SECTION E

STRUCTURAL CHARACTERISTAION OF AN ACTIVE PRINCIPLE ISOLATED FROM *CENTELLA ASIATICA*

Preliminary experiments with *Centella asiatica* has shown that the plant has anticancerous property, both in its crude from and partially purified form. Reports available also support our inferences.[156] So a detailed study was carried out to purify the active principle and to elucidate the structure of the same. We purified the compound and studied the antitumour effect. The compound was found to have excellent antitumour activity which has been established by studies mentioned in this section.

Experimental details

The compound from *Centella asiatica* was partially purified as mentioned in chapter 3 of Section C. In the above studies we found that acetone fraction from the methanol extract was found to have the most significant antitumour property. The acetone fraction was taken for further purification which was carried out with Column Chromatography using acetone:methanol as the solvent system, progressively increasing the polarity. The cytoxicity was studied in each fraction with varying polarity. The fraction eluted in acetone:methanol in the ratio 7:3 showed maximum cytotoxicity while all others showed only negligible cytotoxicity.

This cytotoxic fraction was then analysed for purity using Thin Layer Chromatography[224] (Silica gel-G, acetone:methanol 1:1 as the solvent system). Weighed quantities of silica gel G was made to a homogeneous suspension with distilled water. About 20-30 ml of this slurry was poured on to the TLC glass plate and to spread the slurry uniformly on the glass plate

by tilting the plate. Then it was dried and activated by placing it in hot air oven for 1 h. Sample was applied in a spot (≅ 5 mm in diameter) about one inch away from the side of the plate to be immersed in the solvent system. Spot was dried in between sample applications. About 50 µl of the sample was applied at a time. Spotted TLC plate was placed in the chamber previously saturated with the acetone-methanol (1:1) system. The Rf value was noted in each case.

A single fluorescent spot was observed under UV light in TLC studies using the same solvent system (Rf value 1.1). This fluorescent spot gave a coloured spot in iodine vapour and aluminium chloride reagent (under UV light). Experiments were repeated and the TLC purified fraction was subjected for a detailed study to establish the antitumour effect and structural studies using MS, NMR, R, etc.

Spectral studies using Mass Spectrometer (Hewlett Packard 1100 Series Electrospray Mass Spectrometer) was done in APCI (Atmospheric Pressure Chemical Ionisation) positive mode. IR spectrum was recorded on Jasco FTIR 410 and NMR spectrum on JEOL 300 in $CDCl_3$ (deuteriated chloroform in which the sample was dissolved) with TMS (Tetramethyl Silane) as internal standard. The above structural studies were carried out in the Molecular Biology Unit at the Indian Institute of Science, Bangalore.

Antitumour studies using DLA and EAC

The appropriate concentration of the fraction was detected by cytotoxic studies (refer chapter 2 of section C) and it was found to be 0.025 mg/ g body weight of mice as intraperitoneal injections, prepared in normal

saline (0.9%) on alternate days. The concentration of partially purified fraction was taken as same in Section C (0.075 mg/g body weight).

Lymphoma and Solid tumour were induced in mice using DLA and EAC cell lines as in Chapter 3 of Section C. Mice weighing 21± 1.5g were divided in to three groups consisting of 6 mice in each group.

Normal: Fed with normal lab feed.

Group I: Control (Diseased)- Each mice received intraperitoneal injections of 1X 10^5 lymphoma cells (DLA and EAC) for lymphoma and not treated . For solid tumour, each mice received subcutaneous injections of 5X10^4 lymphoma cells (DLA and EAC) and not treated.

Group II: Each mice received intraperitoneal injections of 1X 10^5 lymphoma cells (DLA and EAC) for lymphoma and treated with partially purified fraction. For solid tumour, each mice received subcutaneous injections of 5X10^4 lymphoma cells (DLA and EAC) and treated with partially purified fraction.

Group III: Each mice received intraperitoneal injections of 1X 10^5 lymphoma cells (DLA and EAC) for lymphoma and treated with purified fraction. For solid tumour, each mice received subcutaneous injections of 5X10^4 lymphoma cells (DLA and EAC) and treated with purified fraction

Body weight, Survival period and Life span were studied in all cases and observations are given below in Tables 1 to Table 7.

Table 1. **Changes in body weight of mice bearing Dalton's Lymphoma (DLA) and Ehrlich's Ascites Lymphoma (EAC)**

Groups	Body weight in grams ± SD	
	DLA	EAC
N	21 99 ± 0.18	20.61 ± 2.19
1	28 00 ± 0.32	28.77 ± 1.19
2	24 77 ± 5.11	23.71 ± 1.18
3	22.01 ± 7.1	22.78 ± 2.18

N – Normal, 1 – Control, 2 – Partially purified fraction, 3 – purified fraction.

⇒*Average of six values in each group ± SD.*

Table 2. **P values between the groups**

Groups	Body weight in grams ± SD	
	DLA	EAC
N X I	P< 0.005	P<0.005
I X II	P<0.005	P<0.005
I X III	P<0.001	P<0.001

Table 3. **Life span of mice bearing Dalton's lymphoma (DLA) and Ehrlich's Ascites Lymphoma (EAC).**

Groups	Mean life span (in days)		% increase in survival period	
	DLA	EAC	DLA	EAC
1	21.33± 2.61	20.31 ± 6.18	100	100
2	34.18 ± 2.11	38.16 ± 3.01	170.78	179.71
3	40.61 ± 2.18	43.71 ± 4.11	210.71	230.18

1 – Control, 2 – Partially purified fraction, 3 – purified fraction.

⇒*Average of six values in each group ± SD.*

Survival period and body weight of mice were brought to normal by treatment with partially purified and purified fraction. Life span was found to be increased in treated mice. But the effect was pronounced with purified form (21 to 40 days for DLA and 20 to 43 days for EAC). Body weight was found to increase in lymphoma bearing mice and there was a significant decrease in body weight in mice treated with the partially purified and purified fraction. The effect produced by purified fraction was found to be more significant (P<0.001) than partially purified fraction.

Table 4. Changes in body weight of mice bearing solid tumours induced by Dalton's lymphoma ascites (DLA)and Ehrlich's ascites cells (EAC)

Groups	Body weight in grams ± SD	
	DLA induced tumour	EAC induced tumour
N	19.71 ± 0.27	19.08 ± 0.78
1	24.05 ± 0.65	25.61 ± 0.88
2	21.78 ± 0.91	20.78 ± 2.11
3	18.61 ± 1.11	19.21± 1.18

N – Normal, 1 – Control, 2 – Partially purified fraction, 3 – purified fraction.

⇒*Average of six values in each group ± SD.*

Table 5. P values between the groups

Groups	Body weight in grams ± SD	
	DLA	EAC
N X I	P< 0.005	P<0.005
I X II	P<0.01	P<0.01
I X III	P<0.005	P<0.005

Table 3. **Life span of mice bearing solid tumours induced by Dalton's lymphoma cells (DLA) and Ehrlich's Ascites Lymphoma cells (EAC)**

Groups	Mean life span (in days)		% increase in survival period	
	DLA	EAC	DLA	EAC
1	18.61 ± 0.18	21.11 ± 2.18	100	100
2	32.17 ± 1.19	34.60 ± 4.18	176.18	169.28
3	37.22 ± 9.1	39.19 ±6.18	200.31	190.61

1 – Control, 2 – Partially purified fraction, 3 – purified fraction.

Table 7. **Mean change in tumour size (cm) in mice bearing solid tumour induced by Dalton's lymphoma ascites (DLA) and Ehrlich's ascites lymphoma cells (EAC)**

Groups	DLA induced tumour *	EAC induced tumour*
1	5.92	6.18
2	2.01	2.56
3	1.01	1.5

1 – Control, 2 – Partially purified fraction, 3 – purified fraction.

* Diameter of the tumour in cm.

⇒*Average of six values in each group ± SD*

There was an increase in body weight in tumour bearing mice. Body weight was found to be decreased by treatment with partially purified and purified fractions (Tables 4&5). Purified fraction gave a significant decrease ($P<0.005$) when compared to partially purified fraction. Life span increased up to 200.31% when treated with purified fraction and partially purified fraction increased the life span only up to 176.16%.(Table 6). Tumour size was decreased significantly from 5.92cm to 1.01cm in DLA induced tumour and 6.18cm to 1.5cm in EAC nduced tumour when treated with purified fraction.

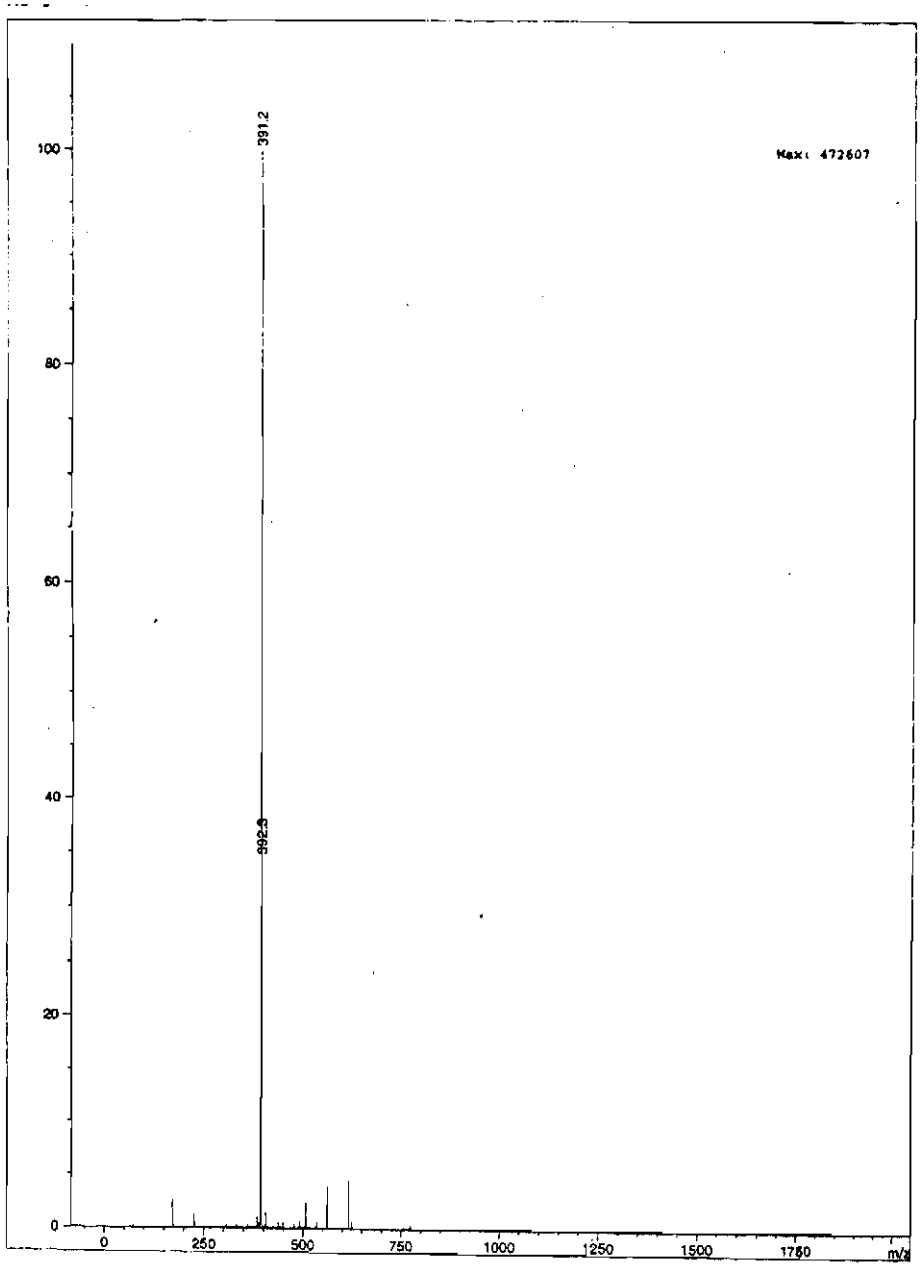

991.2
992.3
Max: 472607
100
80
60
40
20
0
0
250
500
750
1000
1250
1500
1750
m/z

Figure 1

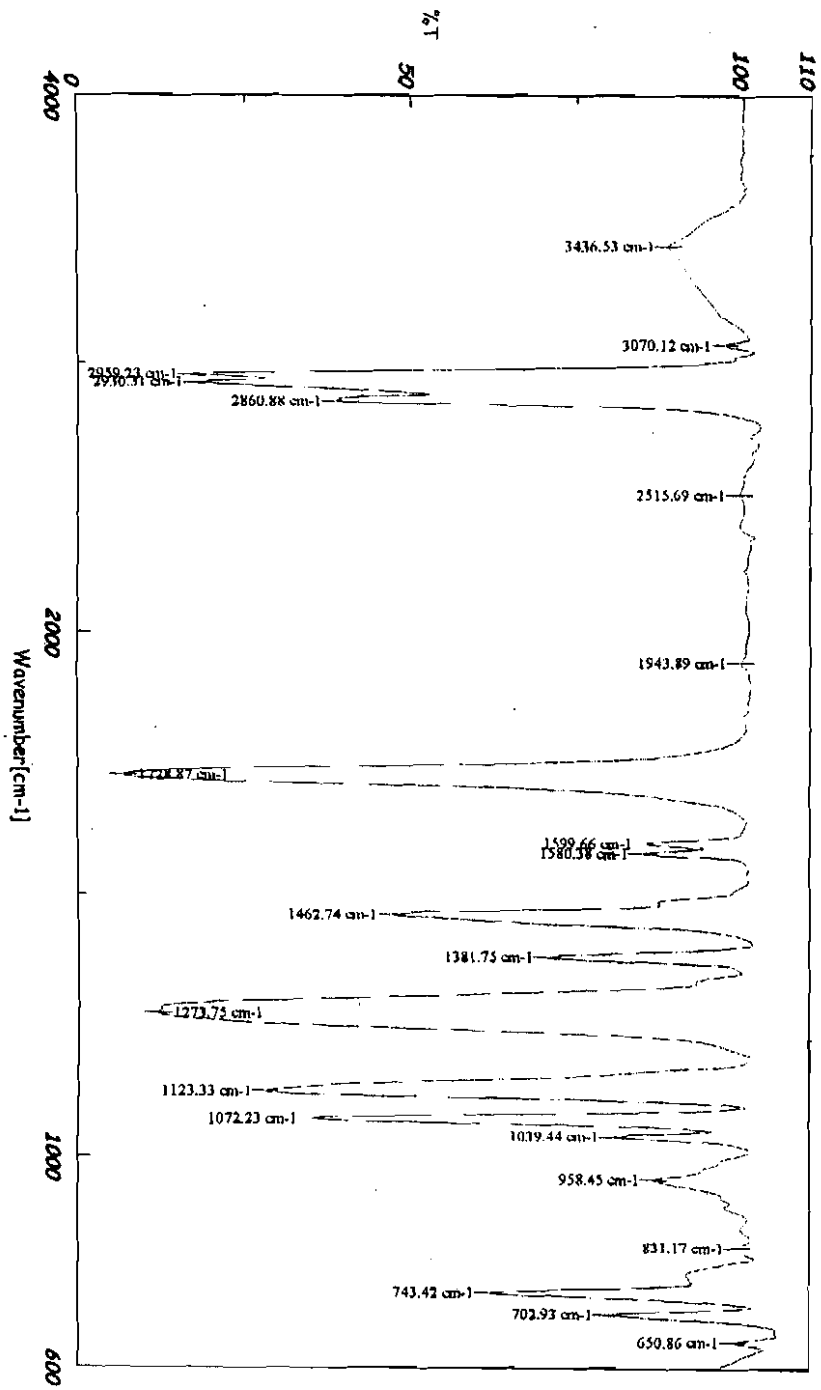
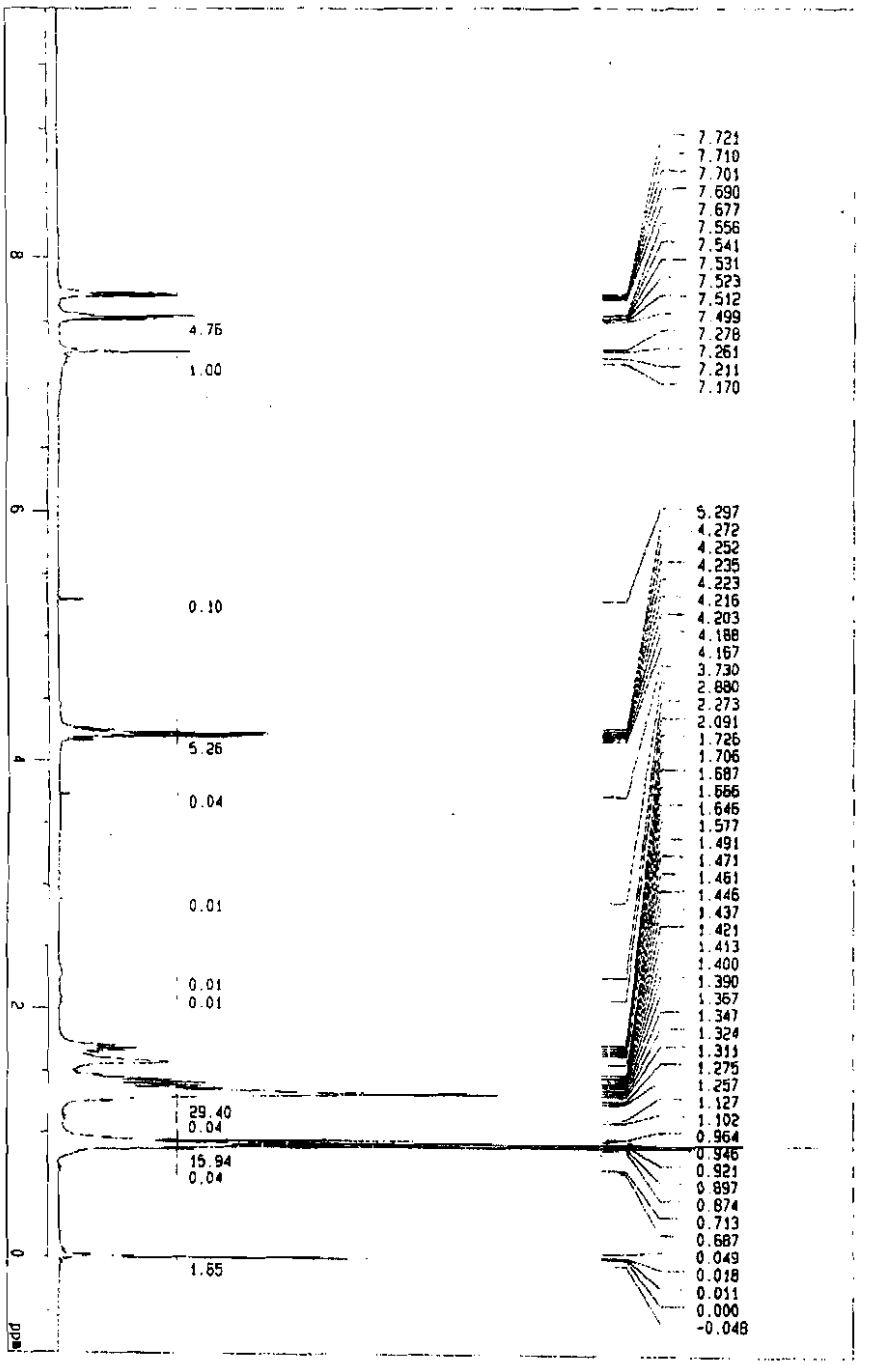

%T
0
50
100
110
4000
3000
2000
1500
1000
600
Wavenumber [cm-1]
3436.53 cm-1
3070.12 cm-1
2959.23 cm-1
2910.31 cm-1
2860.88 cm-1
2515.69 cm-1
1943.89 cm-1
1599.66 cm-1
1580.38 cm-1
1462.74 cm-1
1381.75 cm-1
1273.75 cm-1
1123.33 cm-1
1072.23 cm-1
1019.44 cm-1
958.45 cm-1
831.17 cm-1
743.42 cm-1
702.93 cm-1
650.86 cm-1

Figure 2


7.721
7.710
7.701
7.690
7.677
7.556
7.541
7.531
7.523
7.512
7.499
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7.261
7.211
7.170
5.297
4.272
4.252
4.235
4.223
4.216
4.203
4.188
4.167
3.730
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2.091
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1.706
1.687
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1.471
1.461
1.446
1.437
1.421
1.413
1.400
1.390
1.367
1.347
1.324
1.311
1.275
1.257
1.127
1.102
0.964
0.946
0.921
0.897
0.874
0.713
0.687
0.049
0.018
0.011
0.000
-0.048
4.76
1.00
0.10
5.26
0.04
0.01
0.01
0.01
29.40
0.04
15.94
0.04
1.65
8
6
4
2
0
ppm

Figure 3

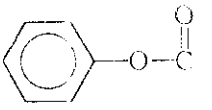
Inferences of spectral studies

Mass spectrum (Figure 1) showed a single peak at 391.2 which indicated that the sample was extremely pure as there was no more additional peaks. The value 392.3 in the spectra is the mass of the isotope that is quite common in ES-MS spectrum.

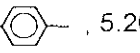
From the IR spectrum (Figure 2), the following details were deduced. The values and their corresponding functional group relations are given below.

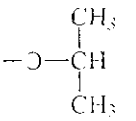
3436.53 cm^{-1}	-OH
3070.12	Aromatic –CH protons
2959.23	Alkyl –CH protons (band stretching frequency)
2930.31	
2860.88	
1728.87	Carbonyl ester group
1599.66	Aromatic region
1580.38	
1381.75	CH-CH stretching frequency
1123.37 to 650.86	Fingerprint region

The spectrum at 2959.23, 2930.31 and 2860.88 shows the presence of an alkyl group. Expected structure representing this region may be $-CH_3$ group. Presence of -OH (3436.53) and aromatic –CH protons (3070.12)



Region at 1599.66 in the spectrum corresponds to an aromatic region and 1381.75 might be due to –CH-CH stretching frequency. 1123.37 to 650.86 has no significance as this corresponds to fingerprint region.

H^1NMR *spectrum (Figure 3) explained some more details* about the structure. The region 4.76 showed the presence of para substituted benzene,  , 5.26 showed the presence of



and 15.94 showed the presence of CH_3's doublet. Region 1.00 represents the $CDCl_3$ peak and 1.65 represents TMS peak.

From the above data and comparisons with reference of National Cancer Institute (NCI), USA and American Chemical Society's subsidiary called Chemical Abstract Sources (CAS) database, it was seen that no compound with this above characters are mentioned as a product from *Centella asiatica* and this is perhaps the first report of the compound. Unfortunately the above spectral data is insufficient to identify and to assign a definite structure of the compound. As this compound has been effective in anticancerous studies we hope that this will make an impact on the treatment for a cure of cancer.