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GC-MS analysis and antimicrobial activity of *Piper longum* Linn. fixed oil

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Abstract
The present study was carried out to investigate the chemical constituents of *Piper longum* Linn. seed oil and to assess its antimicrobial activity. 118 components were detected by GC-MS analysis. Major components are: cubedol (9.93%), 2,4-diphenyl-6-(2-hydroxy-3-tolyl)pyrimidine (8.42%), 2-[2,4-dimethoxybenzylidene]-2H-thiazole(3.36%), chloroacetic acid dodec-9-ynyl ester (3.12%), shyobunone (3.00%).
The antimicrobial activity of the oil was evaluated using the diffusion bioassay against: (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 significant activity against *Staphylococcus aureus* at 100 and 50mg/ml. It also revealed excellent activity against *Escherichia coli* at 100µg/ml. However, only a partial activity was shown against the yeast *Candida albicans* in the range: 100-25mg/ml.It seems that the oil is a lead for further optimization.

Keywords: *Piper longum*, fixed oil, GC-MS, antimicrobial activity

Introduction
Different medicinal plants have shown great potential as leads for drug development and drug discovery. In developing countries, conventional medicines, in most cases, are beyond affordability and consequently phytotherapy retained its popularity.
Piper longum L. is a perennial, slender, climbing plant in the family Piperaceae. The fruits are the most widely used part in ethno-medicine and they are used as remedies for a wide spectrum of pathological conditions [1]. Phytochemical screening of fruits revealed the presence of alkaloids, saponins, carbohydrates, but tannins were not detected [2]. Fruits also contain starch, protein and volatile oil. The alkaloids: piperine, piplatin, piperolactam A, piperolactam B and piperadione were reported from spikes of this species, while the alkaloids: piperine and piper longuminine were detected in roots [3].
Piper longum volatile oil contains caryophyllene as a major constituent beside long chain hydrocarbons, sesquiterpenes, piperine, piplartine, piper nonaline and other constituets [4, 5]. Piperine isolated from *Piper longum* exhibited a central stimulant effect in model animals [6]. In *in vivo* studies, *piper longum* extract restricted liver fibrosis but failed to protect against acute damage of cirrhotic changes [6].
Methyl piperine from *piper longum* significantly decreased total lipid serum cholersterol and hepatic cholesterol in hypercholesterolemic models. A decoction of fruits showed significant antifungal activity in some *in vivo* studies [7] while a decoction of immature fruits largely relieved symptons of chronic bronchitis [8].
Piper longum volatile oil showed antibacterial activity against a panel of human pathogens [9]. The antitubercular activity of *Piper longum* has been demonstrated [10]. The plant was also reported as a cardioprotective agent [11].
The safety profile of *Piper longum* has been investigated and a dose of 1g/Kg body weight demonstrated contraceptive properties without any toxic effects [12].

Materials and Methods
Plant Material
The fruits of *Piper longum* were purchased from the local market – Omdurman, Sudan. The plant was identified and authenticated by Institute of Aromatic and Medicinal Plants-Khartoum, Sudan.

Test Organisms
Piper longum oil was screened for antibacterial and antifungal activities using the standard microorganisms: *Bacillus subtilis* (Gram +ve), *Staphylococcus aureus* (Gram+ve), *Pseudomonas aeroginosa* (Gram –ve), *Escherichia coli* (Gram –ve) and the fungal species *Aspergillus niger* and *Candida albicans*.

Methods
Extraction of oil from *Piper longum* seeds
Dry-powdered fruits of *Piper longum* (200g) were macerated with n-hexane at room temperature for 48h. The solvent was removed under reduced pressure giving the oil. For GC-MS analysis, the oil was esterified.

GC-MS analysis
Piper longum fixed oil was analyzed by gas chromatography – mass spectrometry. A Shimadzo Ultra instrument; RTX-5MS column (30m, length; 0.25mm diameter; 0.25µm, thickness)

was used. Analytical grade helium was used as carrier gas. Chromatographic conditions are displayed below:

Table 1: Oven temperature program

Rate	Temperature(C)	Hold time (mim.$^{-1}$)
-	60.0	0.00
10.00	300.0	0.00

Table 2: Chromatographic conditions

Column oven temperature	1300.0 °C
Injection temperature	280.0 °C
Injection mode	Split
Flow control mode	Linear velocity
Pressure	93.1KPa
Total flow	50.0ml/ min
Column flow	1.50ml/sec
Linear velocity.	44.7cm/sec
Purge flow	3.0ml/min.
Spilt ratio	- 1.0

Measurement of some physical properties

Acid value measurement

The oil (5mg) was placed into a conical flask. (50 ml) of neutralized alcoholic solution was added to the oil. The mixture was heated for 10 minutes in a water bath. Then it was titrated, with shaking against a KOH solution to the end point of the indicator (5 drops of phenolphthalein indicator).
The acid value is calculated by the formula: 56.1 x N x V / M

Where:
V: is the volume of KOH solution used
N : normality of KOH
M: Mass (in g) of the sample
56.1: Equivalent weight of the KOH

Refractive index measurement

Small portion of the oil sample was placed into an automatic refract meter device (at 20°C and wavelength 584nm). The results were displayed on the screen.

Density Measurment

Density was determined by direct measurement of mass and volumen.

Antimicrobial Assay

The disc diffusion bioassay was used to screen the antimicrobial activity of the target oil. Discs of What man No.1 filter paper (6mm in diameter) were used. Mueller Hinton and Sabouraud dextrose agars were the media used for the bacterial and fungal growth respectively. The media was prepared according to the manufacturer instructions. (150µl) suspension of individual test microorganism was spread homogenously on each plate of Mueller Hinton and Sabouraud dextrose agars. Each disk was soaked in a test solution and then placed on the microbial lawns. Four discs were assigned for each plate. The plates were incubated at 37ºC for 24hrs. After incubation, the diameters of the resultant growth inhibition zones were measured in two replicates and averaged.

Results and Discussion

Physical Properties

Some physical properties of the oil are displayed in Table (3).

Table 3: Physical properties of the oil

Physical Property	
Refractive index	1.511
Acid value	2.559
Density	0.98g/ml

GC-MS analysis of *Piper longum*fixed oil

GC-MS analysis of *Piper longum* 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.
The GC-MS spectrum of the studied oil revealed the presence of 118 components (Table 4). The typical total ion chromatograms (TIC) is depicted in Fig.1.

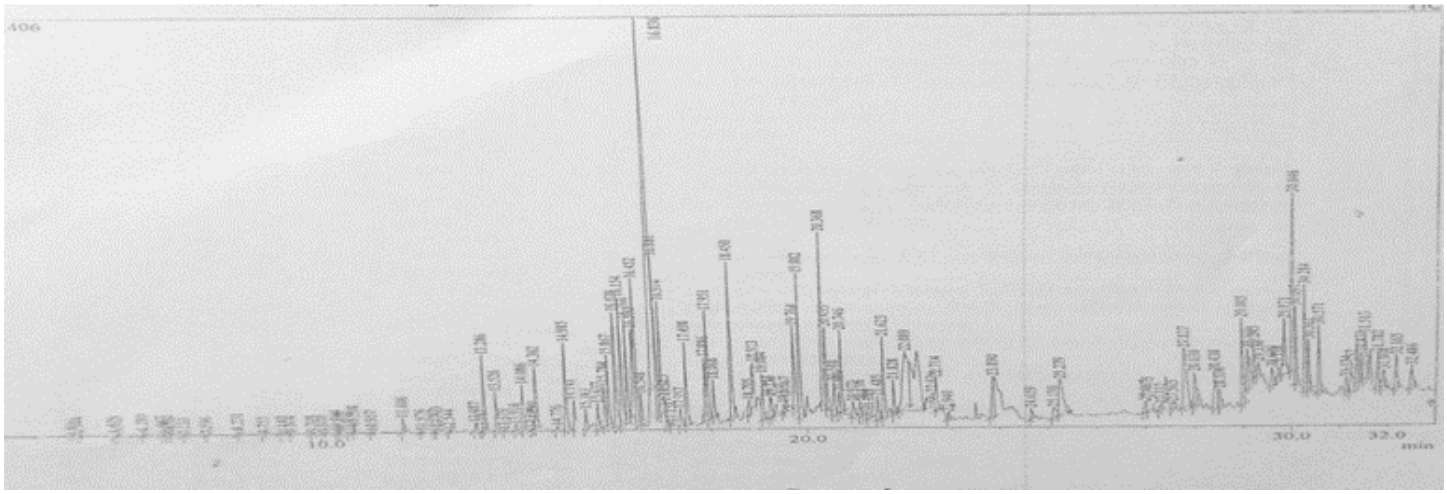

Fig 1: Total ions chromatogram

Table 4: Contituents of *Piper longum* oi

#	R.Time	Area	Area%	Name
1	4.806	15524	0.00	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-me
2	4.944	128492	0.03	.alpha.-Pinene
3	5.679	144587	0.03	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-m
4	5.763	186676	0.04	.beta.-Pinene
5	6.280	256018	0.05	.alpha.-Phellandrene
6	6.687	116448	0.02	o-Cymene
7	6.792	318838	0.06	.beta.-Phellandrene
8	6.856	42710	0.01	Eucalyptol
9	7.133	29660	0.01	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-
10	7.596	51044	0.01	Cyclohexanol, 1-methyl-4-(1-methylethen
11	8.231	275784	0.05	1,6-Octadien-3-ol, 3,7-dimethyl-
12	8.755	45807	0.01	2-Cyclohexen-1-ol, 1-methyl-4-(1-methyle
13	9.140	68618	0.01	5-Caranol, (1S,3R,5S,6R)-(-)-
14	9.304	43238	0.01	(+)-2-Bornanone
15	9.748	78014	0.02	endo-Borneol
16	9.970	93059	0.02	Terpinen-4-ol
17	10.244	155363	0.03	.alpha.-Terpineol
18	10.298	37747	0.01	Dodecane
19	10.498	70022	0.01	Bicyclo[3.1.0]hexan-3-ol, 4-methylene-1-(
20	10.598	425147	0.08	2-Cyclohexen-1-ol, 3-methyl-6-(1-methyle
21	10.957	94841	0.02	1,3-Cyclopentanedione, 2,4-dimethyl-
22	11.606	1049759	0.21	2-Cyclohexen-1-one, 3-methyl-6-(1-methy
23	11.976	82376	0.02	D-Carvone
24	12.237	45995	0.01	Bornyl acetate
25	12.303	433430	0.09	Safrole
26	12.544	101406	0.02	Thymol
27	13.087	634127	0.13	Cyclohexane, 1-ethenyl-1-methyl-2-(1-me
28	13.212	61744	0.01	Bicyclo[3.2.0]heptan-2-one, 6-hydroxy-5-
29	13.286	5938630	1.18	Cyclohexane, 1-ethenyl-1-methyl-2-(1-me
30	13.526	2942925	0.58	.alpha.-Cubebene
31	13.679	48449	0.01	Eugenol
32	13.916	438746	0.09	1,2,4-Metheno-1H-indene, octahydro-1,7
33	14.086	3422945	0.68	Copaene
34	14.240	183284	0.04	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-m
35	14.290	392082	0.08	Furazan-3-amine, 4-(3,4-methylenedioxy

Table 4: Continued

36	14.362	6828046	1.35	.gamma.-Muurolene
37	14.776	271070	0.05	1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7
38	14.985	7925296	1.57	Caryophyllene
39	15.151	2501347	0.50	1H-Cyclopenta[1,3]cyclopropa[1,2]benzen
40	15.387	1740948	0.34	(-)-Aristolene
41	15.637	2156779	0.43	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramet
42	15.784	3413126	0.68	Alloaromadendrene
43	15.867	5433647	1.08	.alpha.-Guaiene
44	16.028	11464549	2.27	.gamma.-Muurolene
45	16.154	12172237	2.41	.beta.-copaene
46	16.266	7625321	1.51	1H-Cycloprop[e]azulene, decahydro-1,1,7-
47	16.393	9600692	1.90	Gisenol
48	16.452	13671984	2.71	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-met
49	16.548	2374323	0.47	.beta.-Bisabolene
50	16.836	50177441	9.93	cubedol
51	16.881	10879903	2.15	6-epi-shyobunol
52	16.974	8927768	1.77	Ledol
53	17.025	255158	0.05	2,5-di-tert-butyl-1,4-benzoquinone

Table 4: Continued

ak#	R.Time	Area	Area%	Name
54	17.057	1530286	0.30	.alpha.-Muurolene
55	17.158	230712	0.05	(-)-Aristolene
56	17.357	1706403	0.34	Cyclohexanemethanol, 4-ethenyl-.alpha.,,a
57	17.498	6831585	1.35	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-,
58	17.886	4572578	0.90	1-Hydroxy-1,7-dimethyl-4-isopropyl-2,7-cy
59	17.951	9341771	1.85	1H-Cycloprop[e]azulen-7-ol, decahydro-1,
60	18.068	2850439	0.56	Caryophyllene oxide
61	18.450	15177964	3.00	Shyobunone
62	18.795	1378669	0.27	cis-Thujopsene
63	18.913	3157823	0.62	2-Hydroxy-4,5-methylenedioxypropiopher
64	19.084	3817514	0.76	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydr
65	19.235	1987274	0.39	Cedrol
66	19.276	1406921	0.28	Selina-6-en-4-ol
67	19.516	750645	0.15	(+)-3-Carene, 2-.alpha.-isopropenyl-
68	19.567	745075	0.15	IR.4S,7S,11R-2,2,4,8-Tetramethyltricyclo{
69	19.764	8311780	1.64	6.beta.Bicyclo[4.3.0]nonane, 5.beta.-iodom
70	19.882	14136700	2.80	Illudol
71	20.368	15762101	3.12	Chloroacetic acid, dodec-9-ynyl ester
72	20.435	6605702	1.31	2,2,6-Trimethyl-1-(2-methyl-cyclobut-2-en
73	20.588	3556691	0.70	2-Cyclohexen-1-one, 3-methyl-6-(1-methyl
74	20.680	1836415	0.36	Milbemycin b, 5-O-demethyl-28-deoxy-6,2
75	20.746	6265646	1.24	1-Isopropenyl-3-propenylcyclopentane
76	20.972	1406868	0.28	trans-p-Mentha-2,8-dienol
77	21.116	1078587	0.21	(-)-Spathulenol
78	21.204	660028	0.13	3-(1,5-Dimethyl-hex-4-enyl)-2,2-dimethyl-
79	21.289	916663	0.18	1a,2,5,5-Tetramethyl-cis-1a,4a,5,6,7,8-hex
80	21.485	1881620	0.37	Caparratriene

Table 4: Continued

81	21.623	7822891	1.55	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
82	21.828	2690148	0.53	Cycloheptane, 4-methylene-1-methyl-2-(2-
83	22.089	16958890	3.36	2-[2,4-Dimethoxybenzylidene]-2H-thiazol
84	22.089	42572856	8.42	2,4-Diphenyl-6-(2-hydroxy-3-tolyl)pyrimi
85	22.714	3190017	0.63	4-Hexen-1-ol, 6-(2,6,6-trimethyl-1-cyclohe
86	22.940	475211	0.09	Naphthalene, decahydro-2,2-dimethyl-
87	23.890	5422423	1.07	7H-Furo[3',2':4,5]furo[2,3-c]xanthen-7-o
88	24.659	962467	0.19	2-methyltetracosane
89	25.130	2116199	0.42	N-Methyl-N-veratryl-p-toluenesulfonami
90	25.279	7657986	1.52	Phenol, 4,4'-methylenebis[2-(1,1-dimethy
91	27.017	1529402	0.30	1,2,3,4,5,8-Hexahydroisoquinoline, 1-[3-h
92	27.075	2317825	0.46	1,3-Benzodioxole, 5,5'-(tetrahydro-3,4-di
93	27.332	895280	0.18	Butanoic acid, 2-methyl-, 2-methoxy-4-(2-
94	27.565	825662	0.16	Phenol, 2,6-dimethoxy-4-(2-propenyl)-
95	27.827	11441498	2.26	Furan,2,5-bis(3,4-dimethoxyphenyl)tetra
96	28.030	4171070	0.83	Isoquinoline, 1,2,3,4-tetrahydro-2-(1'-phe
97	28.438	4847852	0.96	6-Methoxy-3-methyl-2-benzofurancarbox
98	28.538	2043461	0.40	1,3-Benzodioxole, 5,5'-(tetrahydro-3,4-di
99	29.005	7710514	1.53	2-Oxo-2-phenylethyl 2,6-dimethoxybenzo
100	29.116	4808801	0.95	4,7-Dimethoxy-2-methylindan-1-one
101	29.245	1829469	0.36	Benzyloxy(butyl)dimethylsilane
102	29.336	1686855	0.33	4H-1-Benzopyran-4-one, 5-hydroxy-7-me
103	29.609	2149757	0.43	Phenol, 2,6-bis(1,1-dimethylethyl)-4-[(4-h
104	29.738	918631	0.18	4-tert-Butylcatechol, dimethyl ether
105	29.871	6967852	1.38	Phenol, 2,4,6-tris(1-methylethyl)-
106	30.046	17995839	3.56	2,4-Diphenyl-6-(2-hydroxy-3-tolyl)pyrimi
107	30.097	6389471	1.26	Scopoletin, O-pentafluoropropionyl-
108	30.284	9162933	1.81	1-Phosphacyclopent-2-ene, 1,2,3-tripheny
109	30.361	3960195	0.78	Phenol, 4-[2,3-dihydro-7-methoxy-3-meth
110	30.571	5681101	1.12	3,5-Bis(2,5-dimethylphenyl)-2,3-dihydro-

Table 4: Continued

ak#	R.Time	Area	Area%	Name
111	31.134	1266944	0.25	Phenol, 2-methoxy-4-(1-propenyl)-, acetat
112	31.232	3320434	0.66	Phenol, 2-(1,1-dimethyl-2-propenyl)-3,6-di
113	31.431	1943448	0.38	2-Hydroxy-4-isopropyl-7-methoxytropone
114	31.513	6551029	1.30	(+)-Longicamphenylone
115	31.782	3128619	0.62	1-(3-Methoxymethyl-2,5,6-trimethylpheny
116	31.908	2164732	0.43	Imidazo[1,5-b]isoquinolin-1(5H)-one, 2,3,1
117	32.165	3096771	0.61	7,9-Di-tertbutyl-1-oxaspiro[4,5]deca-6,9-di
118	32.486	2867759	0.57	7-Hydroxy-3-methoxy-2-p-methoxyphenyl
		505319922	100.00	

Some important constituents of the oil are discussed below:

Cubedol (9.93%)

The EI mass spectrum of cubedol is shown in Fig. 2.The peak at m/z 222, which appeared at R.T. 16.836 in total ion chromatogram, corresponds to $M^+[C_{15}H_{26}O]^+$. The peak at m/z207 corresponds to loss of a methyl function.

2, 4-Diphenyl-6- (2-hydroxy-3-tolyl) pyrimidine (8.42%)

The EI mass spectrum of 2, 4-Diphenyl-6- (2-hydroxy-3-tolyl) pyrimidine is shown in Fig. 3.The peak at m/z 338(R.T. 22.089 in total ion chromatogram) corresponds: $M^+[C_{23}H_{18}N_2O]^+$.

2-[2, 4-dimethoxybenzylidene]-2H-thiazole (3.36%)

The mass spectrum of 2-[2,4-dimethoxybenzylidene]-2H-thiazole is depicted in Fig. 4.The peak at m/z338 (R.T. 22.089 in total ion chromatogram) corresponds : $M^+[C_{18}H_{14}N_2O_3S]^+$. The peak at m/z323 is due to loss of a methyl function.

Chloroacetic acid, dodec-9-ynyl ester (3.12%)

The EI mass spectrum of 9- dodecynyl chloroacetate is displayed in Fig. 5.The peak at m/z
182, which appeared at R.T. 20.368 in total ion chromatogram, matches: $M^+[C_{14}H_{23}ClO_2]^+$.

Shyobunone (3.00%)

The EI mass spectrum of Shyobunone is shown in Fig. 6.The peak at m/z 220, which appeared at R.T. 18.450 in total ion chromatogram, corresponds $M^+[C_{15}H_{24}O]^+$. The peak at m/z205 corresponds to loss of a methyl function.

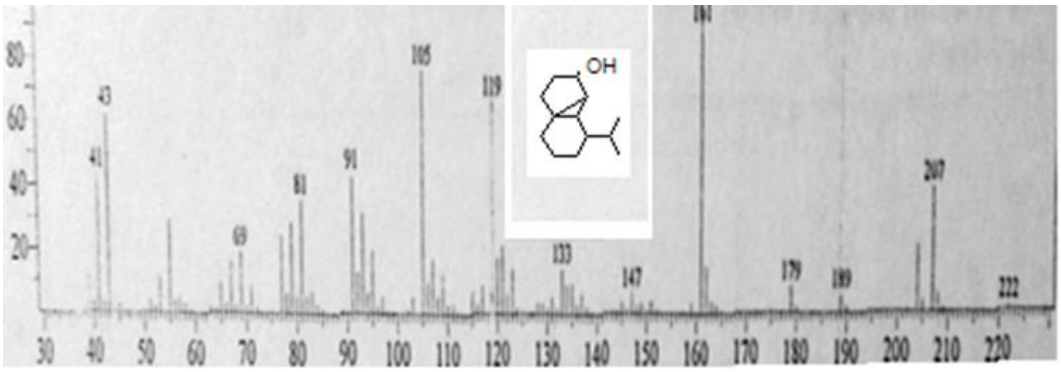

Fig 2: Mass spectrum of Cubedol

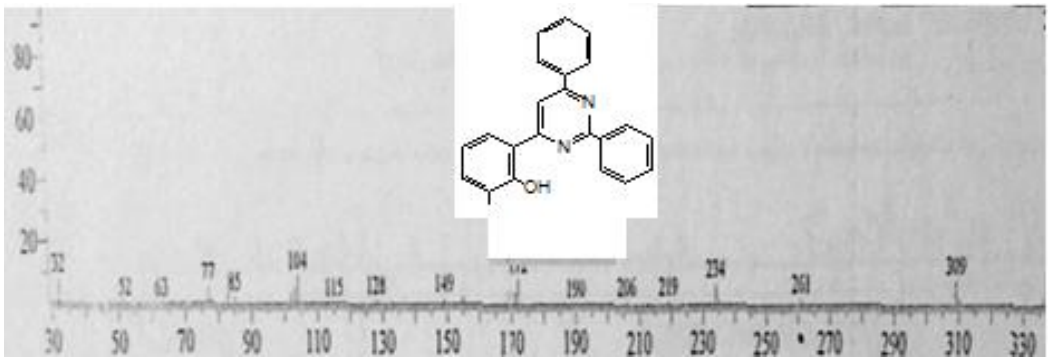

Fig 3: Mass spectrum of 2, 4-diphenyl-6- (2-hydroxy-3-tolyl) pyrimidine

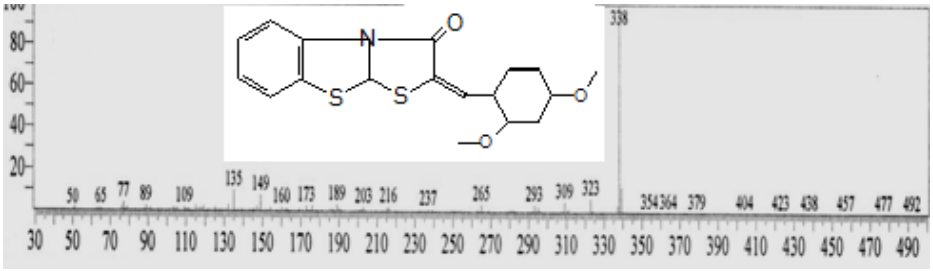

Fig 4: Mass spectrum of 2-[2,4-dimethoxybenzylidene]-2H-thiazole

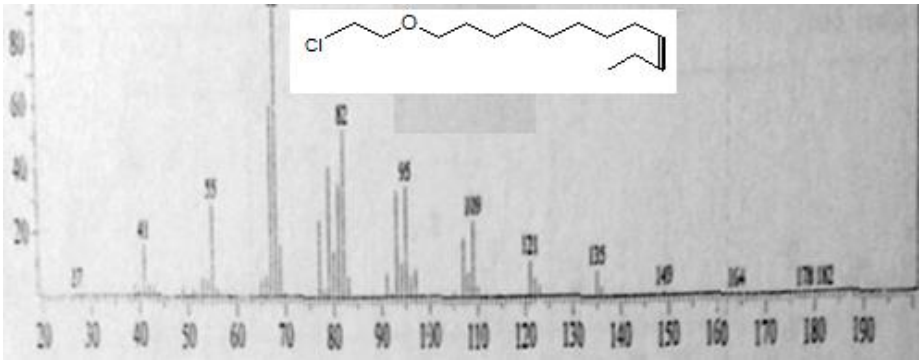

Fig 5: Mass spectrum of chloroacetic acid dodec-9-ynyl ester

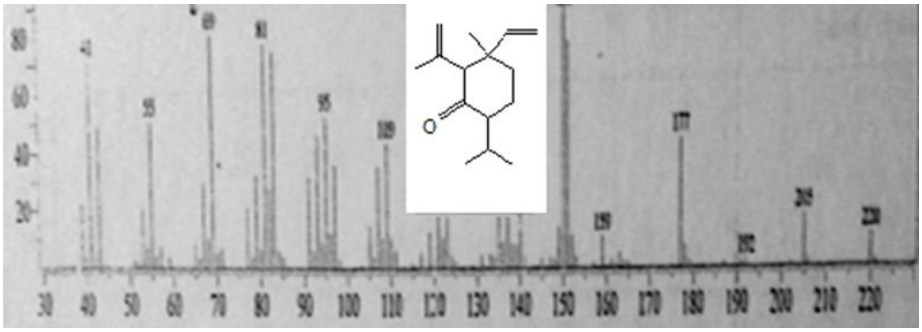

Fig 6: Mass spectrum of shyobunone

The oil was assessed for antimicrobial activity against six standard organisms. Diameters of the growth inhibition zones are displayed in Table 5. The results were interpreted according to the common terms: (<9mm: inative; 9-12mm: partially active; 13-18mm: active;>18mm: very active). Tables (6) and (7) show the antimicrobial activity of standard drugs.

Table 5: Antibacterial activity of *Piper longum* seed oil

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca	An
Piper longum oil	100	19	--	21	16	12	08
	50	16	--	18	13	10	08
	25	14	--	15	11	9	--
	12.5	14	--	12	9	--	--

Table 6: Antibacterial activity of standard chemotherapeutic agents

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

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 significant activity against *Staphylococcus aureus* at 100 and 50mg/ml. It also revealed excellent activity against *Escherichia coli* at 100µg/ml. However, only a partial activity was shown against the yeast *Candida albicans* in the range: 100-25mg/ml (Table 4).

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