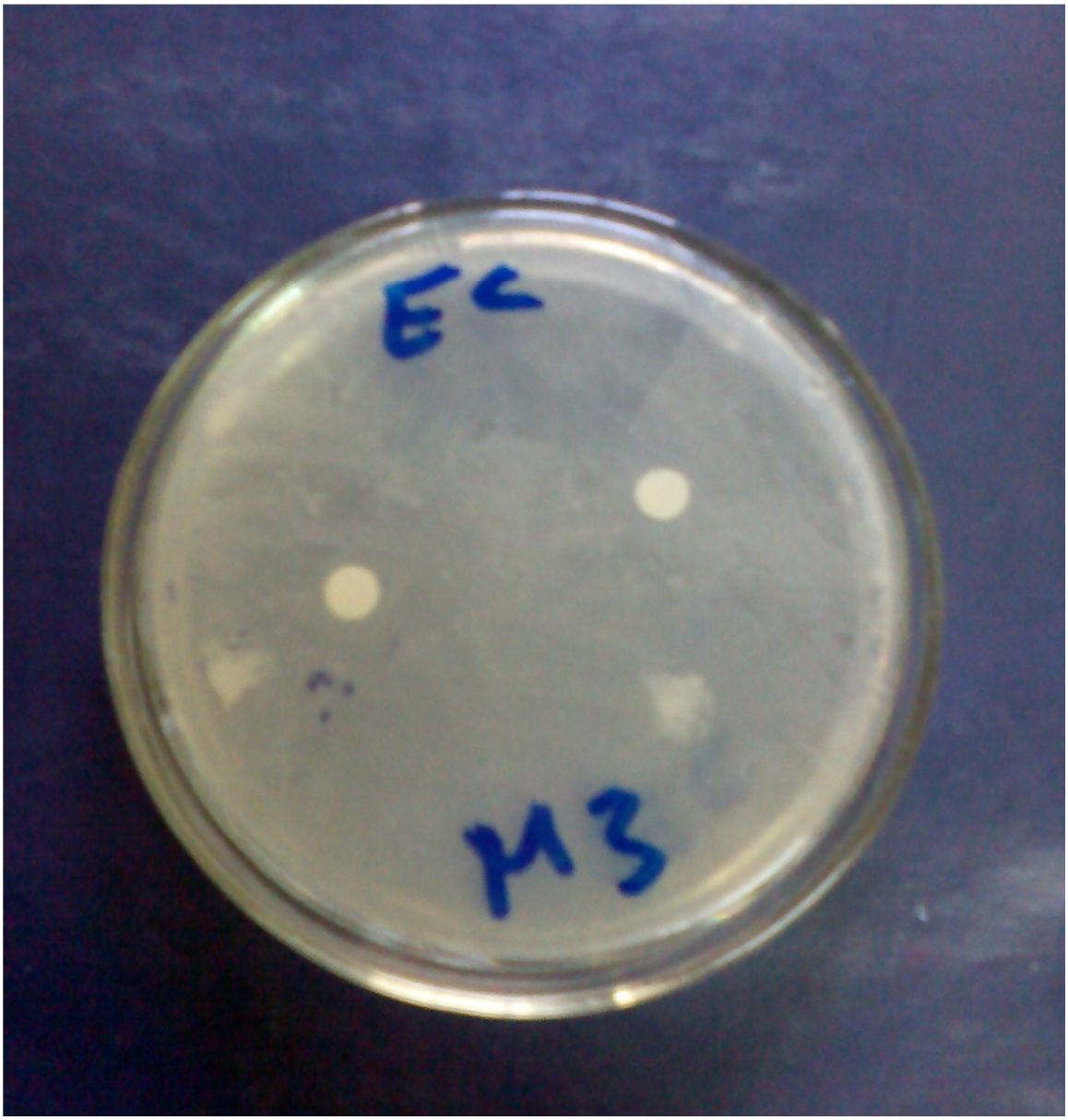
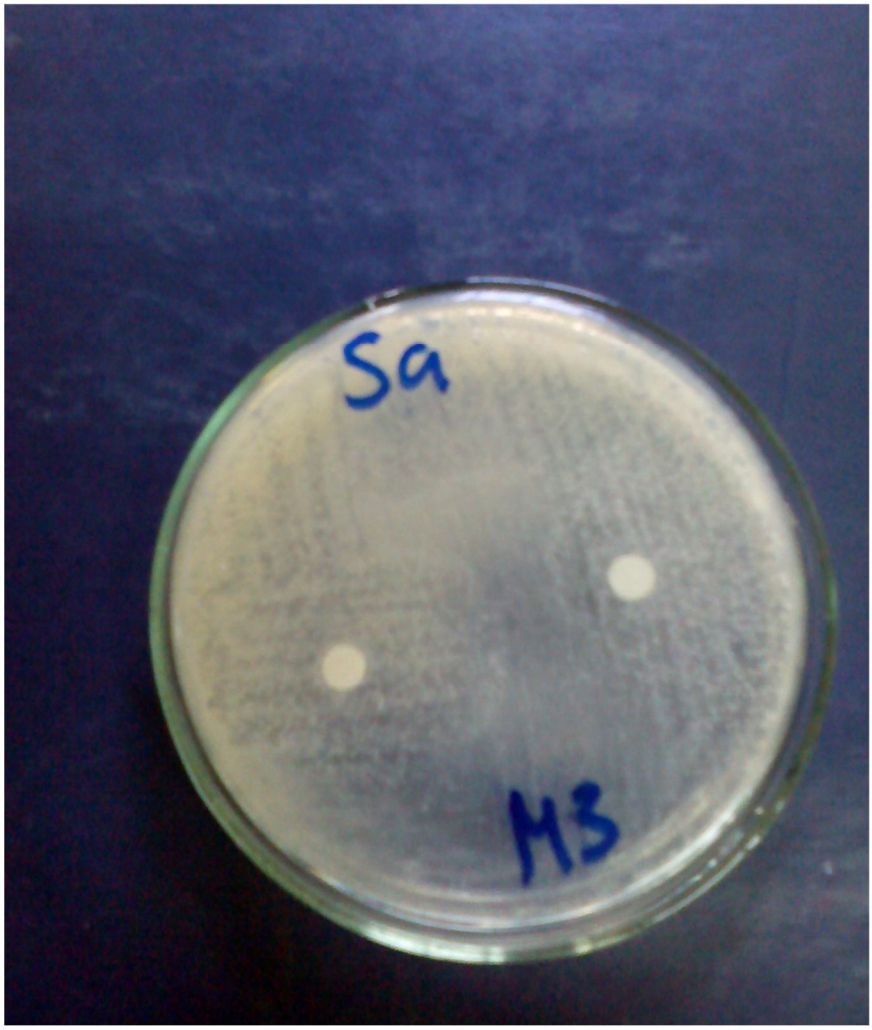


Fig (3-2)antibacterial activity of mango oil- pseudomonas aeruginosa



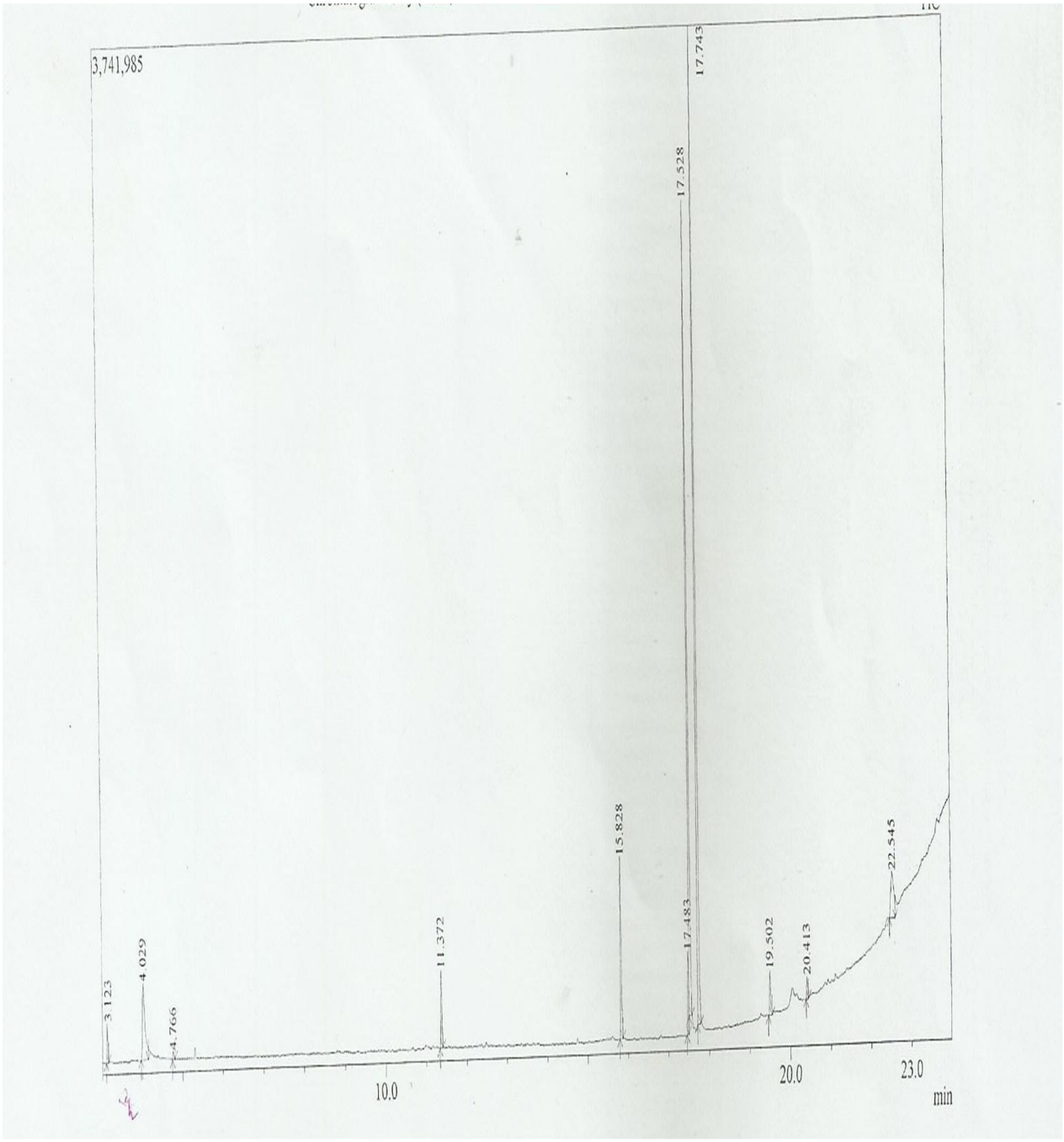
Fig(3-3)Antibacterial activity of mango oil-Escherichiacoli



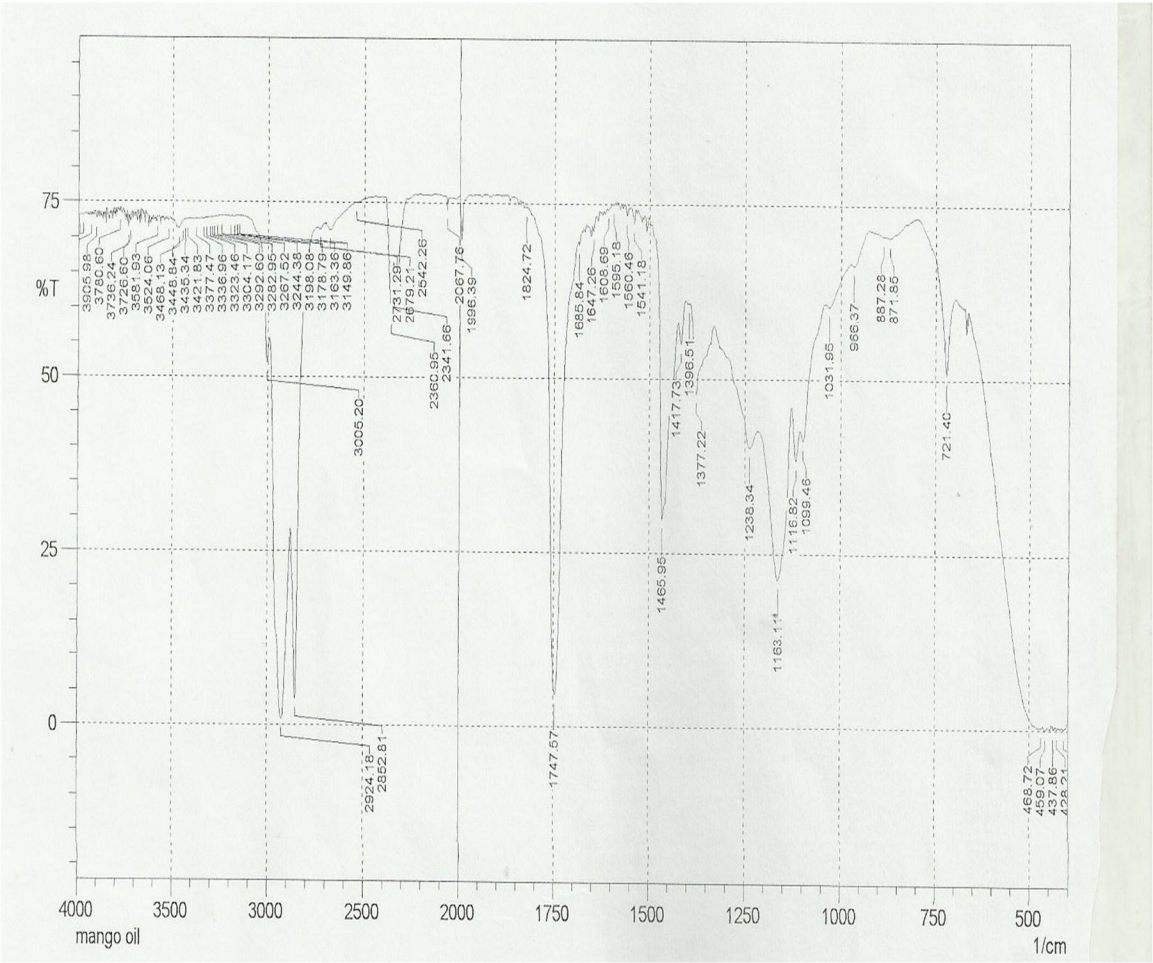
Fig(3-4)antibacterial activity of mango oil staphylococcus aureus

Fig(3-5)antifungal activity of mango oil-candida

intensity

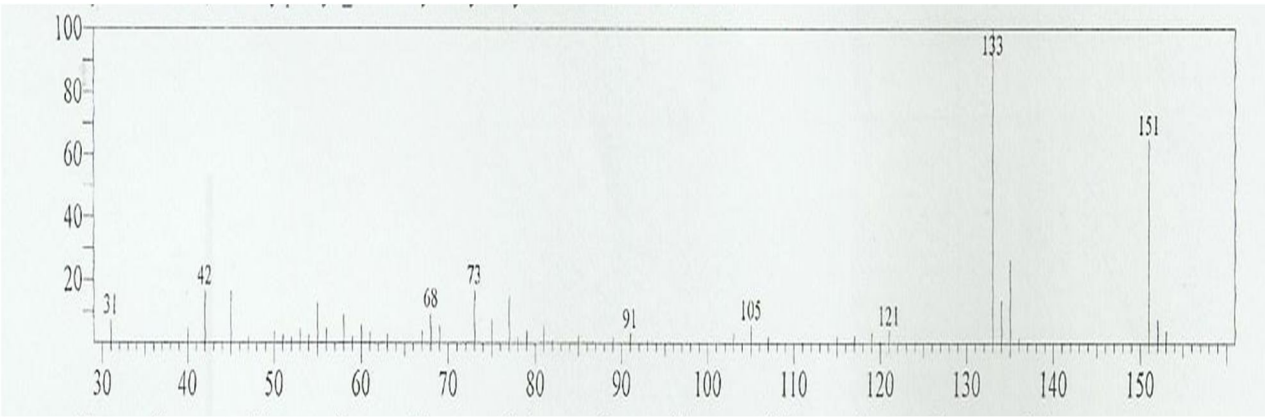


Fig(3-6): GC-MS total ion chromatogram of the fixed oil of mango .



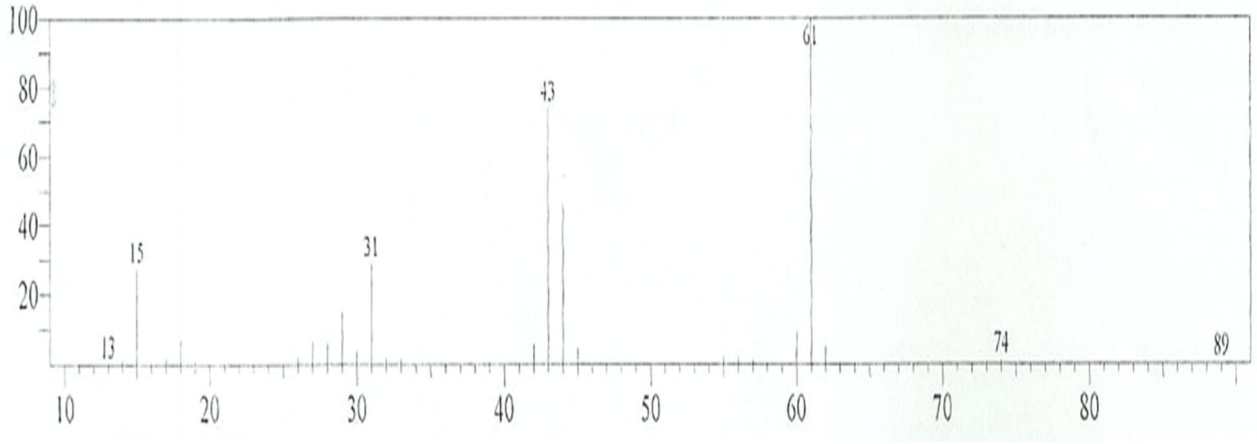
Fig(3-7): FTIR of mango seed oil.

Peak at R.time: 3.123



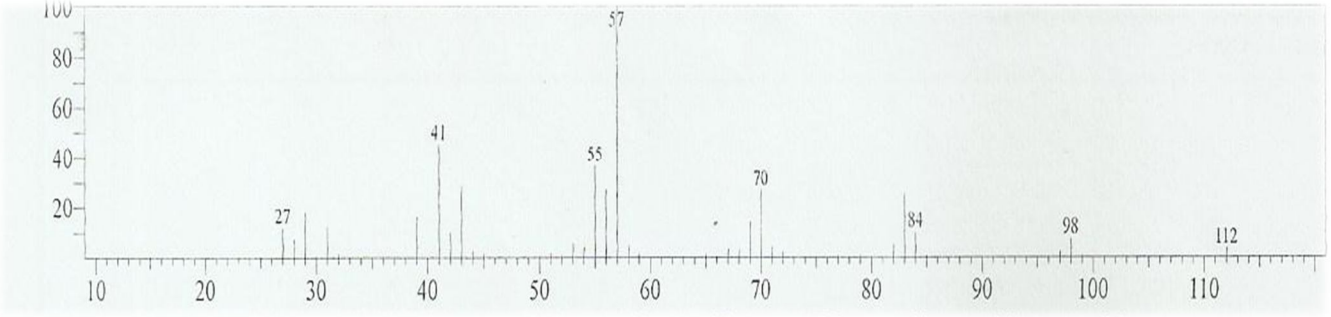
Figure(3-8) : mass spectrum of fragment, oxime, methoxy-phenyl-

Peak at R. Time: 4.29



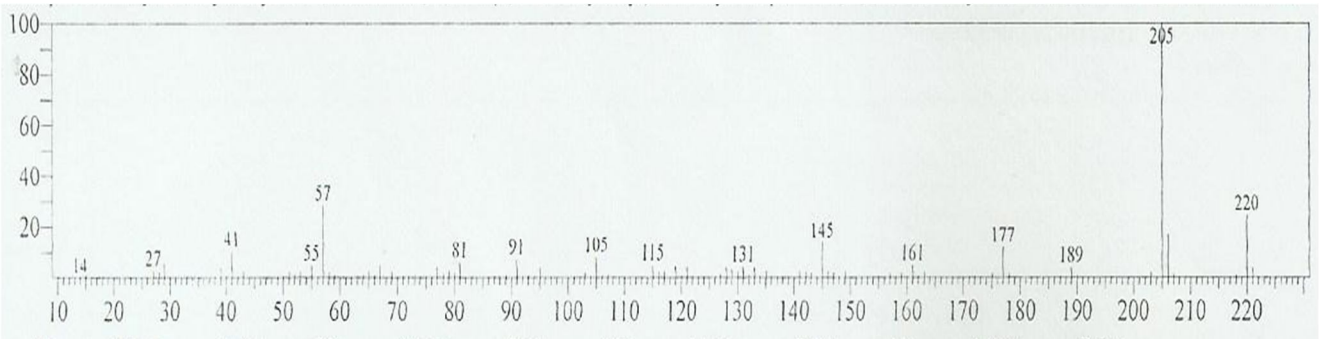
Figure(3-9): mass of fragment, glycerin.spectrum

Peak at R.Time:4.766



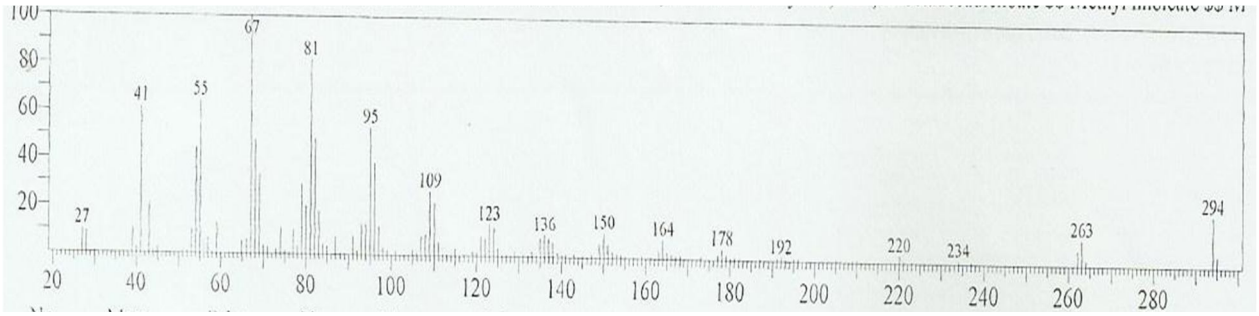
Figure(3-10): mass spectrum of fragment, 1-hexanol,2-ethyl

Peak at R.Time: 11.372



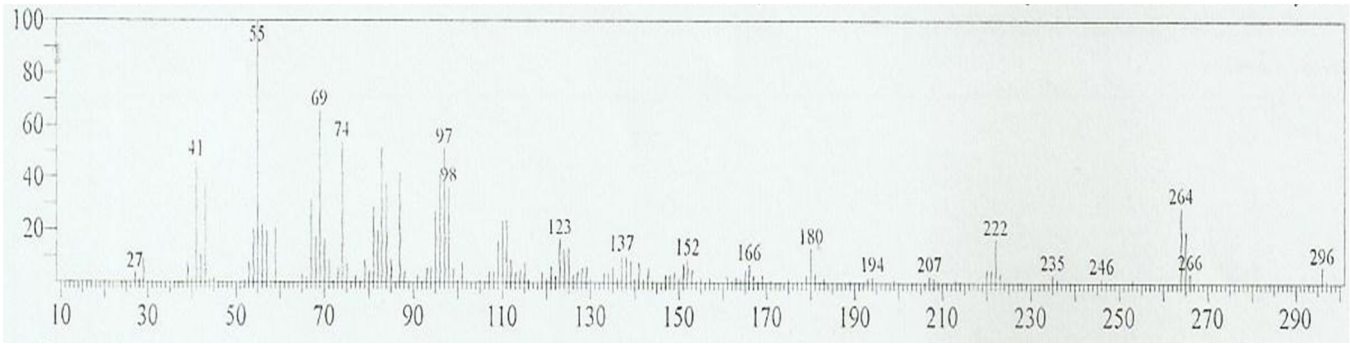
Figure(3-11) :mass spectrum of fragment butylated hydroxytoluene.

Peak at R.Time: 17.483



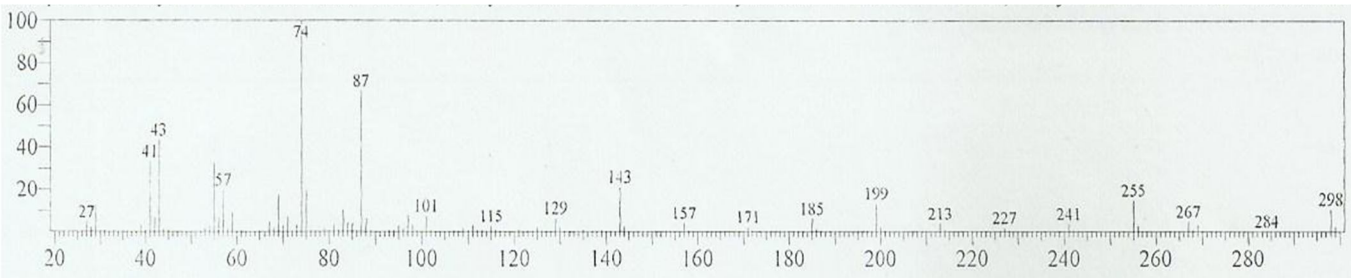
Figure(3-12) : mass spectrum of fragment, 9,12-octadecadienoic acid(z,z), lenoleic acid.

Peak at R.Time: 17.528



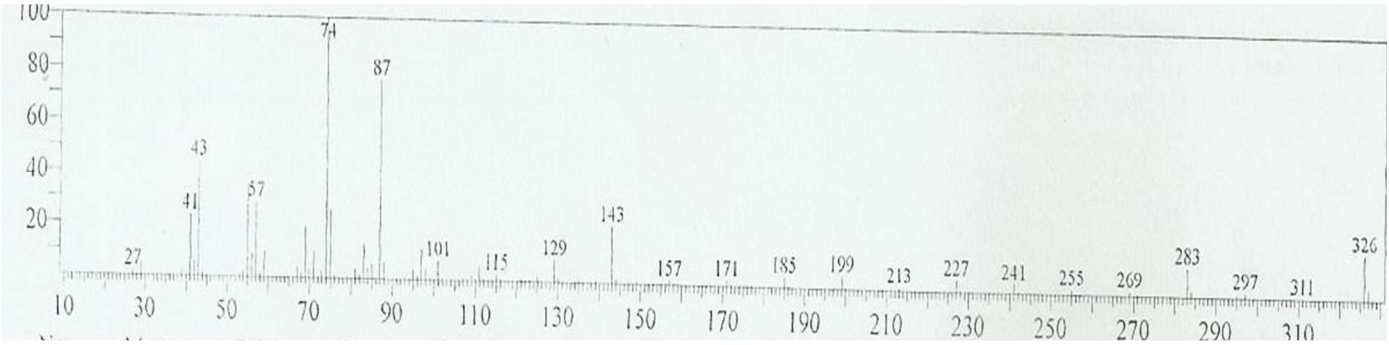
Figure(3-13): Mass spectrum of fragment, 9-octadecenoic acid(z),oleic acid.

Peak at R.Time:17.743



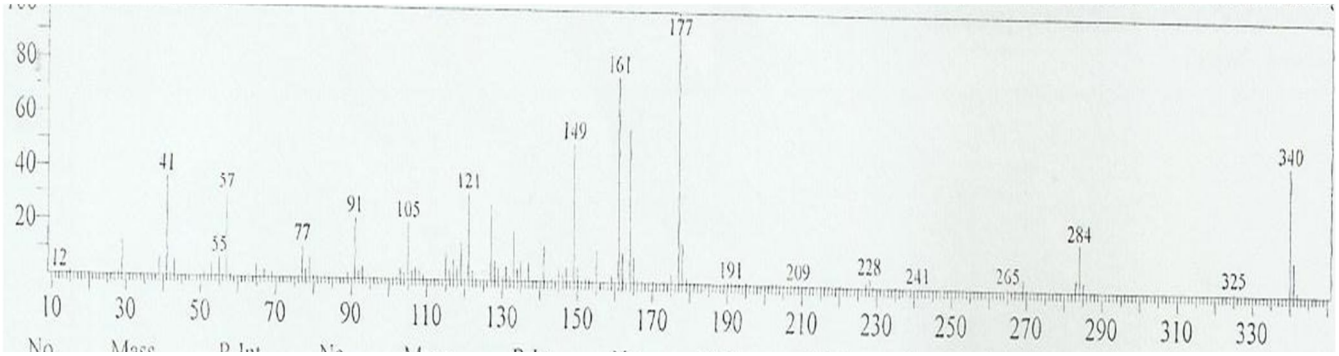
Figure(3-14): Mass spectrum of fragment, methyl stearate, stearic acid.

Peak at R.Time: 19.502



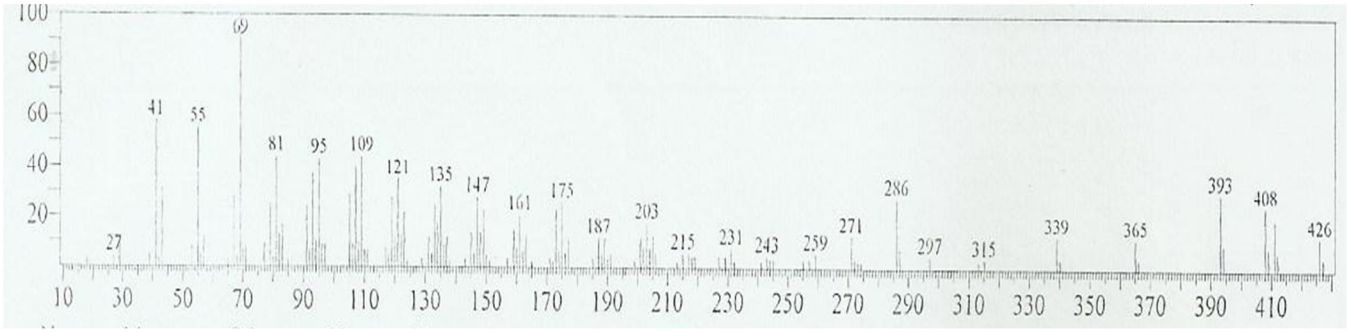
Figure(3-15): Mass spectrum of fragment, methyl 18-methylnonadecanoate.

Peak at R.Time: 20.413



Figure(3-16): Mass spectrum of fragment,phenol,2,2`-methylenebis[6-(1,1-dimethylethyl)-4-methyl-

Peak at R.Time: 22.545



Figure(3-17): Mass spectrum of fragment, 9,19-cyclolanost-24-en-3-ol.

CHAPTER FOUR

DISCUSSION, and suggestion for further work

Discussion

The yield percentage of oil extracted from the mango kernel seed is 12.482 %.the oil yield levels reported in this work for mango seed may be

useful in food industries but not be considered economical as oil seed when compared to other vegetable oil.

-Oil density equal to 0.8958, the lowest compared to the density of water.

-Refractive index equal to 1.467.

-Viscosity equal to 14.186

-Acid value equal 2.805, the low acidity of mango kernel oil indicated that the mango seed was almost free from hydrolytic rancidity brought almost by lipases and enables the direct use of such an oil in industries with out further neutralization.

-Saponification value equal to 190 this indicates that the oil contains a high amount of triglycerides and thus his ability to form soap.

-Iodine value equal to 49.173.

-Peroxide value equal to 0.960 this indicated that mango kernel oil had ahigh quality due to the low level of peroxide value.

-gas chromatography mass spectroscopy(GC-MS)analysis reveals that the main fatty acid compositions in mango seeds oil from sudan, which are characterized in table(3-4).

Mango seed oil is characterized by a high content of stearic acid and oleic acid as a principal fatty acid,There are different amount of minor fatty acid, palmitic, lenoleic acid. the minor

component of anti oxidant (butylated hydroxyl toluene), oxime, methyl 18-methylnonadecanoate,triterpenoid(9,19-cyclolanost-24-en-3-ol).

Fourier transform infrared(FTIR) spectra analysis of mango seed oil indicates that:

The carbon –hydrogen single bond(-c-H) stretching vibration absorption observed at 3005cm^{-1}.

A range of 2852-2924cm^{-1} absorption recorded is characteristic of oxygen hydrogen single bond (-o-H) stretching vibration.

The carbon –oxygen double bond (-c=o) stretching vibration absorption observed at 1747cm^{-1}.

The carbon –carbon double bond(-c=c) stretching vibration absorption observed at 1647cm^{-1}.

A range of 1163-1238cm^{-1} absorption recorded is characteristic of carbon-oxygen single bond (-c-o) stretching vibration.

The carbon-carbon single bond(-c-c) stretching vibration absorption observed at 871cm^{-1}.

The 1465 cm^{-1} absorption is likely to be for CH_2bending(scissoring). And at 721cm^{-1} for CH_2 bending(rocking).

The 1417cm^{-1} absorption is likely to be for CH_3 out of plane bending, and 1377cm^{-1} for CH_3 in plane bending.

-One experiment was conducted to test the effectiveness of mango oil as an antimicrobial for certain types of bacteria and one types of fungi.

Were obtained as a result of negative and this means that the oil is un suitable for use for this type of bacteria due to the high resistance of this type of bacteria.

Suggestion for further work:

This study may open a venues for further application and even production of good hybrids.

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- Physicochemical properties were done according the british pharmacopoeia(2007):
- Oil extraction was carried out according to method descried by (harborne 1984):