4 - RESULTS AND DISCUTIONS

4-Results and discussions

4.1 - The identification and antioxidant activities of Eucalyptus essential oil:

4.1.1 - Chemical composition of the HD and SFE extracts

The overall yields of leaf essential oils of *E. camaldulensis var. brevirostris* obtained by HD and SFE extraction techniques and the extraction time are presented in table (2) and Fig (12). The time of SFE was optimised with respect to the extraction yield by performing extraction at different times. The optimum yield was obtained at 2 h and did not increase upon belonging extraction time.

Extraction method	Yield[a, b] (%)	Extraction time (h)
HD extraction	0.6 (± 0.01)	3
SFE extraction	1.1 (± 0.02)	2

Table (2): Yield of essential oil of leave and extraction time

a- The yield is expressed as the average of three extractions and represents the amount extracted in g / 100 g of fresh leaves.

b- Reported values are the mean ± SD (standard deviation) for three values.

In the above table, it is shown that SFE produces better yields and needs less extraction time than HD. According to GLC-MS analyses their chemical compositions differ qualitatively and quantitatively (see Fig.13 and Table 3). The various compounds which were separated by GLC-MS, were identified by comparison of their mass spectra with NIST library data and by comparison of

using the non polar column (4). The retention index, as defined by Kovats (77), is a measure of relative retention time which uses the normal alkanes (here C_9-C_{23}) as standard references, see fig (14). The Kovats index values were calculated by the following equation

$$I_a = 100N + 100n\left(\frac{t_{R_a} - t_{R_N}}{t_{R(N+n)} - t_{R_N}}\right)$$

t_{R_a} retention time of the component

t_{R_N} retention time of the n-alkanes that bracket the component,

N carbon number of the lower n-alkane

n difference in carbon number of the two n-alkanes that bracket the compound

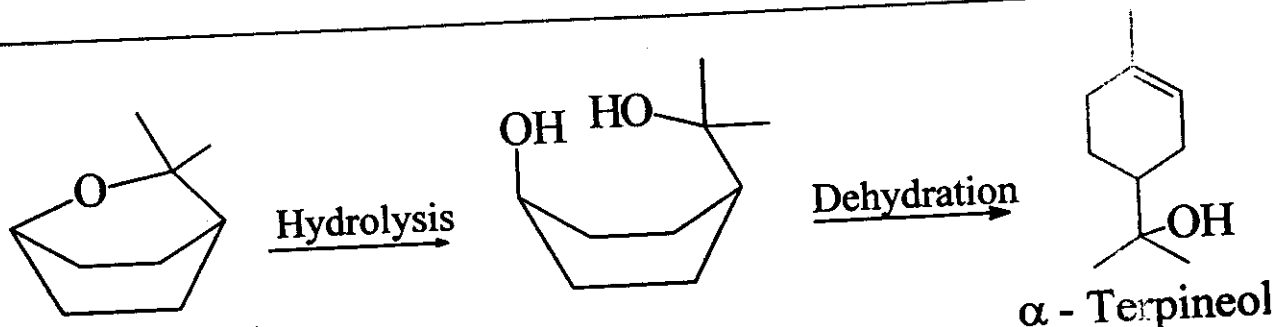
The SFE extract showed 81 GLC signals, the HD oil showed only 66 signals. Most of the compounds could be identified. Because pure standard substances of most of these compounds have not been available, the quantitative calculation was based upon the relative areas of the corresponding GLC signals. α-Pinene (peak no. 2) (4.28 % and 1.49 %), β-pinene (peak no. 5) (5.75 % and 1.41 %), p-cymene (peak no. 9) (24.01 % and 10.61 %), β-phellandrene (peak no. 11) (8.94 % and 4.09 %), pinocarveol (peak no. 25) (1.26 % and 1.24 %), terpinen-4-ol (peak no.32) (4.42 % and 2.92 %), cryptone (peak no. 34) (12.71 % and 9.82 %), cuminaldehyde (peak no. 45) (2.96 % and 2.32 %) and spathulenol (peak no. 78) (14.43 % and 13.14 %) were found to be the main components in HD and SFE oils The. first values correspond to the HD, and the second to SFE extracts. The differences in the concentrations of the individual compounds between HD and SFE extracts are obviously due to the fact that more of the less volatile compounds like sesquiterpenes and heavy oxygenated compounds are extracted by SFE. The present results are in accordance with the previous

findings which reported these components to be the major compounds in the volatile oils of *E. camaldulensisDehan* var. *camaldulensis, E. camaldulensis Dehan.* var *camaldulensis* (HN1) and *E. camaldulensis Dehan.* var *camaldulensis* (SW1) in Australia (89). Furthermore our results are in accordance with those of the same authors in respect to the 1,8-cineol content. The monoterpenoid ether 1,8-cineole is the major and most important volatile component in most of the *Eucalyptus* species, especially in *E. globulus* (79, 83 - 88). In both of the *E. camaldulensis* leaf oil extracts as well as in those extracted by Bolens (89), its concentration is less than 1%. In contrary to that Bignell et al.(30) reported 1,8-cineol (47 % - 66 %), α-pinene (14.89 % - 5.06 %) and aromadendrene (12 % - 1.15 %) to be the main compounds of three different specimen of the same spices in Australia (30): *E. camaldulensis Dehan. Var camaldulensis* (ECLA1), *E. camaldulensis Dehan. Var camaldulensis* (ECLA6) and *E. camaldulensis Dehan. Var camaldulensis* (AG1). The above mentioned finding makes clear how much the compositions of the essential oil of the same species may differ in dependence of the climatic and agricultural conditions. In order to get a more systematic overview about the effect of extraction method on the chemical composition of the leaf oils, the analysed compounds were grouped into compound classes and their yields (based on 100g fresh leaves) were then calculated (Fig 15. and table 4).

The yields of the monoterpene hydrocarbon fraction were almost the same in both the HD extract (0.288g\100g) and the SFE extract (0.242g\100g). The small difference may be due to the fact that these compounds are highly volatile and not completely trapped in the depressurizing step of the SFE extraction. p-Cymene (24.1 % and 10.61 %), β-phellandrene (8.94 % and 4.09 %) , β-pinene (5.75 % and 1.41 %), α-pinene (4.28 % and 1.49 %) and limonene (1.18 % and 0.74%) were found in monoterpene hydrocarbons in HD and SFE extracts respectively. The amount of sesquiterpenes which were extracted with the HD

technique was negligibly low. The SFE method yielded 0.0825g sesquiterpenes /100g fresh leaves; γ-elemene (peak no. 70) (2.43%), aromadendrene (peak no. 72) (3.37%) and allo-aromadendrene (peak no. 73) (1.34%) were found to be the most prominent compounds of this group in the SFE extract. Aromadendrene was reported to be the most significant sesquiterpene in different *E.* species **(82, 88).** It reaches about 12% in *E. camaldulensis Dehnh. var. camaldulensis (ECLA1)* leave oil obtained by vacuum distillation **(89).** The absence of aromadendrene in the HD extract (table 3) may be due to its conversion to the related alcoholic sesquiterpenes during distillation, for example spathulenol (peak no. 78) (14.43 % and 13.14 %, in HD and SFE resp.).

The amount of light oxygenated components with carbon numbers < 15, extracted by SFE, was approxmately twice as high as in the HD extract - 0.418g/100g in comparison to 0.180g/100g fresh leaves. Cryptone was the component of this group with the highest concentration (12.71 and 9.82%, resp.) in HD and SFE extracts. p-Cymen-7-ol was found in considerable amounts (HD: 0.58 %, SFE: 2.25 %). It was identified by comparing its Kovat's index, and mass spectrum with those of authentic compound and with literature values. To our knowledge the occurrence of p-cymen-7-ol in *E.* species was not yet reported. Terpinen-4-ol (peak no.32) and α-terpineol (peak no. 35) showed higher concentrations in the HD (4.42 and 1.83%) than in the SFE extract (2.92 and 1.00% respectively). This may be explained by hydration or oxidation of monoterpenes during distillation, for example the α-terpineol may result from the hydrolysis of 1,8-cineol, followed by dehydration **(87, 90)** according to the following mechanism:



1,8 - Cineole

α - Terpineol

6-Methyl-2,4-heptadienoic acid ethyl ester (peak no. 71) (1.34 %) was found in the SFE extract only; it was identified tentatively by comparing its mass spectrum with NIST library data.

The total yield of the heavy oxygenated components in the SFE extract (0.225g/100g fresh leaves) was approx. twice as high as that in the HD extract (0.108g/100g fresh leaves). Spathulenol (peak no. 78) was the dominant in the heavy oxygenated components in both extracts HD and SFE (14.43 % and 13.14 % reaspectively), α-14-oxy-muurolene (peak no. 89) (0.31 % and 2.34 %) showed the same trend, whereas phytol (peak no. 90) (0.0 and 1.76 %) was only present in SFE extract. The higher yield of heavy oxygenated components in SFE is plausible because more compounds with low volatility in steam are extracted by the SFE method **(5, 90)**. The above presented data are in close agreement with those obtained by **Sankar (8)**, who reported that ginger oil obtained by SFE was superior in comparison to that extracted by HD because of the lower concentration of monoterpene hydrocarbons, the higher amount of sesquiterpenes, the higher quantity of oxygenated compounds in addation to its better sensory characteristics.

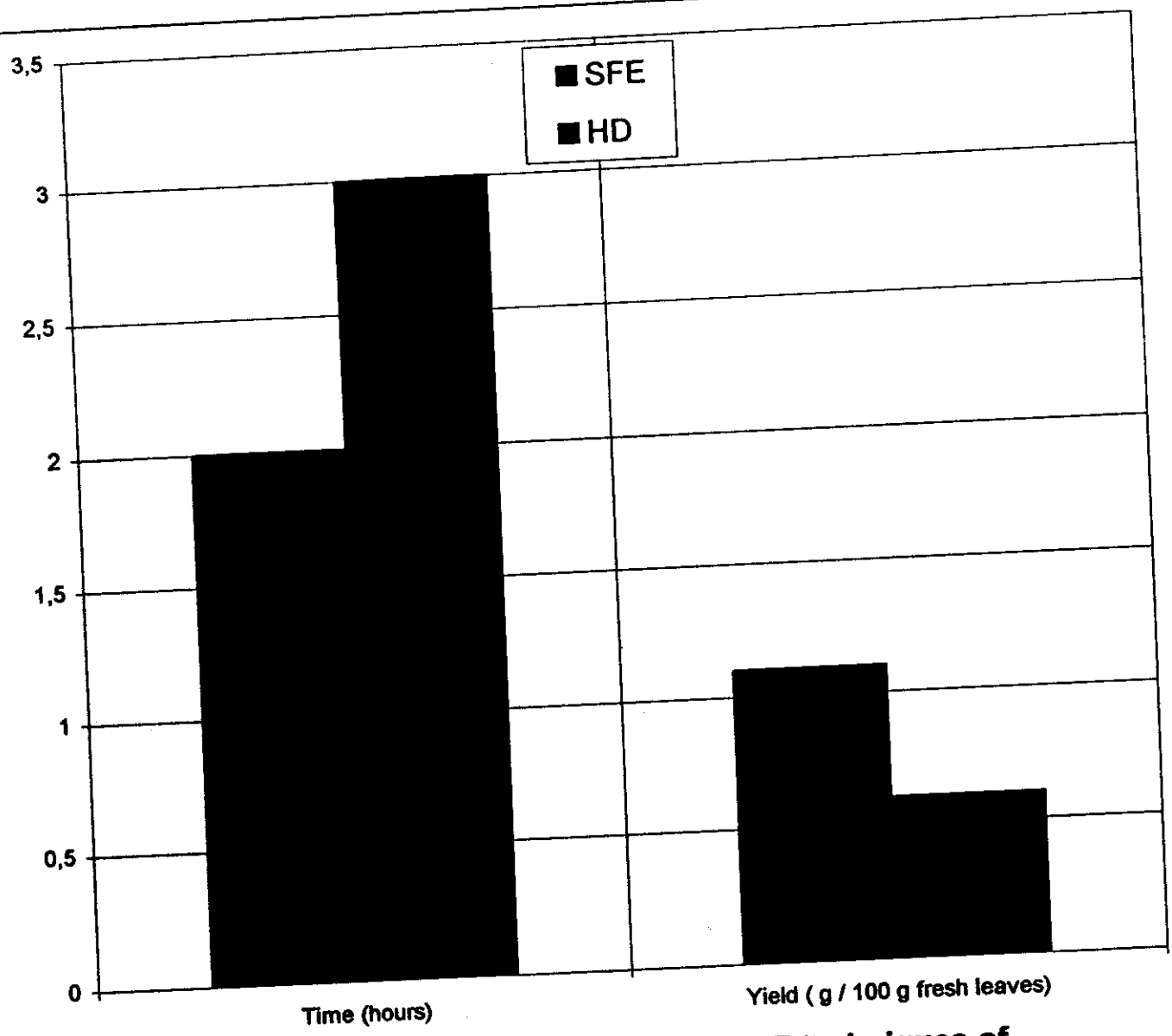


Fig 12: Yield and extraction time of HD and SFE techniques of *E. camaldulensis var. brevirostris*

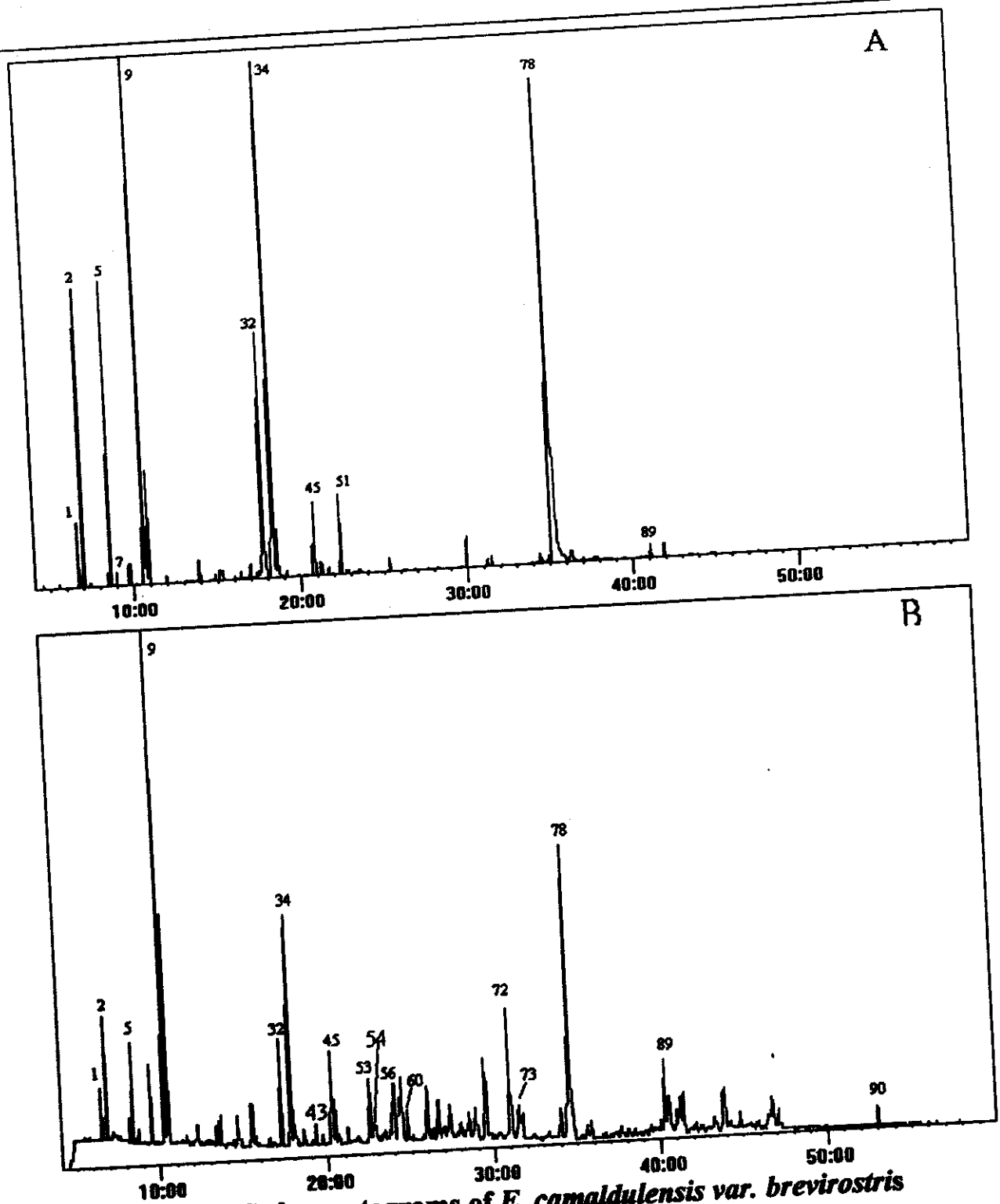


Fig.13: GC-MS chromatograms of *E. camaldulensis var. brevirostris* leaves extracted by HD (A) and SFE (B)

Table (3): Chemical constitution of the volatile oils of *E. camaldulensis* var. *brevirostris* obtained by HD and SFE extraction

Peak No.	KI BPX5	Peak area % HD	SFE	Component	Type	Method of Identification
1	926.28	1.22	0.61	Tricyclene	M	MS & KI
2	935.05	4.28	1.49	α-Pinene	M	MS & KI
3	945.36	0.12	0.31	Verbenene	M	MS & KI
4	976.28	0.28	0.36	Sabinene	M	MS & KI
5	982.98	5.75	1.41	β-Pinene	M	MS & KI
6	991.75	0.34	0.19	Myrcene	M	MS & KI
7	1011.74	1.89	1.22	α-Phellandrene	M	MS & KI
8	1021.05	0.34	0.47	α-Terpinene	M	MS & KI &St
9	1031.57	24.01	10.61	p-Cymene	M	MS & KI
10	1034	1.18	0.74	Limonene	M	MS & KI
11	1036.43	8.94	4.09	β-Phellandrene	LOC	MS & KI
12	1037.65	0.80	0.46	1,8-Cineole	M	MS & KI
13	1048.17	0.04	-	trans-β-Ocimene	LOC	MS & KI
14	1051.41	0.08	-	ArtemisiaKetone	LOC	MS
15	1055.06	-	0.09	3-Thujene-2-ol	M	MS & KI
16	1061.94	0.63	0.14	γ-Terpinene	LOC	MS & KI
17	1090.68	0.16	0.06	Isoterpinolene	LOC	MS & KI
18	1093.11	-	0.46	Linalool	-	MS & KI
19	1098.38	-	0.09	Undecane	LOC	MS & KI
20	1104.78	0.42	0.50	Thujone	LOC	MS & KI
21	1109.92	0.07	0.64	Myrcenol	LOC	MS
22	1126.47	0.11	0.10	Layratol	-	MS
23	1132.35	-	0.79	$C_{10}H_{16}$	-	MS
24	1141.54	-	0.08	$C_{10}H_{14}$	LOC	MS & KI
25	1150.36	1.26	1.24	Pirocarveol	LOC	MS & KI
26	1154.41	0.11	0.32	*trans* Sabinol	LOC	MS & KI
27	1159.92	0.4	0.06	Sabinaketone	LOC	MS & KI
28	1174.26	0.37	0.18	Pinocarvone	-	MS
29	1187.08	-	0.15	$C_{10}H_{14}$	LOC	MS
30	1179.78	0.18	-	exo - Isocamphanon	LOC	MS
31	1183.82	0.39	0.07	$C_{10}H_{12}O$		

Peak No.	KI BPX5	Peak area % HD	SFE	Component	Type	Method of Identification
32	1190.07	4.42	2.92	Terpinen-4-ol	LOC	MS & KI
33	1195.02	-	1.16	p-Cymen-8-ol	LOC	MS & KI
34	1202.58	12.71	9.82	Cryptone	LOC	MS
35	1206.27	1.83	1.00	α-Terpinol	LOC	MS & KI
36	1208.48	0.73	0.72	Myrtenol	LOC	MS & KI
37	1212.33	-	0.51	Unidentified	-	
38	1214.02	0.1	-	$C_{10}H_{14}$	-	MS
39	1218.81	0.37	0.09	*cis*-Sabinenehydrate acetate	LOC	MS & KI
40	1220.29	-	0.12	Ocimenone	LOC	MS & KI
41	1229.88	0.13	0.18	Myrtenylacetate	LOC	MS & KI
42	1233.70	-	0.53	Unidentified	-	
43	1235.54	-	0.34	2-Methyl-6-ethylphenol	LOC	MS & KI
44	1236.90	0.18	0.17	o-Hydroxycumine	LOC	MS
45	1256.82	2.96	2.32	Cuminaldehyde	LOC	MS & KI
46	1258.37	-	1.23	Unidentified	-	
47	1266.42	0.28	0.21	Carvotanacetate	LOC	MS & KI
48	1277.12	0.12	0.63	Unidentified	-	
49	1284.50	0.21	-	Unidentified	-	
50	1291.16	-	0.35	$C_8H_{14}O_2$	LOC	MS
51	1291.88	1.82	-	Piperitone	LOC	MS & KI
52	1301.50	0.1	-	α-Terpinen-7-ol	LOC	MS & KI
53	1305.28	0.58	2.25	p-Cymen-7-ol	LOC	MS & KI& St
54	1310.56	0.65	0.98	Thymol	LOC	MS & KI
55	1323.39	-	0.72	Unidentified	-	
56	1329.03	-	0.64	3,5,7-Nonatriene	-	MS
57	1333.96	-	0.27	*cis*-Carvylacetate	LOC	MS & KI
58	1341.88	-	3.36	1,4-Epidioxy- p-menth-2-ene	LOC	MS
59	1346.41	-	1.88	o-Menthan-3-one	LOC	MS
60	1354.71	-	1.45	Unidentified	-	
61	1356.98	0.27	-	Unidentified	-	
62	1360.37	-	0.13	$C_{10}H_{16}O_2$	LOC	MS
63	1378.49	-	0.17	α-Copaene	S	MS & KI
64	1385.28	-	1.97	Unidentified	-	
65	1392.83	-	0.28	Thumbergol	LOC	MS

Peak No.	KI BPX5	Peak area % HD	SFE	Component	Type	Method of Identification
66	1400.39	-	1.67	Unidentified		
67	1431.07	-	0.70	4,8-Dimethyl-7-nonen-2-one	LOC	MS
68	1443.02	-	2.13	$C_{10}H_{16}O_2$	LOC	MS
69	1466.93	0.23	-	β-Caryophyllene	S	MS & KI
70	1467.72	-	2.43	γ-Elemene	S	MS & KI
71	1470.12	-	1.34	6-Methyl-2,4-heptadienoicacidethylester	LOC	MS
72	1494.82	-	3.57	Aromadendrene	S	MS & KI
73	1503.30	-	1.34	Allo-aromadendrene	S	MS & KI
74	1508.67	0.14	-	Bicyclogermacrene	S	MS & KI
75	1537.60	-	0.15	$C_{14}H_{22}O$	LOC	MS
76	1558.26	0.10	0.21	Elemol	HOC	MS & KI
77	1581.81	-	0.83	$C_{10}H_{16}O_2$	LOC	MS
78	1591.73	14.43	13.14	Spathulenol	HOC	MS& KI&St
79	1593.80	-	1.11	$C_{15}H_{22}$	-	MS
80	1595.45	0.92	1.16	Globulol	HOC	MS & KI
81	1612.98	0.07	0.21	β-Biotol	HOC	MS & KI
82	1618.18	0.26	0.22	γ -10- epi-Eudesmol	HOC	MS & KI
83	1623.37	0.40	0.43	*cis*-Amylcinnamaldehyde	HOC	MS & KI
84	1646.32	0.51	0.12	Khusinol	HOC	MS & KI
85	1659.74	0.06	0.15	α-Santalol	HOC	MS & KI
86	1671.86	0.37	0.47	8-Cedren-13-ol	HOC	MS & KI
87	1690.90	0.09	0.22	α-*trans* Bergamotol	HOC	MS & KI
88	1753.15	0.33	0.06	Aristolone	HOC	MS & KI
89	1769.81	0.31	2.34	α-14-oxy -Muurolene	HOC	MS & KI
90	1970.66	-	1.76	Phytol		

MS: tentative identification by comparison with data obtained from NIST mass spectra library

KI: confirmed by comparison with Kovat's index on DB5 column [4]

St: confirmed by comparison with mass spectrum of authentic compound

M: monoterpene hydrocarbon, LOC: light oxygenated compound

HOC: heavy oxygenated compound, S: sesquiterpene

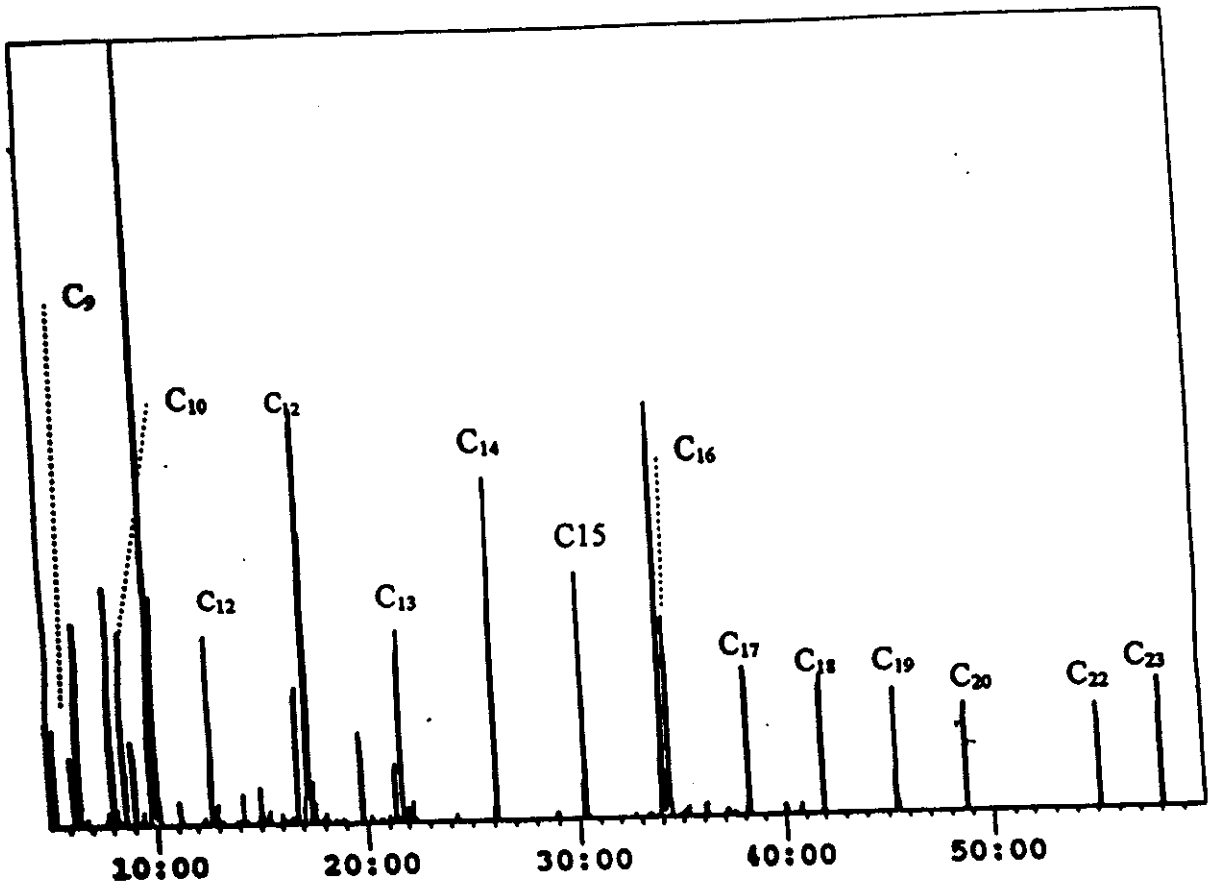


Fig 14: GC-MS chromatograms of standard alkanes (C_9 - C_{23}) and essential oil of *E. camaldulensis var. brevirostris*

Table (4): Effect of extraction techniques on the composition of the main chemical classes in the volatile oil of *E. camaldulensis var. brevirostris* leaves

Compounds	HD		SFE	
	Area %	Yield (g/100g)	Area %	Yield (g/100g)
Monoterpenes	49,02	0,2940	21,64	0,2380
Sesquiterpenes	00,37	0,0022	07,25	0,0800
Light oxygenated compounds	30,35	0,1820	38,71	0,4260
Heavy oxygenated compounds	17,85	0,1070	20,60	0,2270

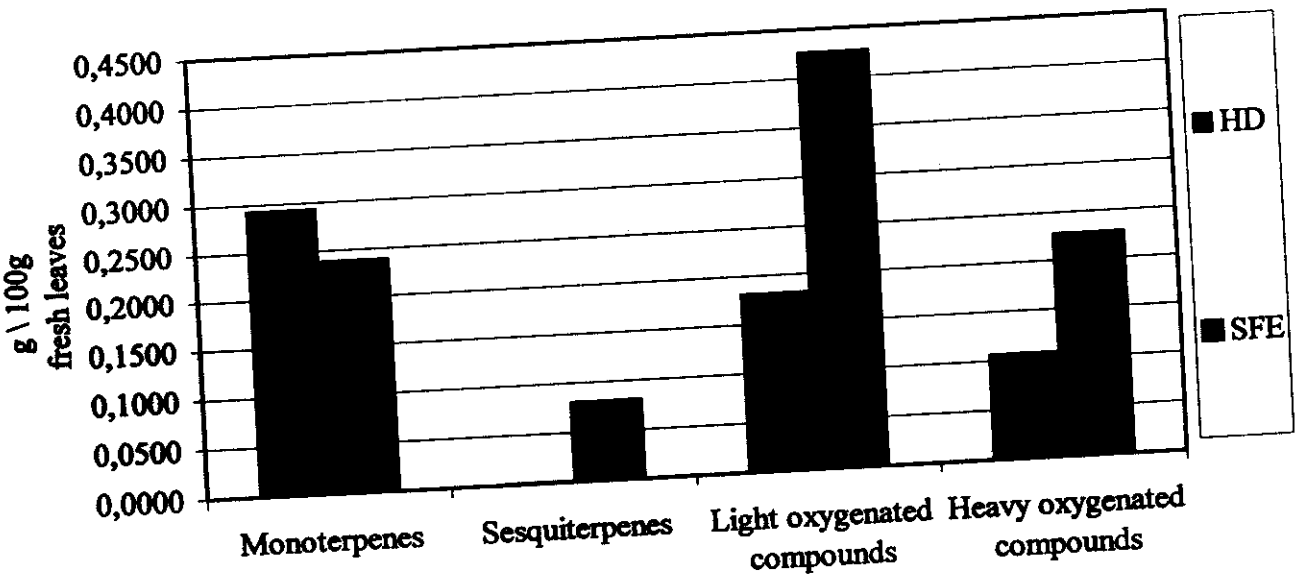


Fig 15: Effect of extraction techniques on the composition of the main chemical classes in the volatile oil of *E. camaldulensis var. brevirostris* leaves

4.1.2 - Antioxidant activity of the HD and SFE extracts

Antioxidants are regarded as compounds capable of delaying, retarding or preventing autoxidation processes. The main justification for using antioxidant is to extend the shelf life of foodstuffs and to reduce nutritional losses by inhibiting and delaying oxidation.

Synthetic antioxidants such as BHT and BHA are commonly used in the food industry because they retard undesirable changes due to oxidation. However, it has begun to be restricted because of their toxicity **(91)** and a trend among consumers towards,, all natural ingredients'' have resulted in a pronounced activity in the field of additives.

Thus in the present study, it was cocentrated on the investigation of the antioxidant activity which extract from the natural source especially (aromatic plant). The antioxidative activities of the HD and the SFE extracts for the studied leaves on the peroxide formation of linoleic acid were measured by the thiocyanante method [79]. In the thiocyanate method ferrous chloride and ammonium thiocyanate are added to a solution of the sample. The amount of ferric thiocyanate complex (red colour) produced after a fixed reaction time with the peroxides present in the sample is determined colorimetrically at 500 nm according to the following equations:

$$Fe^{2+} + \text{peroxides} \longrightarrow Fe^{3+}$$

$$Fe^{3+} + [SCN]^{-} \longrightarrow [FeSCN]^{2+}$$

(red colour complex)

The antioxidative activities of the HD and the SFE extracts were compared with BHA and BHT. Both extracts show antioxidative activities but somewhat lower than that of BHT and BHA. The activity of the SFE is slightly higher than that of the HD extract, (Table 5 and 6, Fig 16).

It was reported that in some essential oils there is a relationship between inhibition of the hydroperoxide formation and the presence of phenols such as thymol and eugenol **(92)** beside other phenolic compounds **(93)**. Therefore obviously the phenolic compounds o-hydroxy cumine (peak no. 44; 0.18 % and 0.17 %), thymol (peak no. 54; 0.65 % and 0.98 %), and 2-methyl-6-ethyl phenol (peak no. 43; 0.34 %], that were found in the HD and SFE extracts respectively of *E. camaldulensis* var. *brevirostris* leaves, contribute to the detected antioxidative activity.

The newly identified compound "p-cymen-7-ol" Peak no. 53, is present in considerable concentration in HD and SFE extracts (0.58% and 2.25%, respectively) as shown in table 3 and so its antioxidant activity was measured and compared to BHA and BHT. As shown in Fig.16 its activity is considerable and very similar that of BHA but the mechanism of antioxidant activity of p-cymen-7-ol is not yet clear and should be investigated.

From the absorbance reading presented in figure 16 "the inhibitory effect" was concluded. The "inhibitory effect" is defined as the selective inhibition of the peroxide formation in a substrate (here linoleic acid) after a certain storage time (12 days). It is calculated by the equation **(94)**.

$$\text{Inhibition}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

A: absorbance reading

The inhibitory effects of the HD and SFE extracts and of p-cymen-7-ol were calculated and illustrated in the table 7 and fig 17. The HD and SFE exhibited 47.81 % and 56.31 % inhibition of the peroxide formation of linoleic acid after 12 days, respectively. p-Cymen-7-ol exhibited 67.23 % inhibition. Peroxide formation was decreased to about 61.67 % by BHA. Thus it may be concluded that the higher inhibition effect of the SFE extract is caused by its higher p-cymen-7-ol concentration.

4.1.3 - Conclusion

From the above mentioned results it is clear that the chemical constitution of the volatile oil of *E. camaldulensis* is affected by the technique of extraction. Both techniques (HD and SFE) yield extracts with similar antioxidant activity, but SFE is to be preferred over HD for antioxidant purposes because the extraction yield of SFE was approx. twice as high as that of the HD extract and the SFE extracts show somewhat superior antioxidant activity.

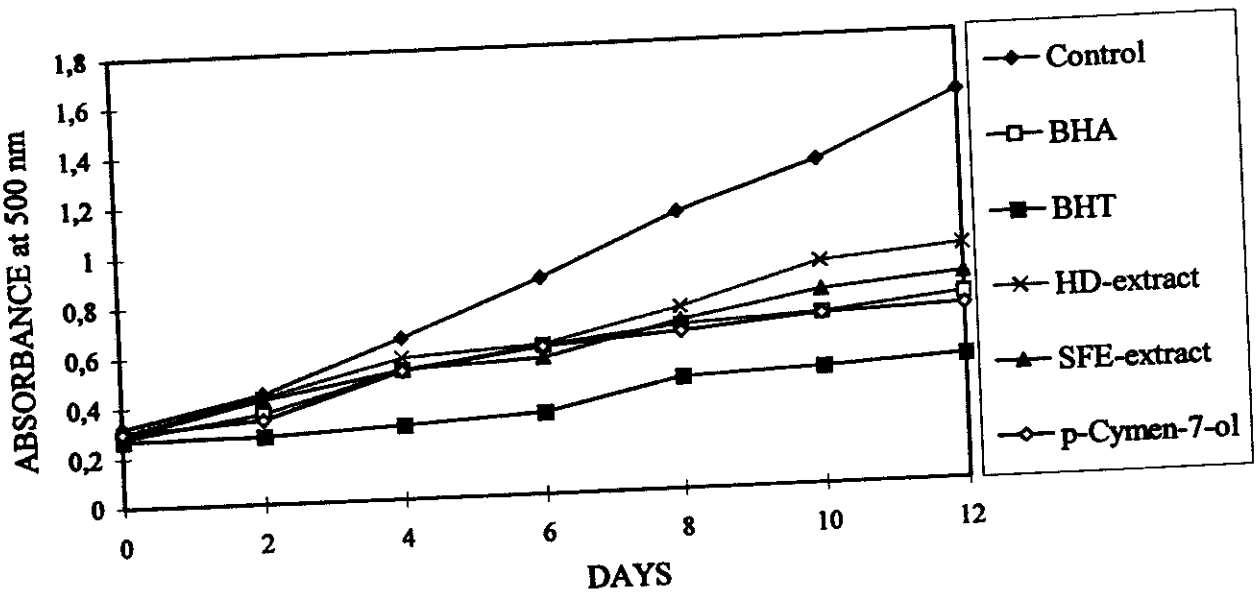


Fig 16: Antioxidant activity of the *E. camaldulensis var. brevirostris* leaves (obtained by HD and SFE), p-cymen-7-ol, BHA and BHT

Table (5): Absorbance reading of the thiocyanate tests of HD extract and SFE extract and p-Cymen-7-ol

time(day)	control	BHA	BHT	HD extract	SFE extract	p-cymen-7-ol
0	0,340	0,276	0,272	0,290	0,288	0,229
	0,300	0,284	0,268	0,310	0,292	0,231
2	0,438	0,369	0,276	0,436	0,423	0,343
	0,446	0,361	0,272	0,424	0,417	0,337
4	0,652	0,522	0,298	0,566	0,516	0,513
	0,648	0,518	0,302	0,574	0,524	0,517
6	0,865	0,596	0,335	0,600	0,545	0,590
	0,875	0,604	0,325	0,610	0,555	0,596
8	1,116	0,664	0,452	0,734	0,681	0,634
	1,108	0,660	0,448	0,730	0,679	0,636
10	1,302	0,684	0,465	0,892	0,784	0,686
	1,298	0,696	0,475	0,896	0,776	0,686
12	1,554	0,755	0,508	0,950	0,827	0,702
	1,558	0,745	0,492	0,940	0,833	0,708

Table (6): Evaluation of antioxidant activities of the HD and SFE extracts and of p-Cymen-7-ol

Days	Control	BHA	BHT	HD extract	SFE extract	p-cymen-7-ol
0	0,320 ± 0,002	0,280 ± 0,004	0,270 ± 0,002	0,300 ± 0,010	0,290 ± 0,002	0,300 ± 0,001
2	0,442 ± 0,004	0,365 ± 0,004	0,274 ±0,002	0,430 ± 0,006	0,420 ± 0,003	0,340 ± 0,003
4	0,650 ± 0,002	0,520 ± 0,002	0,300 ± 0,002	0,570 ± 0,004	0,520 ± 0,004	0,515 ± 0,002
6	0,870 ± 0,005	0,600 ± 0,004	0,330 ±0,005	0,605 ± 0,005	0,550 ± 0,005	0,593 ± 0,003
8	1,112 ± 0,004	0,662 ± 0,002	0,450 ± 0,002	0,732 ± 0,002	0,680 ± 0,001	0,635 ± 0,001
10	1,300 ± 0,002	0,690 ± 0,006	0,470 ± 0,005	0,894 ± 0,002	0,780 ± 0,004	0,686 ± 0,000
12	1,556 ± 0,002	0,750 ± 0,005	0,500 ± 0,08	0,945 ± 0,005	0,830 ± 0,003	0,705 ± 0,003

Reported values are the mean of two analysis; ± values are the corresponding standard deviation

Table (7): The inhibitory effects on the peroxide formation of linoleic acid time period: 12 days

Components	Inhibition %
BHT	81.4
p-cymen-7-ol	67.2
BHA	61.7
SFE extract	56.3
HD extract	47.8

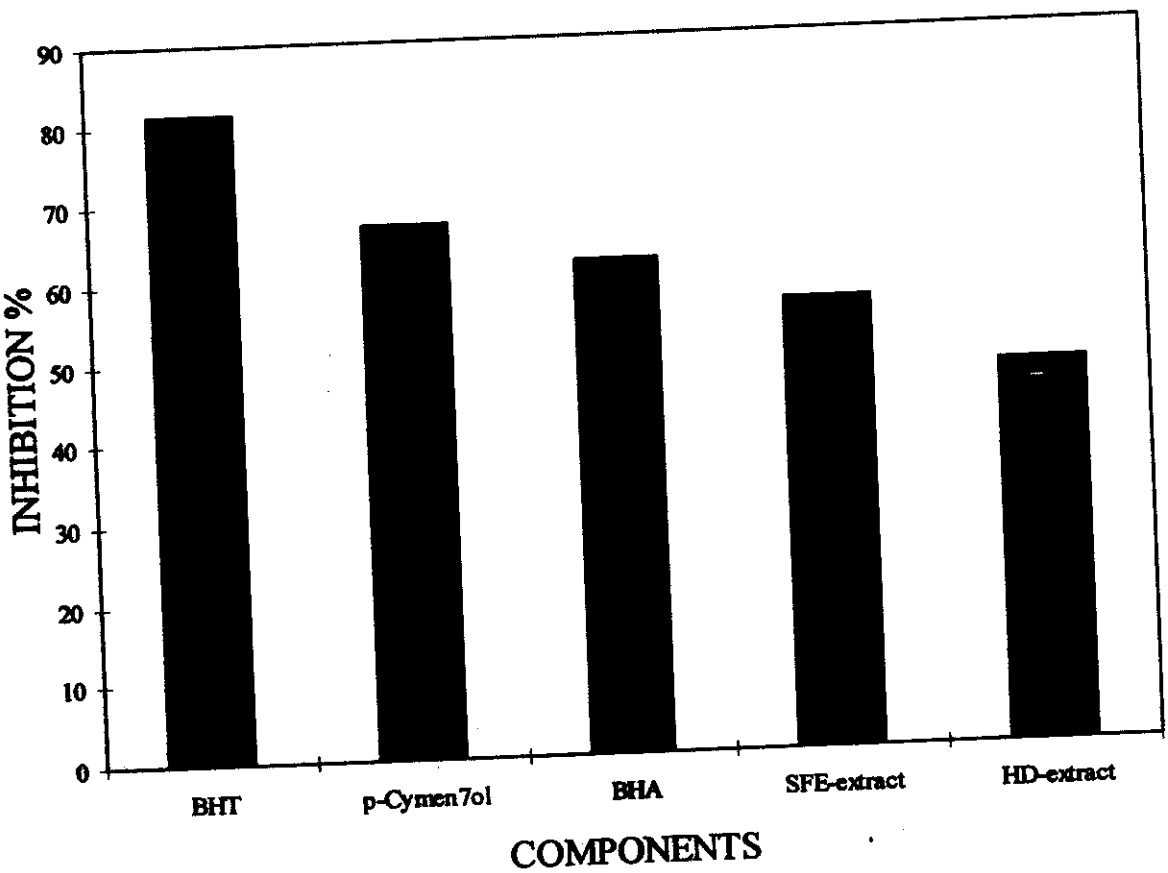


Fig 17: Inhibitory effect of the *E. camaldulensis* leaf oils (obtained by HD and SFE), BHA, BHT and p-Cymen-7-ol substrate: linoleic acid, time perriod : 12 days

4.2 - The antioxidant activities and identification of non volatile compounds of *E. camaldulensis var. brevirostris* leaves:

4.2.1- The antioxidant activities of non volatile compounds:

The nonvolatile components were obtained by solvent extraction and SFE modified with 10 and 15 % ethanol.

Table (8): the yields of different organic solvent extractions from hydrodistillated and lyophilised *E. camaldulensis* var. *brevirostris* leaves

Solvent	Yield (g / 100 g) ± SD
Hexane (digestion)	**3.0 ± 0.2**
Hexane (Sox)	**7.4 ± 0.6**
Methylene chloride(Sox) residue after hexane (Sox) Ex.	**8.4 ± 0.5**
Ethanol (digestion)	**14 ± 0.5**
Ethanol (Sox)	**20 ± 0.3**

The yields are the average of three extractions and represent the amounts extracted in grams / 100 g of lyophilised leaves

SD: Standard deviation

Sox.: Soxhlet; Ex.: Extraction

The extracted yields after Soxhlet extraction are higher than those of solvent digestion because by the first method compounds are extracted repeatedly while the solvent digestion is a one step extraction. Furthermore the amount of the yield is directly proportional to the polarity of the solvent. Accordingly, Soxhlet extraction with ethanol results in the highest yield, while digestion with hexane gave the lowest yield.

The antioxidant activities of the above obtained solvent extracts (table 8) were determined by the thiocyanate method **(3.7 and 4.1.2)**. Each extract was added to linoleic acid and incubated for 12 days at 40 C° under normal atmospheric conditions.

The antioxidant activities of the extracts, obtained by Soxhlet and solvent digestion methods were evaluated and it was observed that solvent digestion gave slightly higher antioxidant activity than Soxhlet extraction.

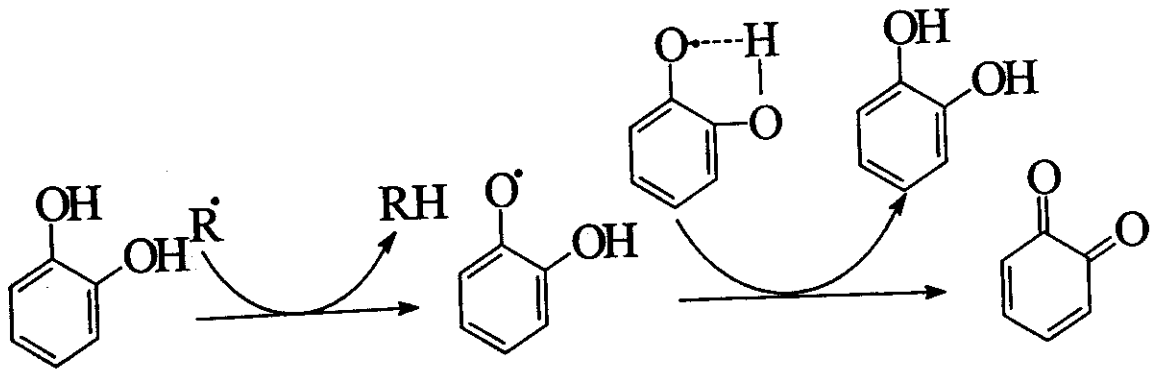
The antioxidative activities of the hexane (digestion) extract and the hexane extract (Soxhlet) were compared with BHA and BHT. Both extracts show more or less the same antioxidant activities, but slightly higher than BHA and lower than BHT (see Fig. 18 and table 9, 10).

The antioxidative activities of the ethanol digestion extract and the ethanol extract (Soxhlet) were compared with BHA and BHT (see figure 19 and table 11, 12). The activity of the ethanol digestion is higher than that of the ethanol extract (Soxhlet) extract, BHA and BHT.

The ethanolic digestion extract showed higher antioxidant activity than that of hexane digestion extract (see fig. 20 and table 13, 14). It could be noted that the extracts obtained by a non-polar solvent are less effective as an antioxidant. These results are in agreement with those reported by **Pin-Der Duh et al (95).** who found that the antioxidant activities of ethanolic extracts of peanut hulls are higher than those of hexane extracts.

The above mentioned result indicates that all extracts [hexane (digestion) extract, hexane (Sox.) extract, ethanol (Sox.) extract and ethanolic(digestion) extract] of *E. camaldulensis* leaves all have antioxidant activity. The highest activity was found for the ethanol (digestion). It is higher than that of BHA and equal to the inhibitory effect of BHT. The yields of the SFE, modified with 10 % ethanol and with 15 % ethanol are 7,2g ± 0,3 /100 g fresh leaves and 10g ± 0,6 /100 g fresh leaves, respectively. Increasing of the modifier

result revealed that the mixture has considerable antioxidant activity compared with that of the ethanol (digestion) extract (**fig. 23 and tables 19, 20**). Generally, the grade of inhibition of lipid oxidation is depending on the chemical structure of the antioxidant compound. The acidic compounds incorporating phenolic groups, especially in ortho position as ellagic acid and gallic acid, have been repeatedly implicated as effective antioxidants. The efficiency of ortho diphenols is in part due to the stabilisation of the aryloxyl radical by hydrogen bonding **(99)** or by regeneration of the diphenol **(97)** as indicated in fig (24).



Mechanism of the regeneration of an ortho-diphenol

FIG (24)

These results are in accordance with those obtained by Duve and White **(100)**. They found that the antioxidant activity of the methanol extract was higher than that of the petroleum ether extract of Ogle oats. These extracts were added to soybean oil and their effectiveness was compared with BHT **(100)**.

The antioxidant activities of SFE extract (modified with 15 % ethanol) may be explained by their content of 5-Hydroxy-7, 4′ dimethoxy-8-methyl flavone (F2) and 5-Hydroxy-7, 4′ dimethoxy flavone (F1) (see 4.2.4) in the main fraction of

HPLC chromatogram of SFE extract (modified with 15 % ethanol)of *E. camaldulensis var. breviostris* (fig. 25). Because the corresponding HPLC fractions have antioxidant activities similar the same of BHA (fig. 26 and tables 21,22).

The inhibition of lipid oxidation can only be completely prevented by the total exclusion of oxygen from the system. Thus the inhibitory effect means the decrease of the rate of peroxides formation in lipids by suppressing the rate of initiation reactions.

The inhibitory effect of the ethanol (digestion) extract and SFE extract (modified with 15 % ethanol) was calculated according to the equation of inhibition (see 4.1.2).

The inhibitory effect of the ethanol (digestion) extract and of the SFE extract (modified with 15 % ethanol) after 12 days on the peroxide formation of linoleic acid was calculated as 81.7 % and 72.5 %, respectively. In comparison the inhibitory of BHA and BHT was 61.67 % and 81.39 % (fig. 27 and table 23). The mixture with the same amount of ellagic acid (22.4 %) and gallic acid (4.6 %) as in ethanol (digestion) extract exhibited 65.3 % inhibition of peroxide formation after 12 days. Thus, it may be concluded that most of remarkable inhibitory effect of the ethanol (digestion) extract is caused by its content of ellagic acid and gallic acid (fig. 27 and table 23) besides other active components.

These results show that among the tested extraction methods the ethanol (digestion) extraction and SFE (modified with15 % ethanol) are the most effective ones. Therefore, extracts prepared by these two methods were subjected to different instrumental analysis to identify the antioxidative major components.

Table (9): Absorbance readings of the thiocyanate tests of hexane (Sox.) extract and hexane (digestion) extract

time(days)	control	BHA	BHT	Hexane sox.	Hexane digestion
0	0,340	0,276	0,270	0,306	0,305
	0,300	0,284	0,268	0,312	0,309
2	0,440	0,369	0,280	0,357	0,377
	0,450	0,361	0,274	0,361	0,373
4	0,650	0,525	0,300	0,435	0,400
	0,655	0,520	0,302	0,425	0,404
6	0,871	0,596	0,326	0,524	0,452
	0,875	0,604	0,330	0,520	0,458
8	1,118	0,670	0,442	0,591	0,487
	1,112	0,665	0,446	0,589	0,497
10	1,304	0,686	0,471	0,615	0,559
	1,302	0,690	0,475	0,609	0,553
12	1,560	0,751	0,504	0,685	0,670
	1,550	0,745	0,502	0,695	0,680

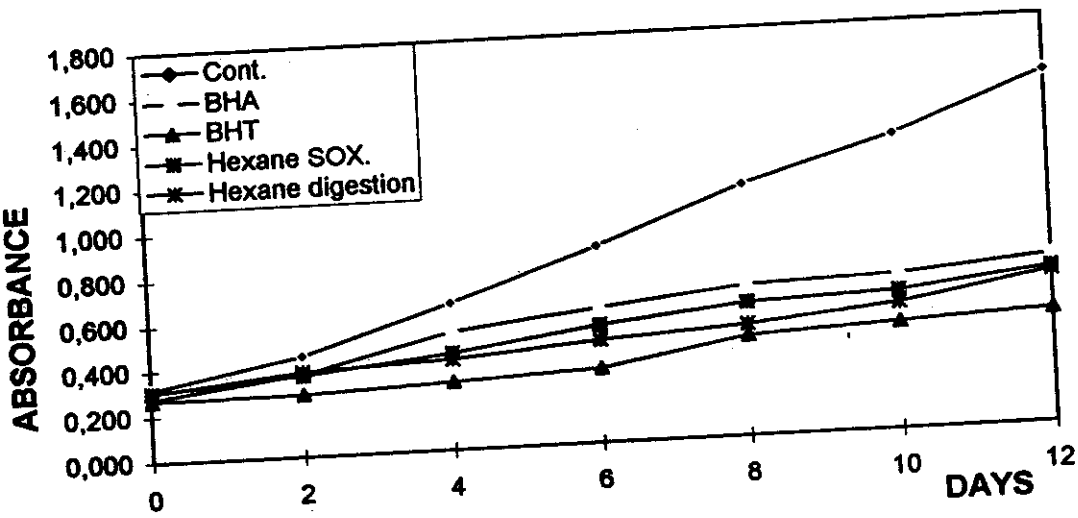


Fig (18): The antioxidant activity of the hexane (digestion) extract and hexane (Soxhlet) extract of E. *camaldulensis var. brevirostris.*

able (10): Evaluation of antioxidant activities of the hexane xtract(Sox.) and hexane (digestion) extract

)ays	Control	BHA	BHT	Hexane (digestion)	Hexane (Sox.)
				0,	0,309 ± 0,003
					),359 ±0,002
					,430 ± 0,005
					,522 ± 0,002
					590 ± 0,001
					612 ± 0,003
					90 ± 0,005

re the

Table (11): Absorbance readings of the thiocyanate tests of ethanol (digestion) extract and ethanol (Sox.) extract

ime(day)	control	BHA	BHT	Ethanol (digestion)	Ethanol (Sox)
0	0,340	0,276	0,270	0,222	0,310
	0,300	0,284	0,268	0,218	0,304
2	0,440	0,369	0,280	0,253	0,373
	0,450	0,361	0,274	0,251	0,377
4	0,650	0,525	0,300	0,273	0,400
	0,655	0,520	0,302	0,277	0,404
6	0,871	0,596	0,326	0,296	0,458
	0,875	0,604	0,330	0,294	0,452
8	1,118	0,670	0,442	0,383	0,495
	1,112	0,665	0,446	0,377	0,489
10	1,304	0,686	0,471	0,416	0,552
	1,302	0,690	0,475	0,414	0,560
12	1,560	0,751	0,504	0,448	0,679
	1,550	0,745	0,502	0,442	0,671

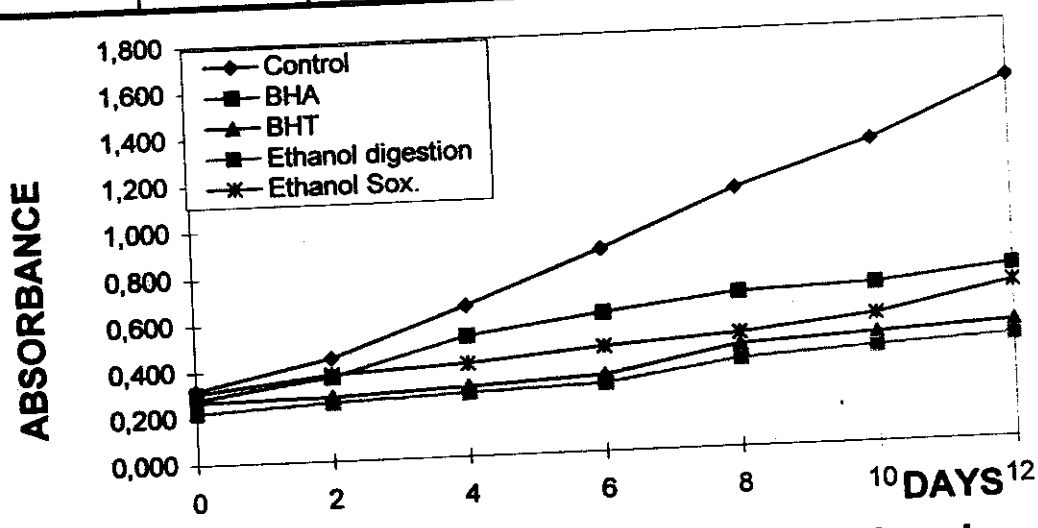


Fig (19): The antioxidant activity of ethanol (digestion) extract and ethanol extract (Sox.) of *E. camaldulensis* var. *brevirostris* leaves

Table (12): Evaluation of antioxidant activities of ethanol (digestion) extract and ethanol extract (Sox.)

Days	Control	BHA	BHT	Ethanol digestion	Ethanol Sox.
0	0,320± 0,020	0,280 ± 0,004	0,269 ± 0,001	0,220 ± 0,002	0,307 ± 0,003
2	0,445± 0,005	0,365 ± 0,004	0,277 ± 0,003	0,252 ± 0,001	0,375 ± 0,002
4	0,653± 0,002	0,523 ± 0,003	0,301 ± 0,001	0,275 ± 0,002	0,402 ± 0,002
6	0,873 ± 0,002	0,600 ± 0,004	0,328 ±0,002	0,295 ± 0,001	0,455 ± 0,003
8	1,115 ± 0,003	0,668 ± 0,002	0,444 ± 0,002	0,380 ± 0,003	0,492 ± 0,003
10	1,303 ± 0,001	0,688 ± 0,002	0,473 ± 0,002	0,415 ± 0,001	0,556 ± 0,004
12	1,555 ± 0,005	0,748 ± 0,003	0,503 ± 0,001	0,445 ± 0,003	0,675 ± 0,004

Reported values are the mean of two analysis; ± values are corresponding standard deviation

Table (13): Absorbance readings of the thiocyanate tests of ethanol (digestion) extract and hexane (digestion) extract

time(days)	control	BHA	BHT	Ethanol (digestion)	Hexane (digestion)
0	0,340	0,276	0,270	0,222	0,305
	0,300	0,284	0,268	0,218	0,309
2	0,440	0,369	0,280	0,253	0,377
	0,450	0,361	0,274	0,251	0,373
4	0,650	0,525	0,300	0,273	0,400
	0,655	0,520	0,302	0,277	0,404
6	0,871	0,596	0,326	0,296	0,452
	0,875	0,604	0,330	0,294	0,458
8	1,118	0,670	0,442	0,383	0,487
	1,112	0,665	0,446	0,377	0,497
10	1,304	0,686	0,471	0,416	0,559
	1,302	0,690	0,475	0,414	0,553
12	1,560	0,751	0,504	0,448	0,670
	1,550	0,745	0,502	0,442	0,680

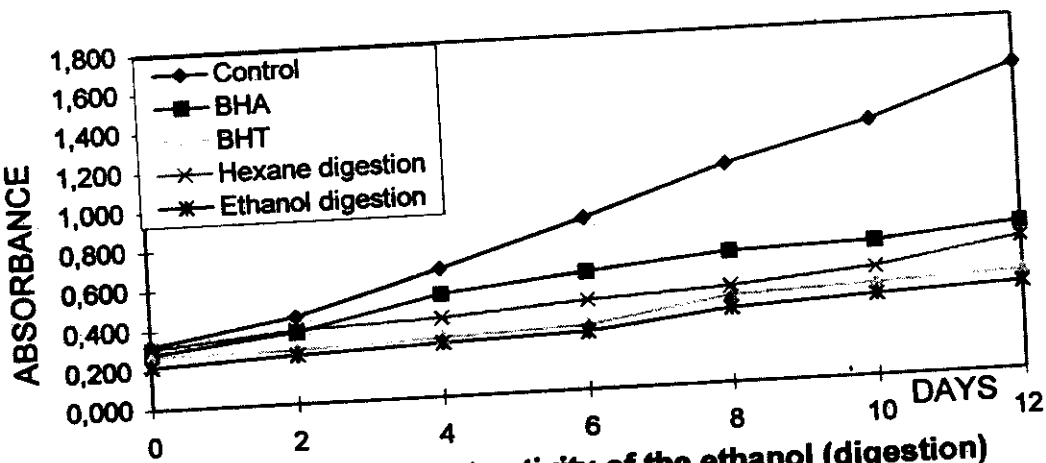


Fig (20): The antioxidant activity of the ethanol (digestion) extract and hexane (digestion) extract *of E. camaldulensis* var. *brevirostris* leaves

Table (14): Evaluation of antioxidant activities of ethanol (digestion) extract and hexane (digestion)extract

Days	Control	BHA	BHT	Ethanol (digestion)	Hexane (digestion)
0	0,320 ± 0,020	0,280 ± 0,004	0,269 ± 0,001	0,220 ± 0,002	0,307 ± 0,002
2	0,445 ± 0,005	0,365 ± 0,004	0,277 ± 0,003	0,252 ± 0,001	0,375 ± 0,002
4	0,653 ± 0,002	0,523 ± 0,003	0,301 ± 0,001	0,275 ± 0,002	0,402 ± 0,002
6	0,873 ± 0,002	0,600 ± 0,004	0,328 ±0,002	0,295 ± 0,001	0,455 ± 0,003
8	1,115 ± 0,003	0,668 ± 0,002	0,444 ± 0,002	0,380 ± 0,003	0,492 ± 0,005
10	1,303 ± 0,001	0,688 ± 0,002	0,473 ± 0,002	0,415 ± 0,001	0,556 ± 0,003
12	1,555 ± 0,005	0,748 ± 0,003	0,503 ± 0,001	0,445 ± 0,003	0,675 ± 0,005

Reported values are the mean of two analysis; ± values are corresponding standard deviation

Table (15): Absorbance readings of the thiocyanate tests of SFE extract (modified with 15 % and 10 % ethanol) and methylene chloride extract

time(days)	control	BHA	BHT	SFE 10%	SFE 15%	CH_2Cl_2 (sox)
0	0,340	0,276	0,270	0,296	0,308	0,309
	0,300	0,284	0,268	0,304	0,312	0,315
2	0,440	0,369	0,280	0,377	0,369	0,368
	0,450	0,361	0,274	0,383	0,363	0,372
4	0,650	0,525	0,300	0,423	0,398	0,373
	0,655	0,520	0,302	0,425	0,402	0,387
6	0,871	0,596	0,326	0,512	0,463	0,459
	0,875	0,604	0,330	0,508	0,457	0,450
8	1,118	0,670	0,442	0,587	0,537	0,557
	1,112	0,665	0,446	0,593	0,543	0,563
10	1,304	0,686	0,471	0,702	0,626	0,570
	1,302	0,690	0,475	0,698	0,634	0,560
12	1,560	0,751	0,504	0,776	0,651	0,632
	1,550	0,745	0,502	0,784	0,649	0,648

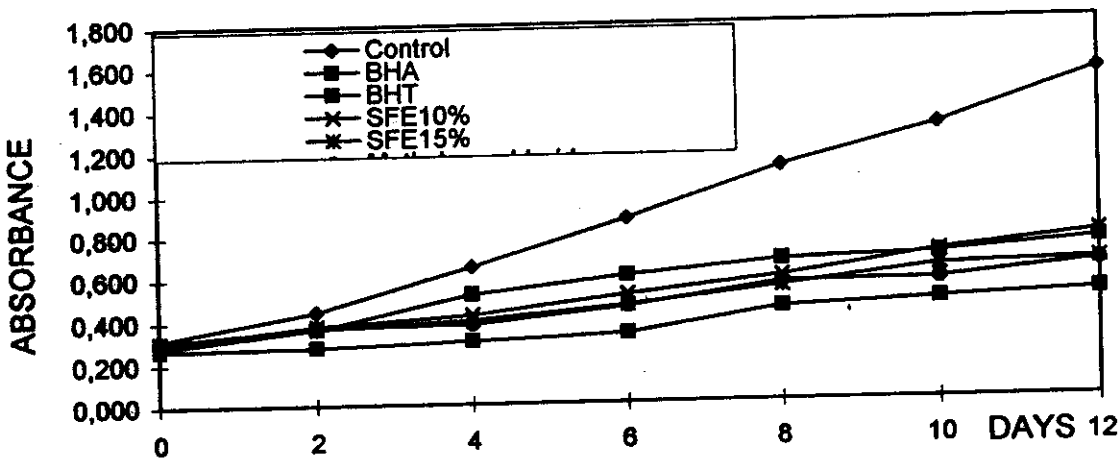


Fig (21): The antioxidant activity of the SFE (modified with 10 % and 15 % ethanol) extract and methylene chloride (Sox.) extract of *E. camaladulensis* var. *brevirostris* leaves

Table (16): Evaluation of antioxidant activities of the SFE extract (modified with15 % ethanol) and the SFE extract (modified with10 % ethanol) and the methylene chloride extract (Sox.)

Days	Control	BHA	BHT	SFE 10%	SFE 15%	CH_2Cl_2(sox.)
0	0,320 ± 0,020	0,280 ± 0,004	0,269 ± 0,001	0,300 ± 0,004	0,310 ± 0,002	0,312 ± 0,003
2	0,445 ± 0,005	0,365 ± 0,004	0,277 ± 0,003	0,380 ± 0,003	0,366 ± 0,003	0,370 ± 0,002
4	0,653 ± 0,002	0,523 ± 0,003	0,301 ± 0,001	0,424 ± 0,001	0,400 ± 0,002	0,380 ± 0,007
6	0,873 ± 0,002	0,600 ± 0,004	0,328 ±0,002	0,510 ± 0,002	0,460 ± 0,003	0,454 ± 0,005
8	1,115 ± 0,003	0,668 ± 0,002	0,444 ± 0,002	0,590 ± 0,003	0,540 ± 0,003	0,560 ± 0,003
10	1,303 ± 0,001	0,688 ± 0,002	0,473 ± 0,002	0,700 ± 0,002	0,630 ± 0,004	0,565 ± 0,005
12	1,555 ± 0,005	0,748 ± 0,003	0,503 ± 0,001	0,780 ± 0,004	0,650 ± 0,001	0,640 ± 0,008

Reported values are the mean of two analysis; ± values are corresponding standard deviation

Table (17): Absorbance readings of the thiocyanate tests of ethanol digestion extract and SFE extract (modified with 15 % ethanol)

time(days)	control	BHA	BHT	SFE (15%)	Ethanol (digestion)
0	0,340	0,276	0,270	0,308	0,222
	0,300	0,284	0,268	0,312	0,218
2	0,440	0,369	0,280	0,369	0,253
	0,450	0,361	0,274	0,363	0,251
4	0,650	0,525	0,300	0,398	0,273
	0,655	0,520	0,302	0,402	0,277
6	0,871	0,596	0,326	0,463	0,296
	0,875	0,604	0,330	0,457	0,294
8	1,118	0,670	0,442	0,537	0,383
	1,112	0,665	0,446	0,543	0,377
10	1,304	0,686	0,471	0,626	0,416
	1,302	0,690	0,475	0,634	0,414
12	1,560	0,751	0,504	0,651	0,448
	1,550	0,745	0,502	0,6490	0,4420

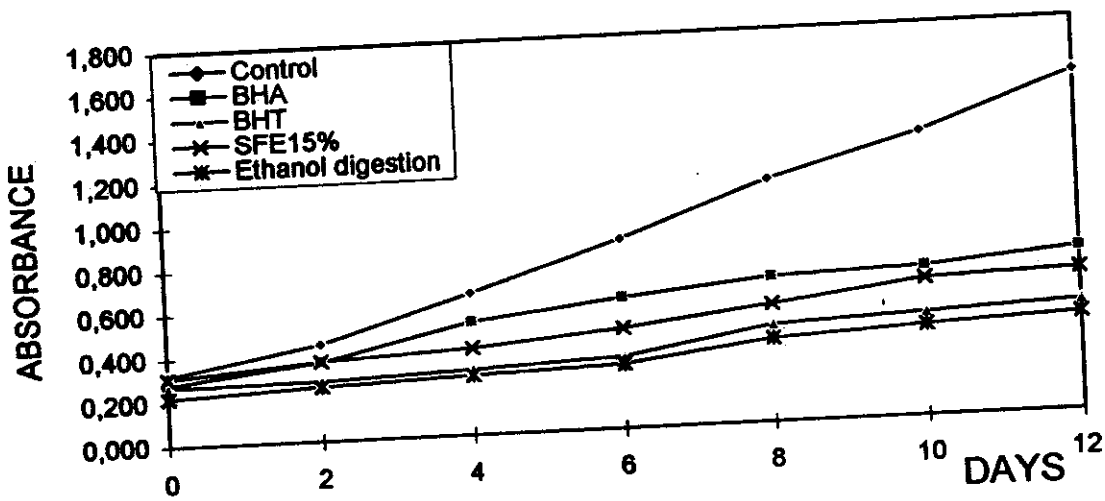


Fig (22): Antioxidant activities of ethanol digestion extract and SFE ex. (Modified with 15 % ethanol) of *E. camaldulensis var. brevirostri* s leaves

Table (18): Evaluation of antioxidant activities of the ethanol (digestion) extract and the SFE extract (modified with 15 % ethanol)

Days	Control	BHA	BHT	SFE 15%	Ethanol digestion
0	0,320 ± 0,020	0,280 ± 0,004	0,269 ± 0,001	0,310 ± 0,002	0,220 ± 0,002
2	0,445 ± 0,005	0,365 ± 0,004	0,277 ± 0,003	0,366 ± 0,003	0,252 ± 0,001
4	0,653 ± 0,002	0,523 ± 0,003	0,301 ± 0,001	0,400 ± 0,002	0,275 ± 0,002
6	0,873 ± 0,002	0,600 ± 0,004	0,328 ±0,002	0,460 ± 0,003	0,295 ± 0,001
8	1,115 ± 0,003	0,668 ± 0,002	0,444 ± 0,002	0,540 ± 0,003	0,380 ± 0,003
10	1,303 ± 0,001	0,688 ± 0,002	0,473 ± 0,002	0,630 ± 0,004	0,415 ± 0,001
12	1,555 ± 0,005	0,748 ± 0,003	0,503 ± 0,001	0,650 ± 0,001	0,445 ± 0,003

Reported values are the mean of two analysis; ± values are corresponding standard deviations

Table (19): Absorbance readings of the thiocyanate tests of ethanol (digestion) extract and mixture of ellagic acid (22.4 %) and gallic cid (4.6 %)

time (days)	control	BHA	BHT	Ellagic acid/ gallic acid	Ethanol digestion
0	0,340	0,276	0,270	0,300	0,222
	0,300	0,284	0,268	0,304	0,218
2	0,440	0,369	0,280	0,389	0,253
	0,450	0,361	0,274	0,385	0,251
4	0,650	0,525	0,300	0,393	0,273
	0,655	0,520	0,302	0,401	0,277
6	0,871	0,596	0,326	0,504	0,296
	0,875	0,604	0,330	0,496	0,294
8	1,118	0,670	0,442	0,560	0,383
	1,112	0,665	0,446	0,560	0,377
10	1,304	0,686	0,471	0,640	0,416
	1,302	0,690	0,475	0,644	0,414
12	1,560	0,751	0,504	0,734	0,448
	1,550	0,745	0,502	0,726	0,442

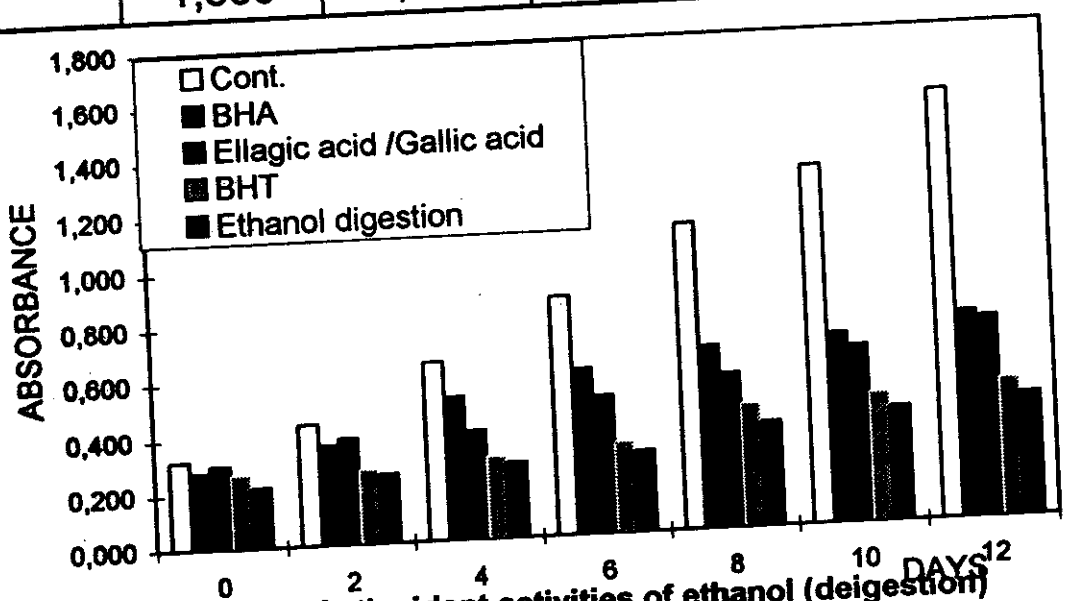


Fig (23): Antioxidant activities of ethanol (deigestion) extract and mixture of ellagic acid [22.4 %] and gallic acid [4.6 %] of *E. camaldulensis* var. *brevirostris* leaves

Table (20): Evaluation of antioxidant activities of ethanol (digestion) extract and mixture of [ellagic acid (22.4 %) and gallic acid (4.6 %)]

Days	Control	BHA	BHT	Ellagic /Gallic	Ethanol digestion
0	0,320 ± 0,020	0,280 ± 0,004	0,269 ± 0,001	0,302 ± 0,002	0,220 ± 0,002
2	0,445 ± 0,005	0,365 ± 0,004	0,277 ± 0,003	0,387 ± 0,002	0,252 ± 0,001
4	0,653 ± 0,002	0,523 ± 0,003	0,301 ± 0,001	0,397 ± 0,004	0,275 ± 0,002
6	0,873 ± 0,002	0,600 ± 0,004	0,328 ±0,002	0,500 ± 0,004	0,295 ± 0,001
8	1,115 ± 0,003	0,668 ± 0,002	0,444 ± 0,002	0,560 ± 0,000	0,380 ± 0,003
10	1,303 ± 0,001	0,688 ± 0,002	0,473 ± 0,002	0,642 ± 0,002	0,415 ± 0,001
12	1,555 ± 0,005	0,748 ± 0,003	0,503 ± 0,001	0,730 ± 0,004	0,445 ± 0,003

Reported values are the mean of two analysis; ± values are corresponding standard deviation

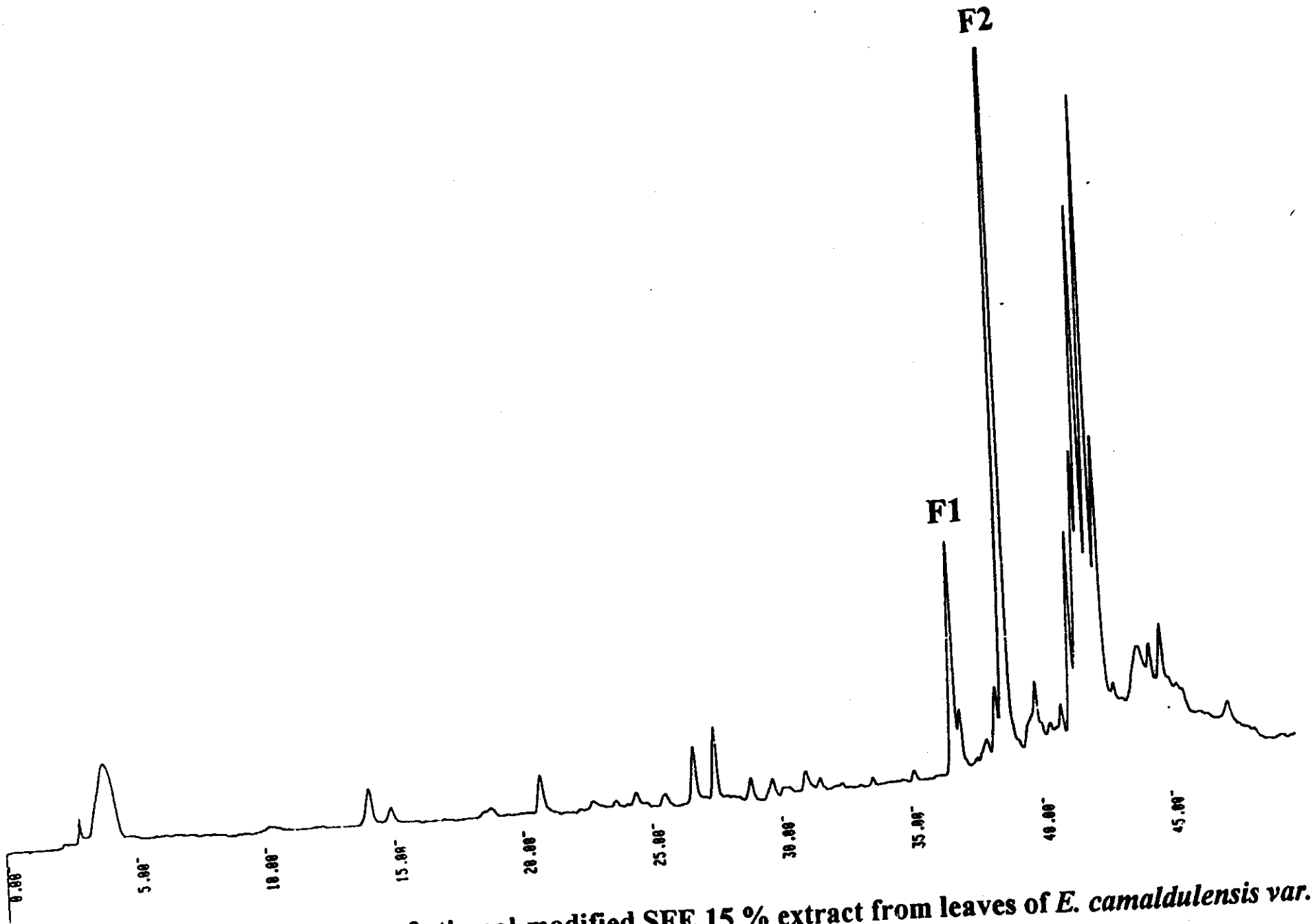


Fig (25): HPLC chromatogram of ethanol-modified SFE 15 % extract from leaves of *E. camaldulensis var. breviostris*; detection at 280 nm

Table (21): Absorbance reading of the thiocyanate tests of two main HPLC fractions (F1, F2) in SFE extract (modified with 15 % ethanol)

time(day)	control	BHA	BHT	F1	F2
0	0,340	0,276	0,272	0,267	0,270
	0,300	0,284	0,268	0,273	0,278
2	0,445	0,369	0,276	0,355	0,370
	0,441	0,361	0,274	0,345	0,374
4	0,660	0,522	0,298	0,510	0,530
	0,650	0,520	0,304	0,514	0,540
6	0,880	0,600	0,340	0,580	0,602
	0,870	0,604	0,330	0,584	0,604
8	1,120	0,670	0,460	0,659	0,660
	1,116	0,660	0,450	0,651	0,670
10	1,310	0,684	0,465	0,670	0,710
	1,312	0,696	0,475	0,672	0,716
12	1,558	0,751	0,510	0,740	0,750
	1,560	0,745	0,494	0,730	0,754

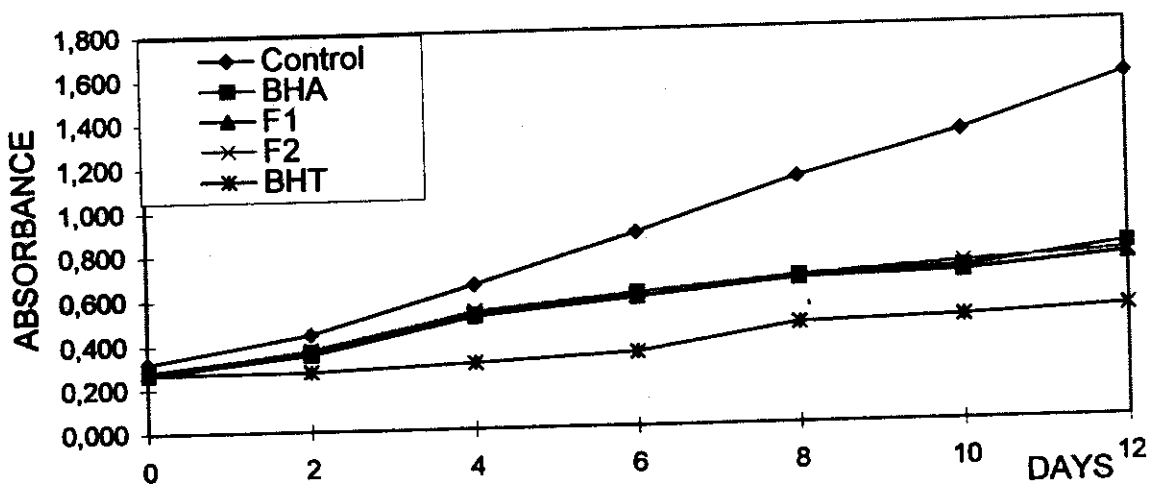


Fig (26): Antioxidant activities of HPLC fractions F1 and F2 in SFE (15 % modified with ethanol) extract of *E. camaldulensis* var. *brevirostris* leaves

Table (22): Evaluation of antioxidant activities of the HPLC fractions (F1 and F2) in the SFE extract (modified with 15 % ethanol)

)ays	Control	BHA	BHT	F1	F2
0	0,320 ± 0,020	0,280 ± 0,004	0,270 ± 0,002	0,270 ± 0,003	0,274 ± 0,004
2	0,443 ± 0,002	0,365 ± 0,004	0,275 ± 0,001	0,350 ± 0,005	0,372 ± 0,002
4	0,655 ± 0,005	0,521 ± 0,001	0,301 ± 0,003	0,512 ± 0,002	0,535 ± 0,005
6	0,875 ± 0,005	0,602 ± 0,002	0,335 ±0,005	0,582 ± 0,002	0,603 ± 0,002
8	1,118 ± 0,002	0,665 ± 0,005	0,455 ± 0,005	0,655 ± 0,004	0,665 ± 0,005
10	1,311 ± 0,001	0,690 ± 0,006	0,470 ± 0,005	0,671 ± 0,001	0,713 ± 0,003
12	1,559 ± 0,001	0,748 ± 0,003	0,502 ± 0,008	0,735 ± 0,005	0,752 ± 0,002

Reported values are the mean of two analysis; ± values are corresponding standard deviation

Table (23) : The inhibitory effect of the SFE extract (modified with 15 % ethanol), mixture of ellagic acid and gallic acid and ethanol (digestion) extracts on linoleic acid after 12 days

pounds	Inhibition(%)
BHA	61.7
Gallic acid & Ellagic acid	65.4
SFE extract (modified with 15 % ethanol)	72.5
BHT	81.4
Ethanol (digestion) extract	81.7

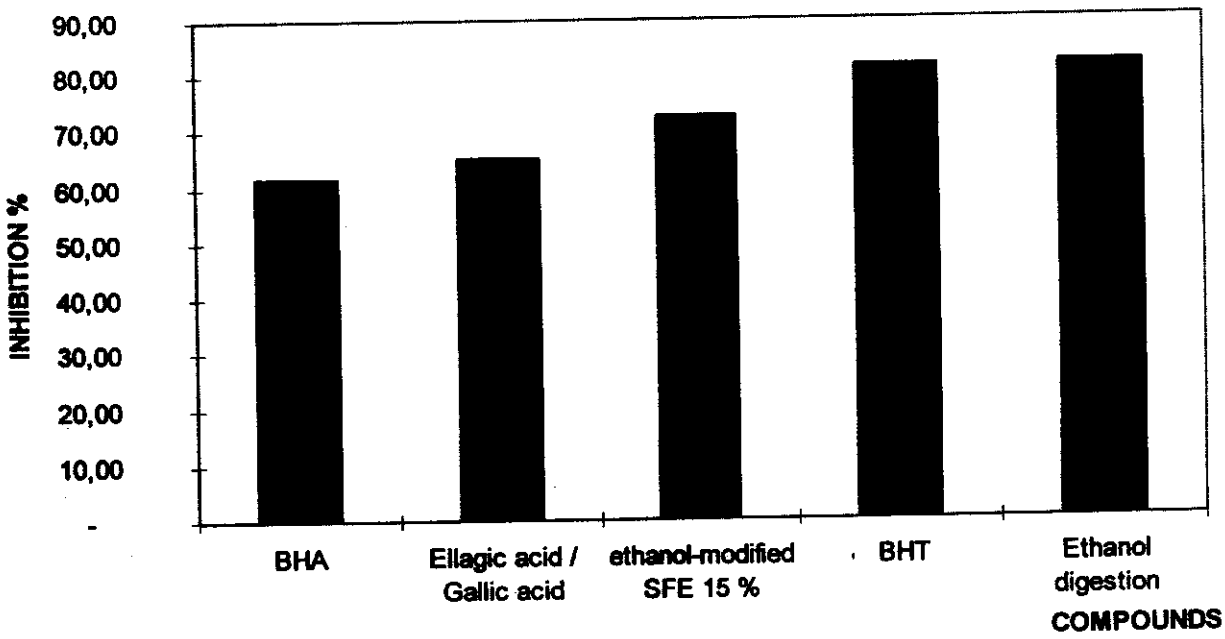


Fig (27): The inhibition effect of the SFE (15 % modified with ethanol) extract, ethanol (digestion) extract and mixture of gallic acid and ellagic acid

4.2.2 - Identification of the antioxidant components in the ethanol (digestion) extract of *E. camaldulensis var. brevirostris* leaves

The separation of antioxidant components by HPLC was carried out on a reversed phase C_{18}-column by gradient elution with water / acetic acid / methanol, eluting compounds were monitored with UV detector at the wave length λ = 280 nm. Mobile phase consisted of 3.5 % acetic acid in water and methanol (conditions see 3.5.1). Acetic acid was added in order to facilitate the separation, to eliminate peak tailing and to prevent the deprotonation of phenolic hydroxy groups **(78).** A typical HPLC chromatogram of an ethanol (digestion) extraction is presented in fig (28). Two main signals are observed at retention times approx. of 3.5 **(I)** and 19.5 **(II)** min.

The corresponding fractions **(I** and **II)** were collected by using semi-preparative HPLC. The components **(I** and **II)** were identified by using the following methods:

4.2.2.1 - Retention time comparison

Injection of standard compounds under identical HPLC conditions showed that gallic acid coeluts with compound **I** and ellagic acid with compound **II.**

4.2.2.2 - UV analysis

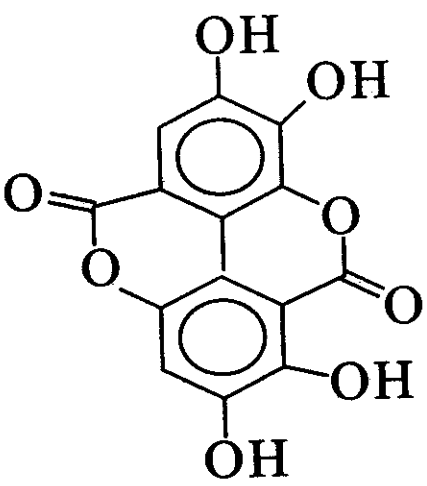
In order to confirm these tentative identifications UV spectra of the HPLC fractions **I** and **II,** were taken and compared with those of the authentic acids (see fig.29, 30).

The corresponding UV spectra for compound I and gallic acid are identical and have maximum absorbance at λ_{max} 268 nm, where those of fraction II and ellagic acid show absorbance maxima at 256 and 365 nm, thus the results of the retention time comparison is confirmed by the UV spectra.

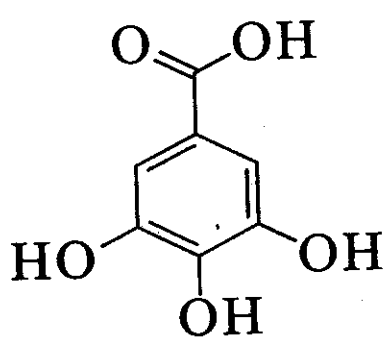
4.2.2.3 -GC-MS analysis of the TMS derivatives

TMS derivatives were prepared from the lyophilised HPLC fractions **I** and **II** and compared with those of gallic acid and ellagic acid by GC-MS analysis (fig. 31, 32). Fig. (31) shows that the TMS-derivatives of fraction (**I**) gives a mass spectrum with a molecular ion peak at m/z 458 and fragment peaks m/z 355, m/z 281, m/z 73 which equals to that of the TMS derivative of standard gallic acid. This indicates that fraction **I** consistes of gallic acid. Fig. (32) shows that the TMS-derivatives of fraction **II** gives a mass spectrum with a molecular ion peak at m/z 590 and fragment peaks m/z 281, m/z 73 which equals to that of the TMS derivative of standard ellagic acid. This indicates that the compound II is ellagic acid. These results confirm the results obtained by retention time comparison and UV spectra.

Based on the these results (retention time comparison, UV and GC-MS analysis of the main HPLC fractions **I** and **II**), it is clearly shown that these two compounds are gallic acid and ellagic acid, see figure (33).



Ellagic acid



Gallic acid

Fig 33: The structure formulas of gallic acid and ellagic acid

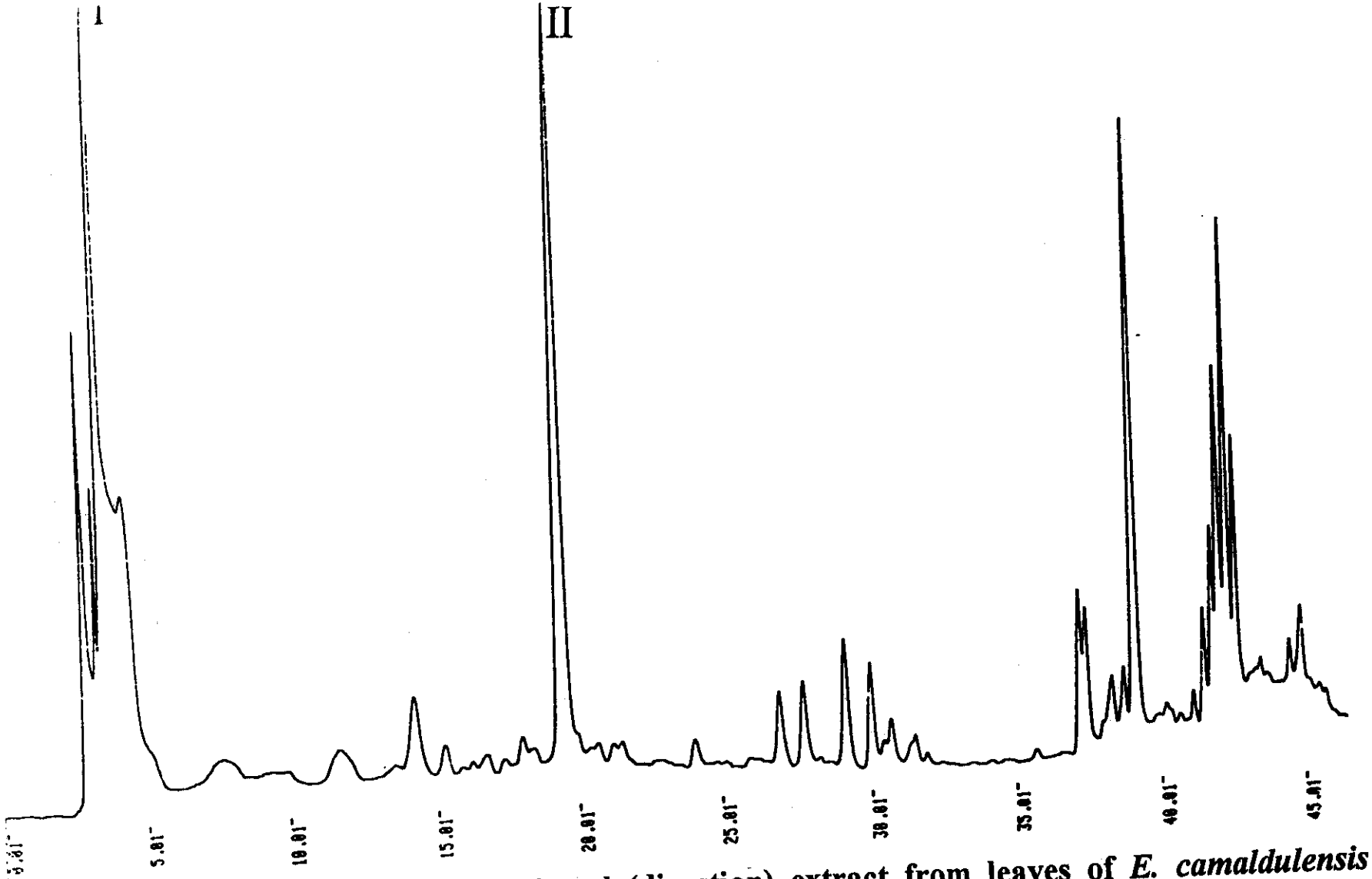


Fig (28): HPLC chromatogram of ethanol (digestion) extract from leaves of *E. camaldulensis var. breviostris*; detection at 280 nm

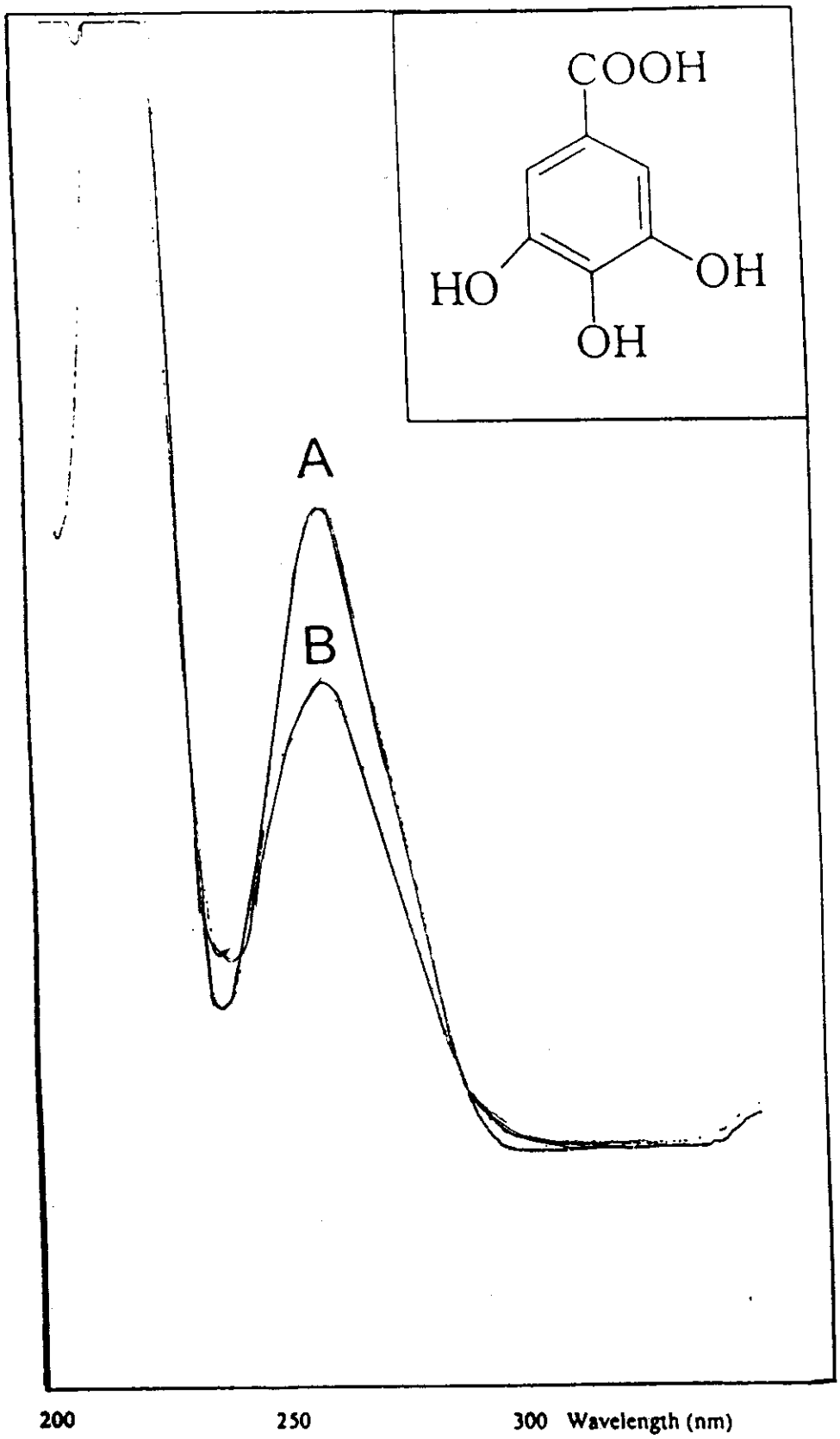


Fig (29): UV spectra of standard gallic acid (A) and of corresponding HPLC fraction (B)

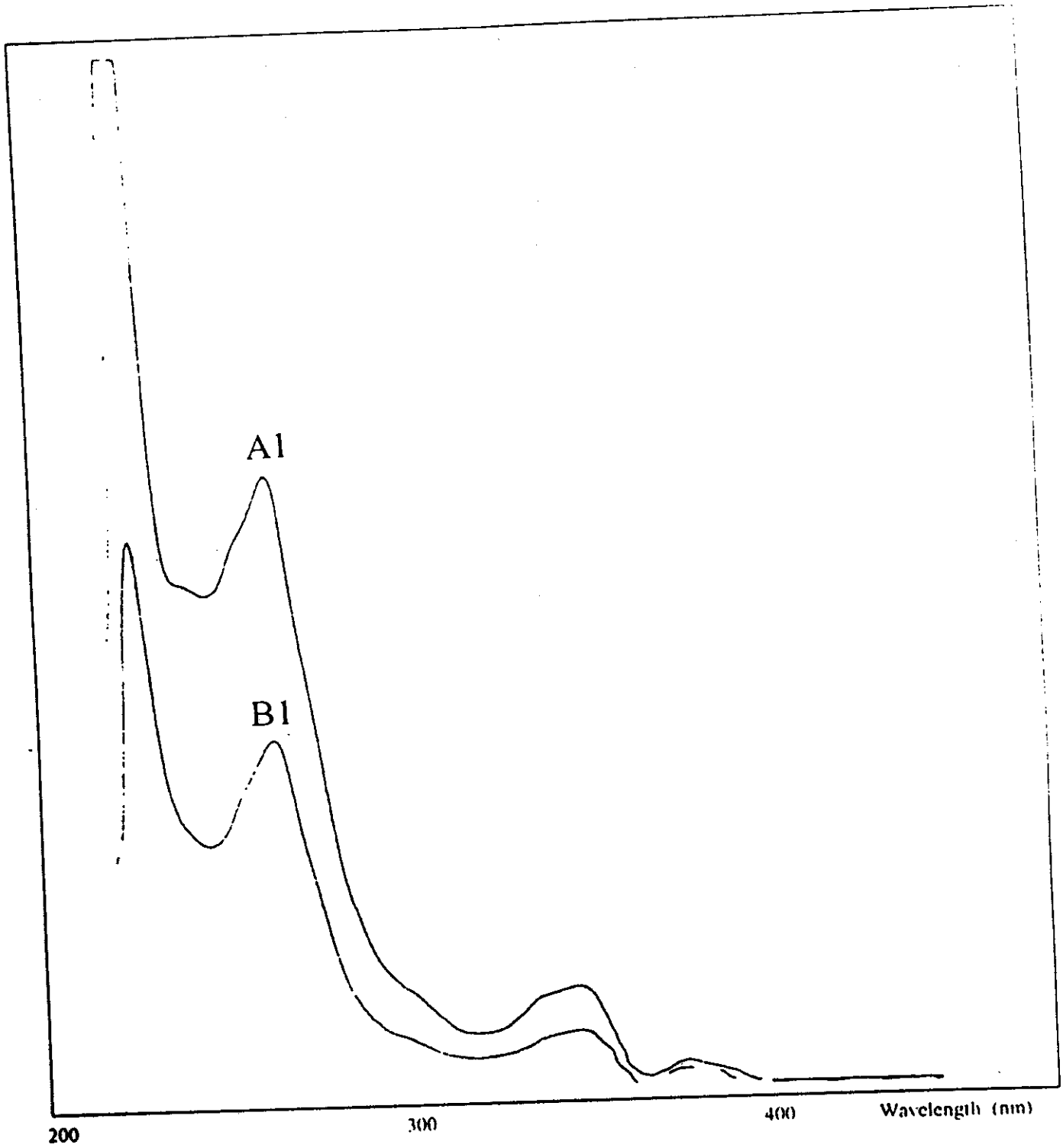


Fig (30): UV spectrum of standard ellagic acid (A1) and of corresponding HPLC fraction (B1)

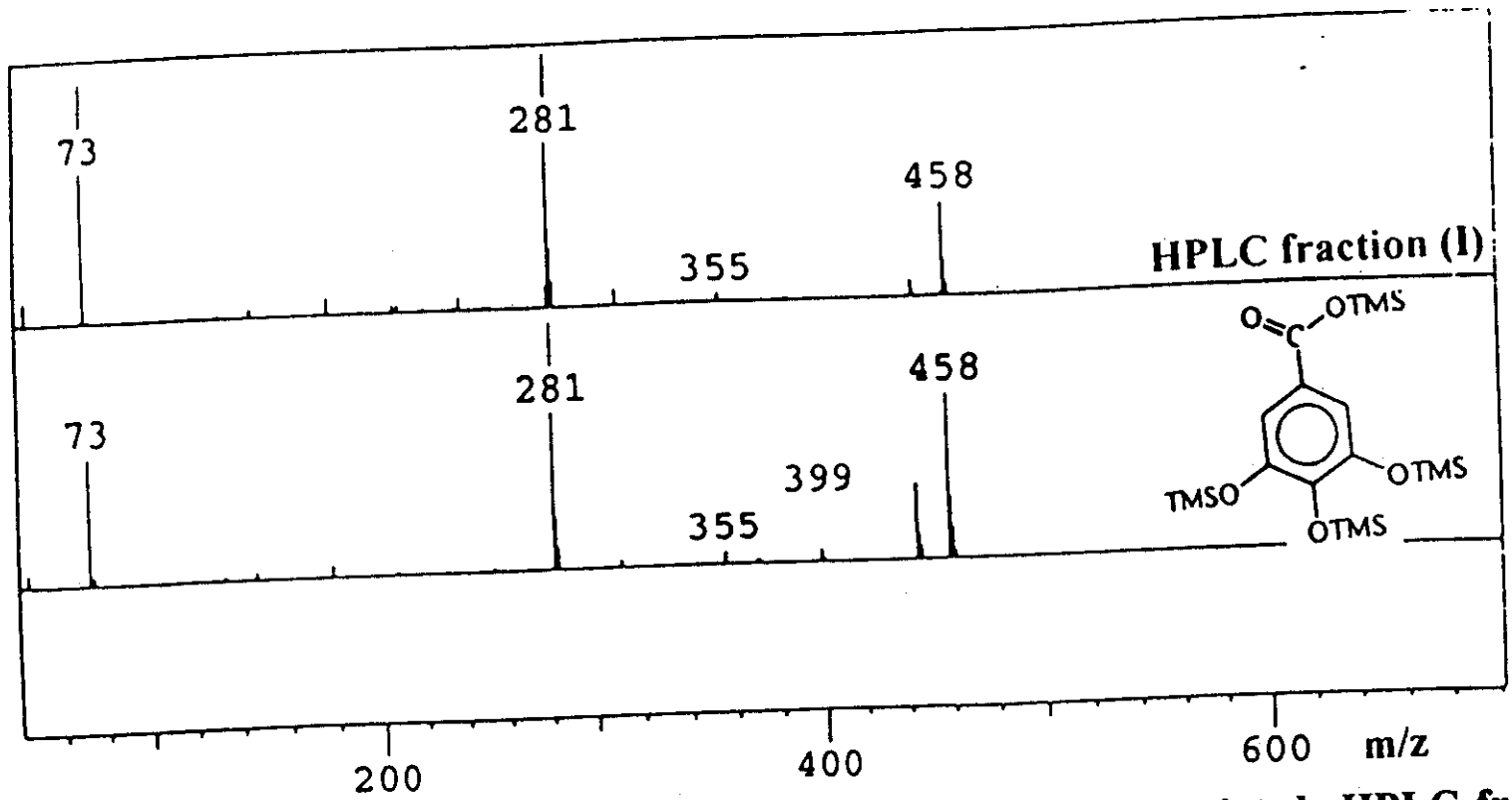


Fig (31): GC-MS spectra of the standard TMS gallic acid and trimethylsilylated HPLC fraction (I)

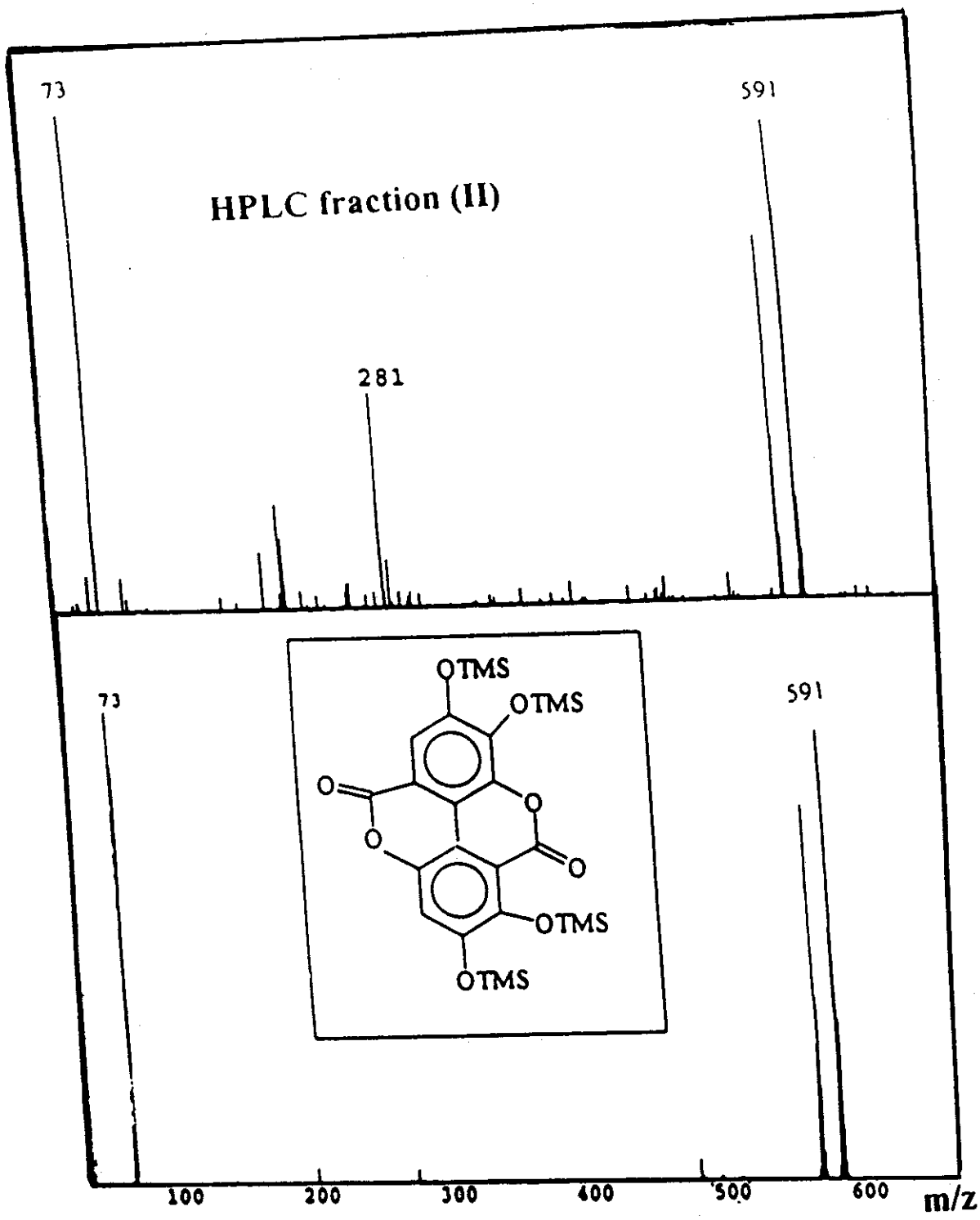


Fig (32): GC-MS spectra of standard TMS-ellagic acid and trimethylsilylated HPLC fraction (II)