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Isolation, characterization of bio-active molecules and its antineoplastic and antioxidant activities of *Justicia beddomei* leaves



By

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Bapuji Pharmacy College-Rajiv Gandhi University of health Sciences

INDIA



INTRODUCTION

- **Present work has been divided in to two parts**

Part – A: Devoted to study the medicinal plant

CHAPTER - 1	Introduction
CHAPTER-2	Survey of Medicinal Plants with special reference to antioxidant and anticancer.
CHAPTER-3	Phytochemical investigation
CHAPTER-4	Isolation and characterization of compounds from *Justicia bedommei*.

Part – B: Devoted to study of Antioxidant and Antineoplastic activities of Crude extract and Isolated molecules of JB.

CHAPTER -5	Antioxidant activity and Antineoplastic activities of crude extracts and isolated molecules

CHAPTER-1

- Growth of the world population
- India is second largest populated country in the world
- There is a great need for planning to control the hazards
- Need of research to control/ prevention cause of death by cancer

American Cancer Society
Estimated New Cases for the Four Major Cancers by Sex and Age Group, 2015

	All ages	Younger than 45	45 and Older	Younger than 65	65 and Older
All sites, men	848,200	52,580	795,620	370,130	478,070
All sites, women	810,170	86,520	723,650	392,100	418,070
Colon & rectum, men	69,090	3,590	65,500	29,420	39,670
Colon & rectum, women	63,610	3,260	60,350	22,800	40,810
Lung & bronchus, men	115,610	1,590	114,020	38,000	77,610
Lung & bronchus, women	105,590	1,850	103,740	33,500	72,090
Breast, women	231,840	24,630	207,210	132,250	99,590
Prostate	220,800	1,330	219,470	94,660	126,140

Projected cases are based on incidence data during 1995-2011 from 49 states and the District of Columbia, as reported by the North American Association of Central Cancer Registries (NAACCR).
Note: Estimates should not be compared with those from previous years.

American Cancer Society, Surveillance Research, 2015

CHAPTER-2

PLANT PROFILE	
Name of the Plant	Justicia beddomei
Family	Acanthaceae
Distribution	All parts of Karnataka, Kerala
Chemical constituents	Isolated pure compounds are not reported so for
Biological activities	Anti-inflammatory, antispasmodic, antibacterial, diuretic, anthelmintic activity and leaves are good for irritable cough and for bleeding in the diarrhoea



Justicia beddomei

CHAPTER-3

Phytochemical investigation

- Plant authentication
- Hot Soxhlet extraction
- Detection of secondary metabolites from aerial parts of JB.

Table : Summary of yield, colour and consistency of different extracts

Sl. No.	Extract	Yield (for 800 g)	Colour	Consistency
1	Petroleum ether	50.00	Yellow	Oily
2	Chloroform	40.00	Dark brown	Oily viscous liquid
3	Ethanolic	35.00	Brownish yellow	Powder
4	Aqueous	25.00	Dark brown	Powder

Table : Summary of phytochemical investigations

SL No	Extract	Constituent
1	Petroleum ether	Steroids, saponin glycosides, fixed oils and fats
2	Chloroform	Alkaloids, steroids, saponin glycosides and coumarins
3	Ethanolic	Alkaloids, **Triterpenoids**
4	Aqueous	carbohydrates, proteins, gums and mucilages

Isolation and characterization of compounds in leaves of *Justicia beddomei*

- Chromatographic separation using silica gel (100-200 mesh)

- Chromatographic separation using silica gel (60-120 mesh)

Column chromatography of the ethanolic fraction over silica gel (100-200) mesh, each fraction 100 ml)zz

Number of fractions	Eluent	Residue on evaporation	Yield in mg
1-6	Petroleum ether	No residue	-
	Petroleum ether: Chloroform		
7-12	90 :10	No residue	-
13-18	80:20	No residue	-
19-24	70:30	No residue	-
25-30	60:40	No residue	-
31-36	50:50	No residue	-
37-42	40:60	No residue	-
43-48	30:70	No residue	-
49-54	20:80	No residue	-
55-60	10:90	No residue	-
61-66	Chloroform	No residue	-
	Chloroform: Ethyl acetate	No residue	
67-72	90 :10	No residue	-
73-78	80:20	No residue	-
79-84	70:30	No residue	-
85-90	60:40	No residue	-
91-96	50:50	No residue	-
97-102	40:60	No residue	-
103-108	30:70	No residue	-
109-114	20:80	No residue	-
115-120	10:90	No residue	-
121-126	Ethyl acetate	No residue	-
	Ethyl acetate : Methanol	No residue	
127-132	90 :10	No residue	-
133-138	80:20	No residue	-
139-144	70:30	No residue	-
145-150	60:40	No residue	
151-156	50:50	No residue	-
157-162	40:60	Brown crystalline solid **A1**	1000
163-168	30:70	Shiny brown crystalline solid **A2**	1250
169-174	20:80	Brown crystalline solid **A3**	530

Purification of JBF1 (60-120 mesh)

Number of fractions	Eluent	Residue on evaporation	Yield in mg
	Ethyl acetate : Methanol	Light brown solid	Negligible
1-6	90 :10	Light brown solid	Negligible
7-12	80:20	Light brown solid	Negligible
13-18	70:30	Light brown solid	Negligible
19-24	60:40	Light brown crystalline solid	400
25-30	50:50	No residue	---
31-36	40:60	No residue	---
37-42	30:70	No residue	---
43-48	20:80	No residue	---
49-54	10:90	No residue	---

Purification of **JBF2** (60-120 mesh)

Number of fractions	Eluent	Residue on evaporation	Yield in mg
	Ethyl acetate : Methanol	Light brown solid	Negligible
1-6	90 :10	Light brown solid	Negligible
7-12	80:20	Light brown solid	Negligible
13-18	70:30	Light brown solid	Negligible
19-24	60:40	Light brown solid	Negligible
25-30	50:50	Pale brown crystalline solid	200

Characterization of pure compound

- **Melting point**
- **Ultraviolet spectrum**
- **Infrared spectrum**
- **Nuclear magnetic resonance spectrum: 1H NMR and 13C NMR**
- **Mass spectrum**

Spectral properties of compound JBF1

Ultraviolet spectrum (fig 6.1a):

1. UV *λmax* (MeOH): 400.50 nm
2. UV *λmax* (MeOH): 222.00 nm

Characterization of pure compound

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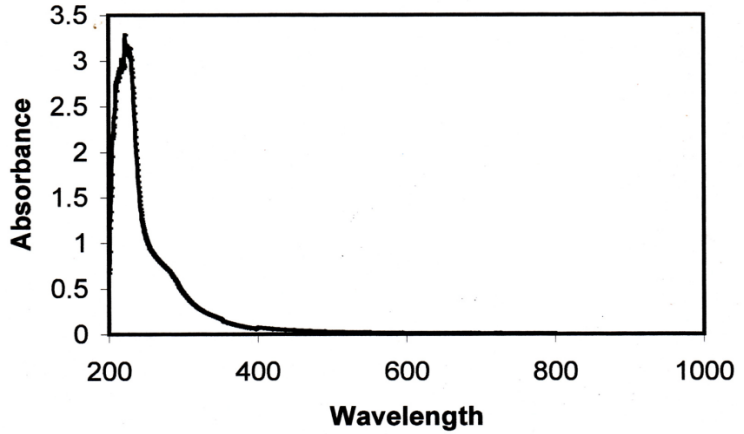


Fig. 6.1a

A1

No.	Wavelength (nm.)	Abs.
1	400.50	0.0779
2	222.00	3.2856

Infrared spectrum (JBF1)

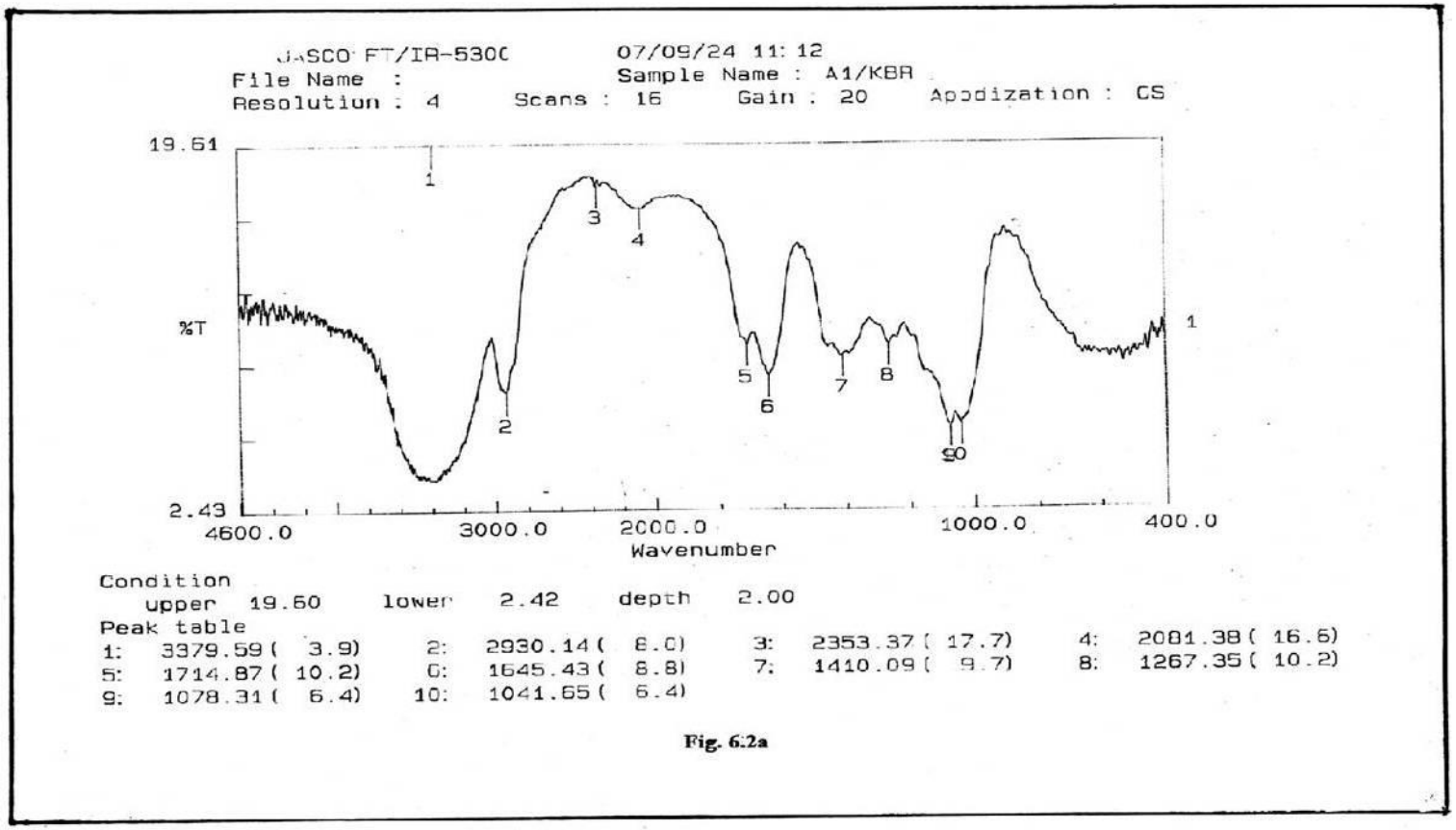


Fig. 6.2a

The IR spectrum shows the characteristic bands for the presence of **hydroxyl group (-OH) at 3379 cm-1** and **unsaturated-C=C-groups at 2081 cm-1** and **C-H stretching at 2930 cm-1,carbonyl (C=O) at 1714 cm-1**.

Proton magnetic resonance spectrum (JBFI)

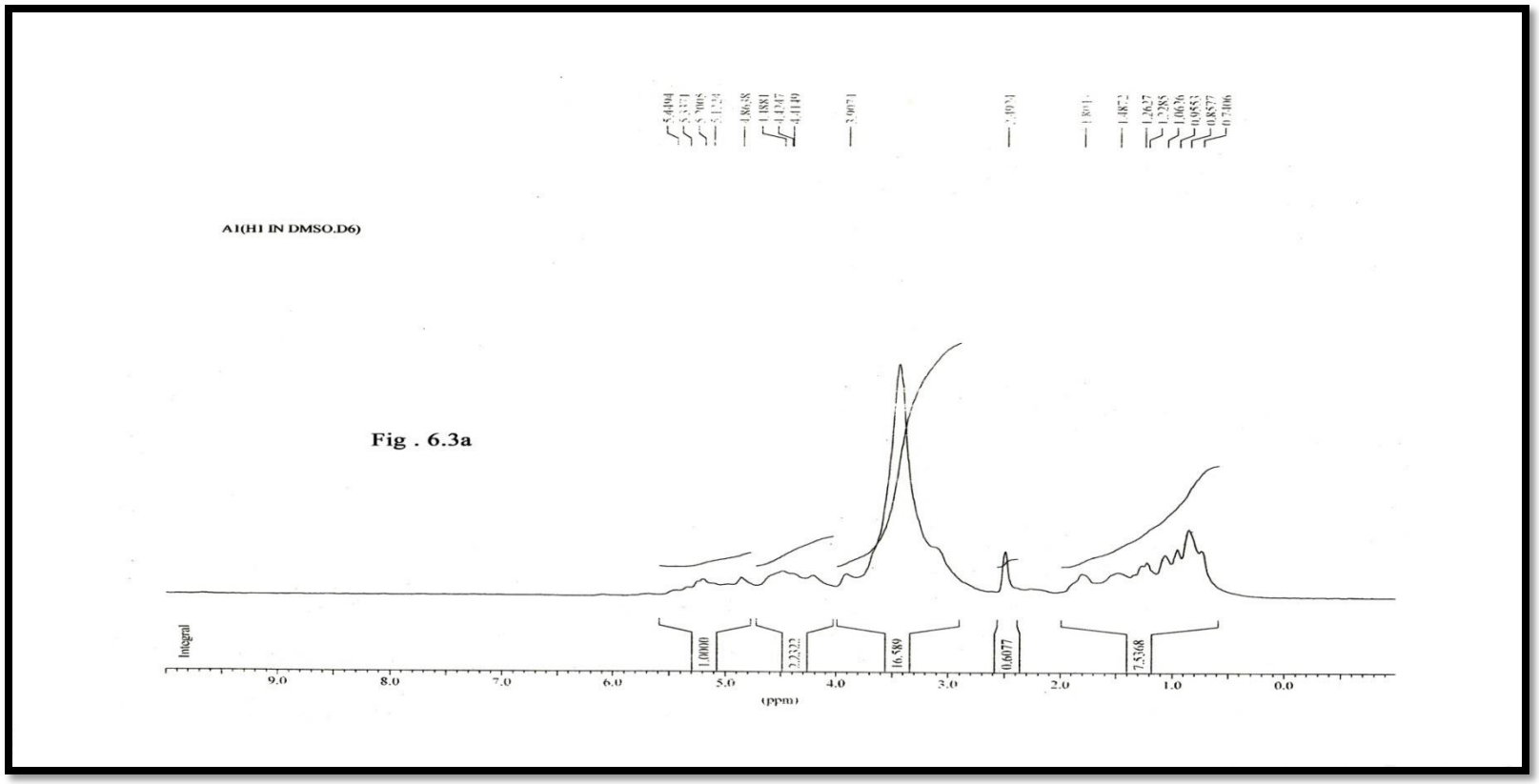
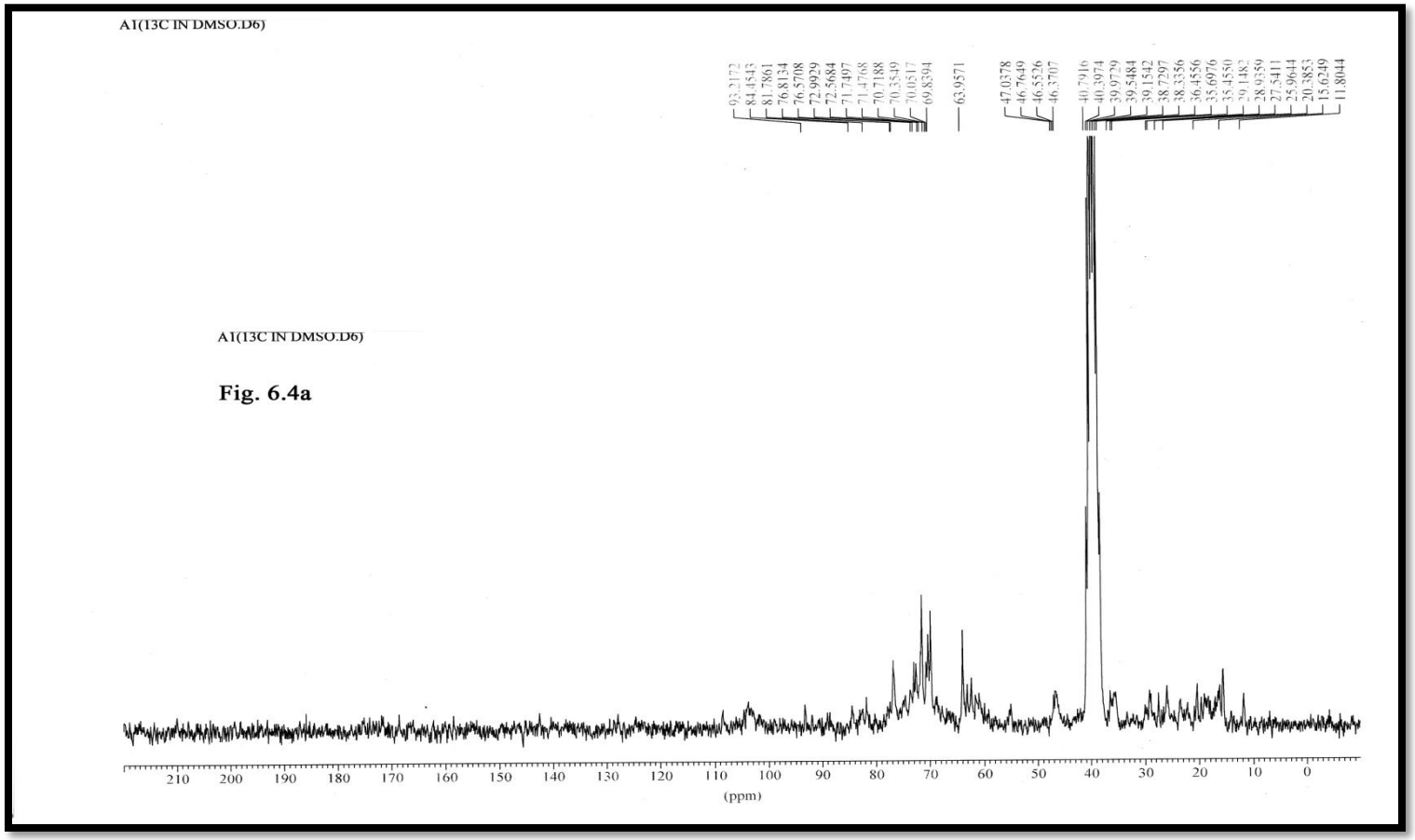


Fig . 6.3a

- Three anomeric protons at δ 5.49, 5.20 and 4.86
- The bunch of signals between δ 3.50 to 3.90 indicating the presence of glucosidic protons
- The triplet at δ 4.42 is to an unsaturated proton
- Other signals at δ 1.80, 1.48, 1.26, 1.22, and 1.06 were due to the methylene and methine protons,
- Signals at δ 0.95, 0.8 and 0.74 were due to the presence of three methyl groups.

^{13}C NMR spectrum (JBF1)



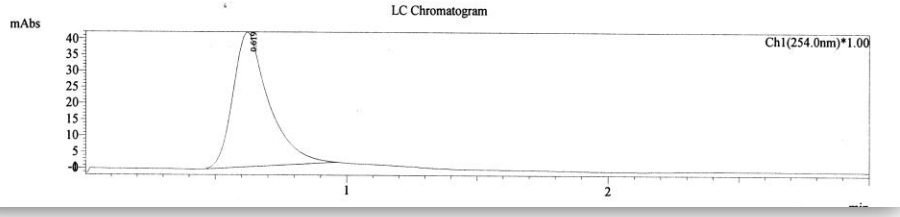
- In the 13C NMR spectrum the signal at δ 170.00 is due to a carbonyl carbon probably a lactone ring
- δ 126.00 and 142.00 due to a carbon-carbon double bond
- δ 108.46, 103.76 and 93.21 due to three anomeric carbon atoms supports the assignments made in the 1H NMR .

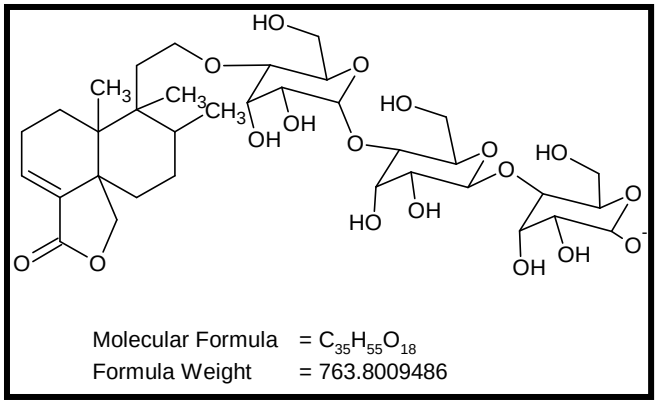
Mass spectrum (JBFI)

Compound JBFI

LCMS-2010A DATA REPORT
SHIMADZU

User	: Admin
Sample	: A1
Inj. Volume	: 5.000
Data Name	: C:\LCMSsolution\User\Data\A1-ESI-NEG1.qld
Method Name	: C:\LCMSsolution\User\Method\JAY-4-APCI.qlm





Molecular Formula = $C_{35}H_{55}O_{18}$
Formula Weight = 763.8009486

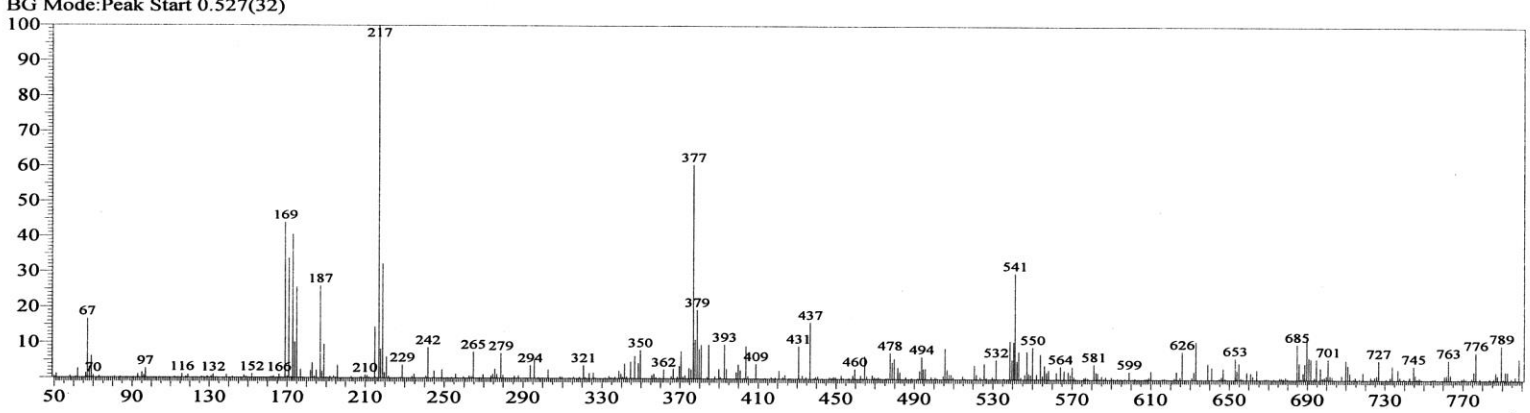
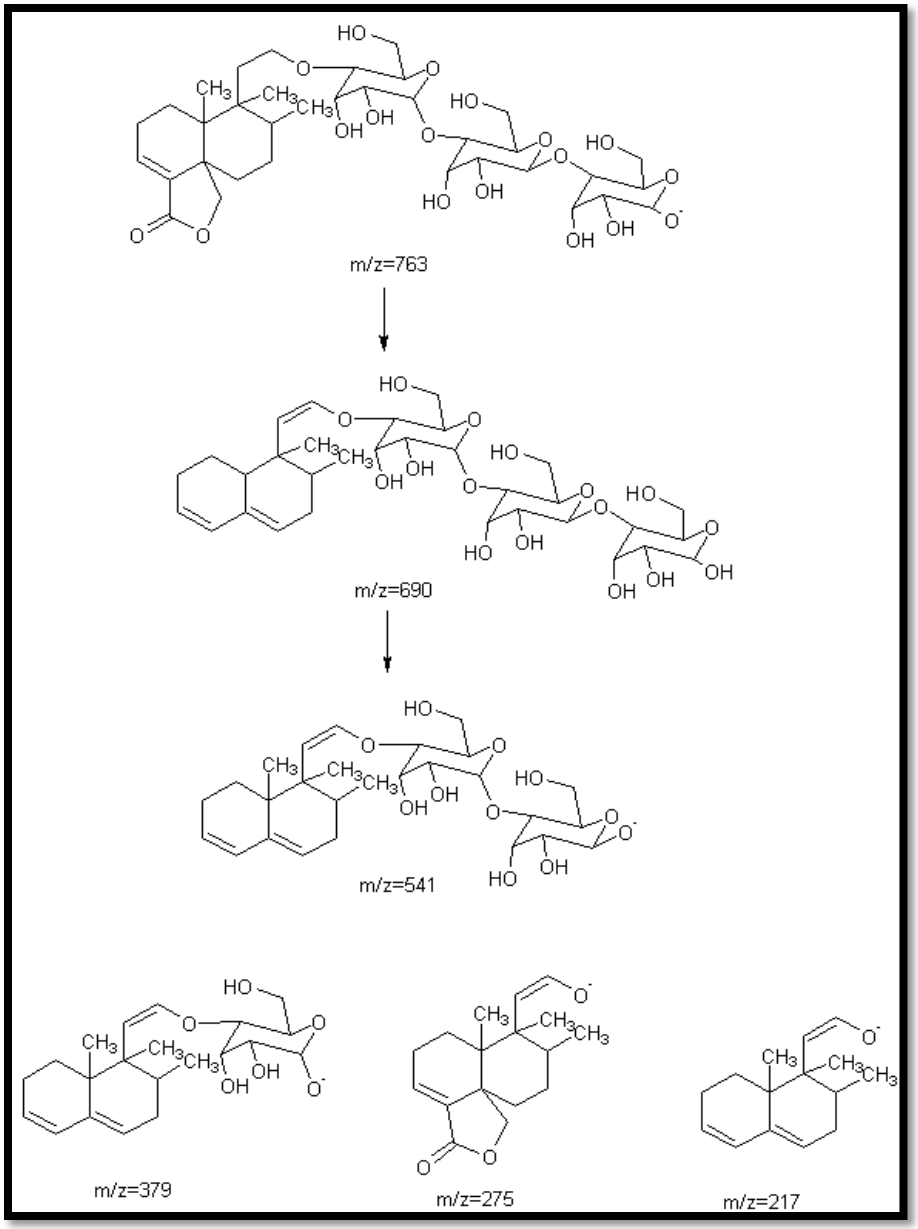


Fig. 6.5a

Mass spectral Fragmentation (JBFi)



Spectral properties of compound JBF2

Ultraviolet spectrum (fig 6.1b):

1. UV λmax (MeOH): 400.50 nm
2. UV λmax (MeOH): 230.00 nm

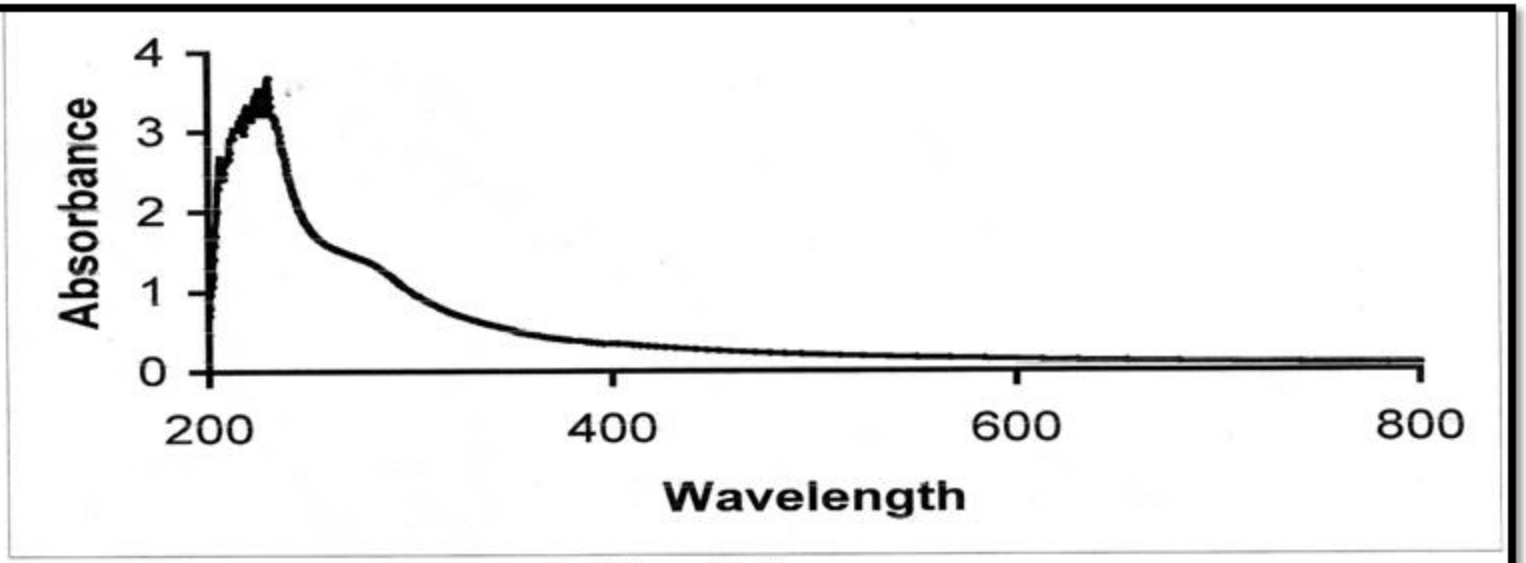
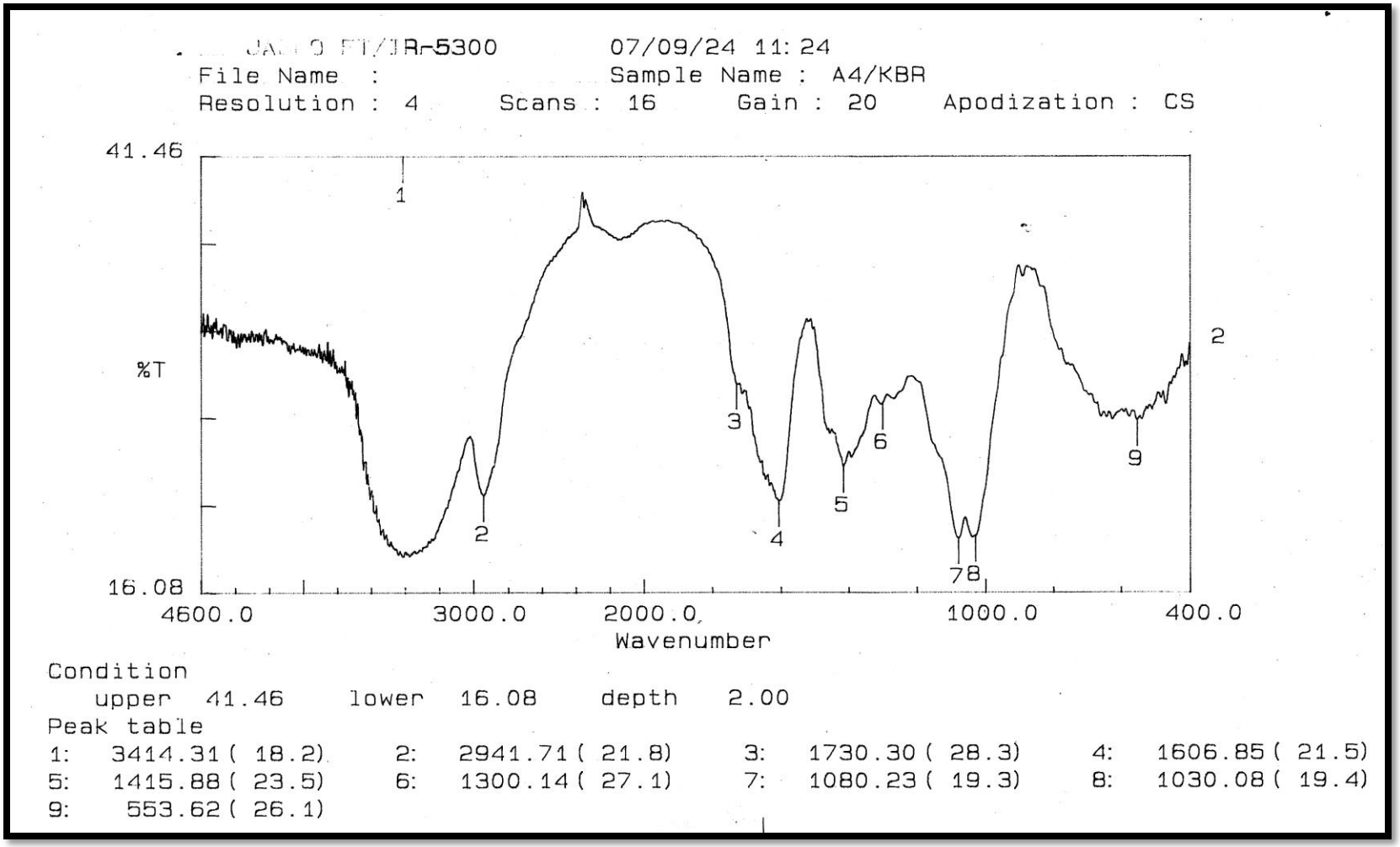


Fig. 6.1b

JBF2

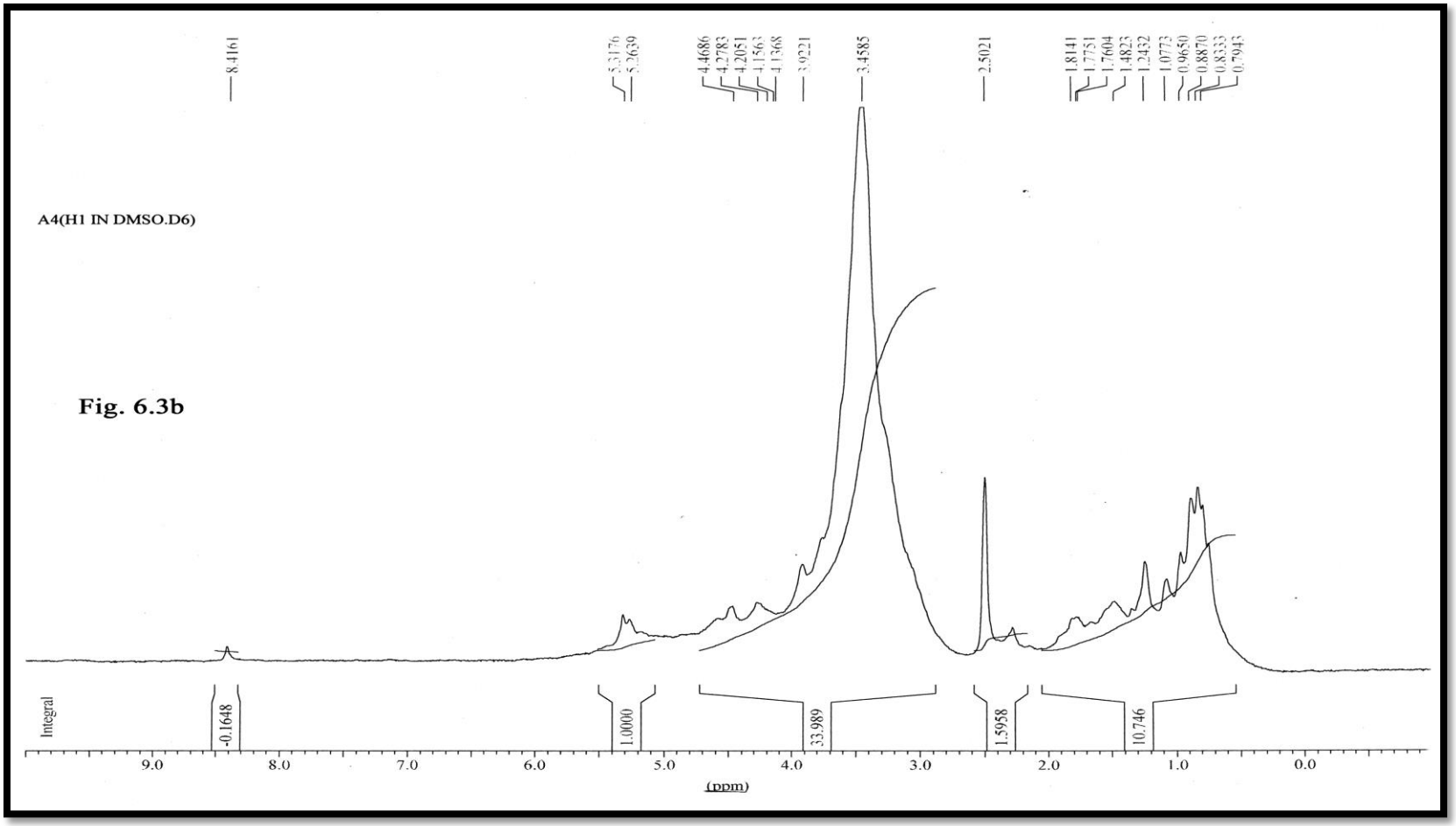
No.	Wavelength (nm.)	Abs.
1	400.50	0.3477
2	230.00	3.6747

Infrared spectrum (JBF2)



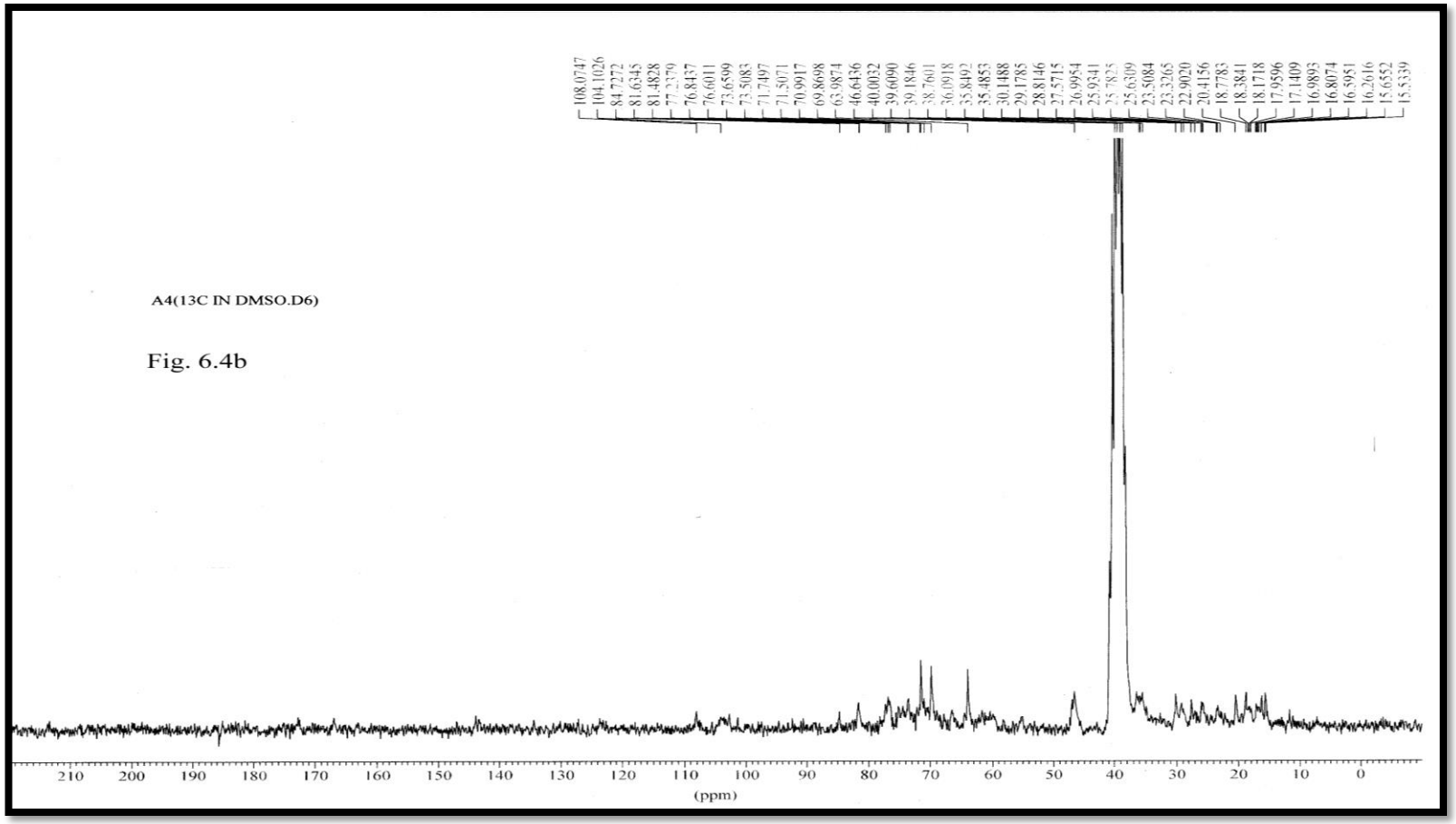
- **The presence of hydroxyl group (-OH) at 3414 cm-1**
- **Unsaturated (C=C) groups at 1606 cm-1**
- **Isopropyl group at 1300 cm-1.**

Proton magnetic resonance spectrum (JBF2)



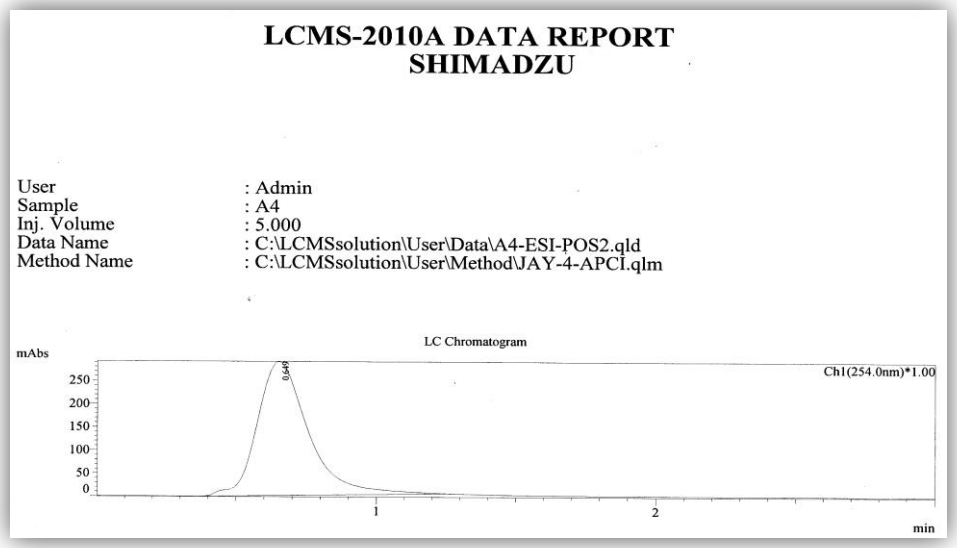
- The δ 0.75, 0.79, 0.83, 0.88, 0.96, 1.07 and 1.24 due to the methyl groups in a triterpenoid nucleus
- The brand of signals at δ 3.45 is due to the hydroxyl group and the hydroxyl methyl protons of the two sugars units
- The signals at δ 4.20 and 4.27 were due to anomeric protons of the sugar unit
- The multiplet signal at δ 4.1 is due to the hydroxyl methyl group
- The unsaturated proton appeared as a multiplet at δ 5.34.

^{13}C NMR spectrum (JBF2)

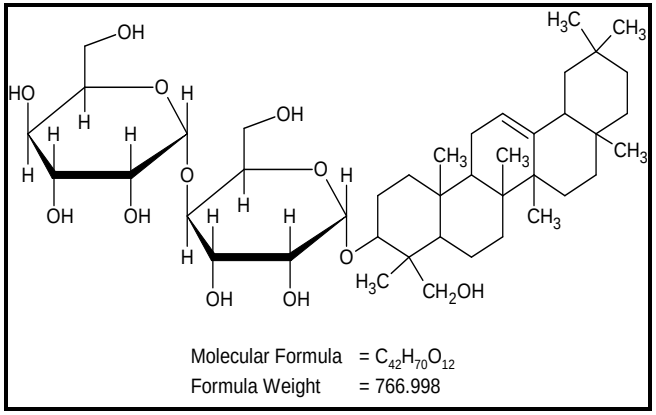


- δ 102.76 and 101.37 for the two anomeric carbon atoms
- δ 108.07 and δ 135.00 for the unsaturated carbon atoms
- δ 84.72, 76.84, 75.14, 71.74, 66.50 correspond to one glucose unit
- δ 81.68, 76.60, 74.41, 71.50 and 66.38 were due to the second glucose unit
- δ 64.53 and 77.23 were due to hydroxyl methyl carbon and the hydroxyl methyl carbon atoms respectively.

Mass spectrum (JBF2)



Compound JBF2



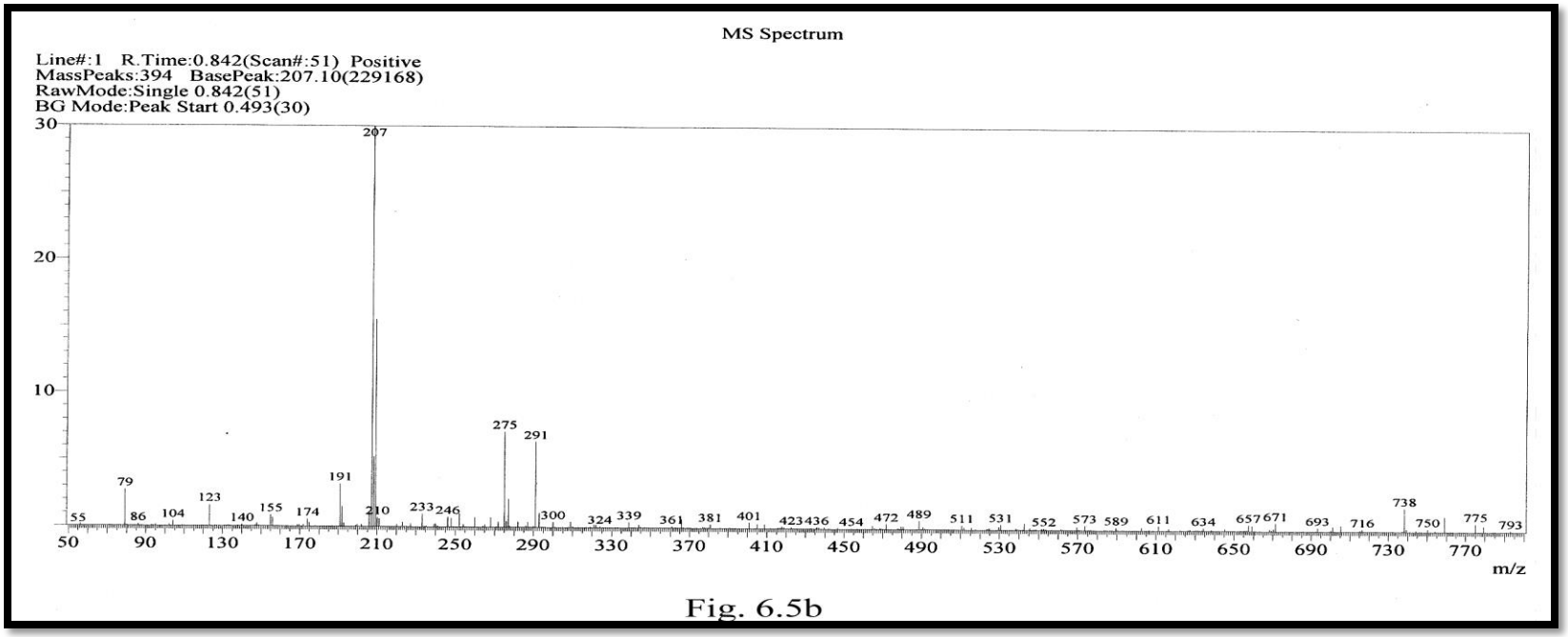
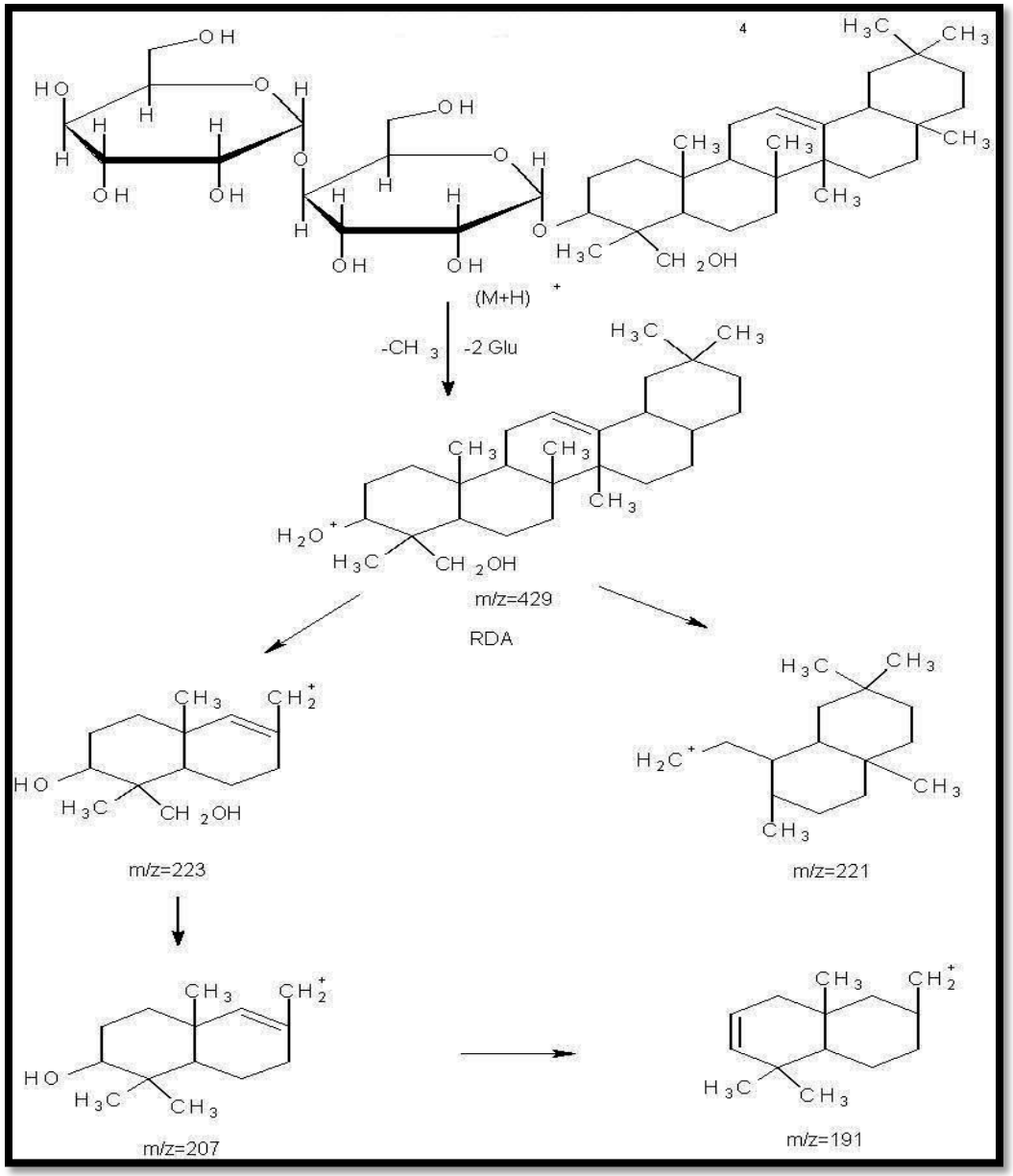


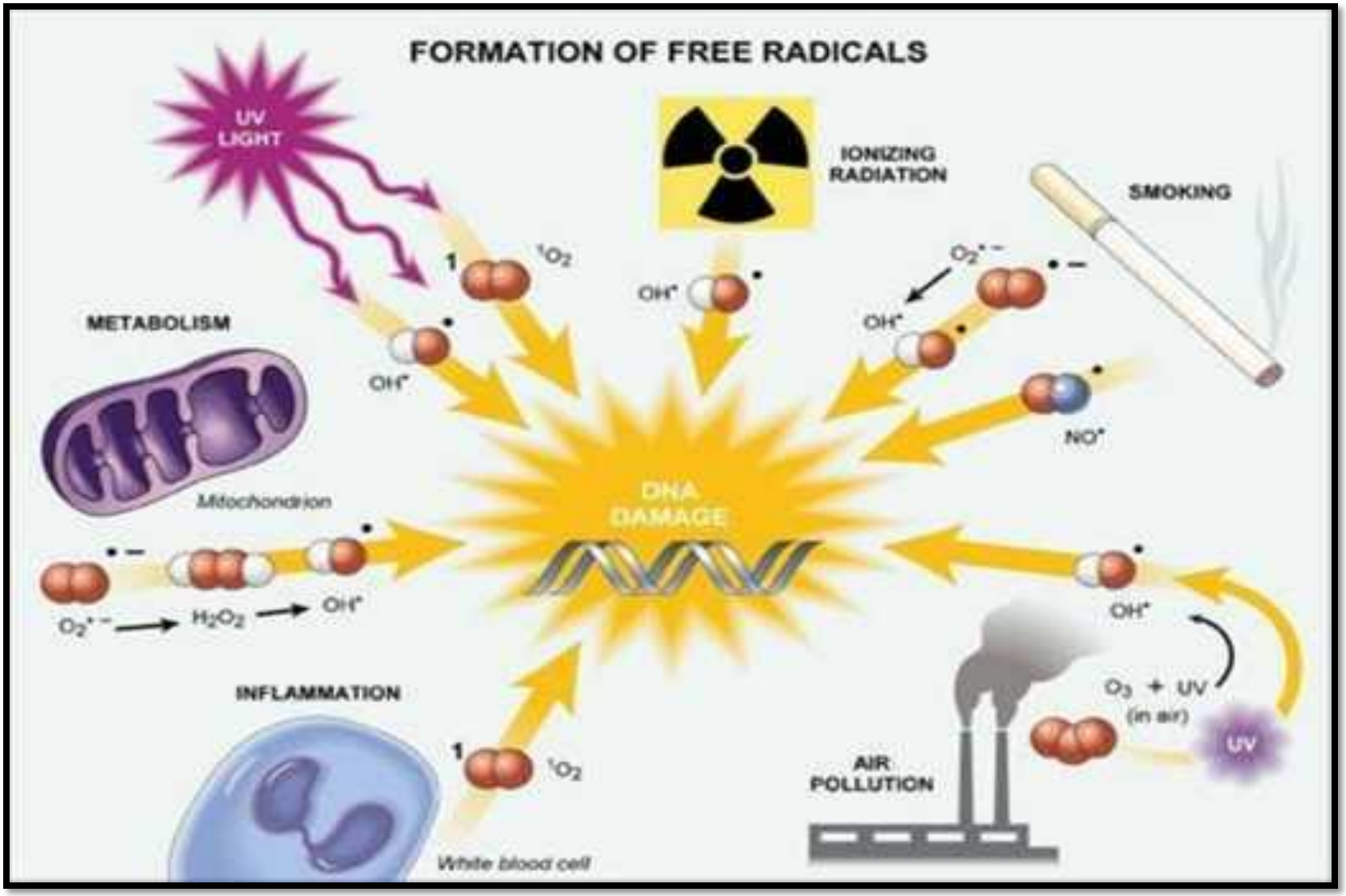
Fig. 6.5b

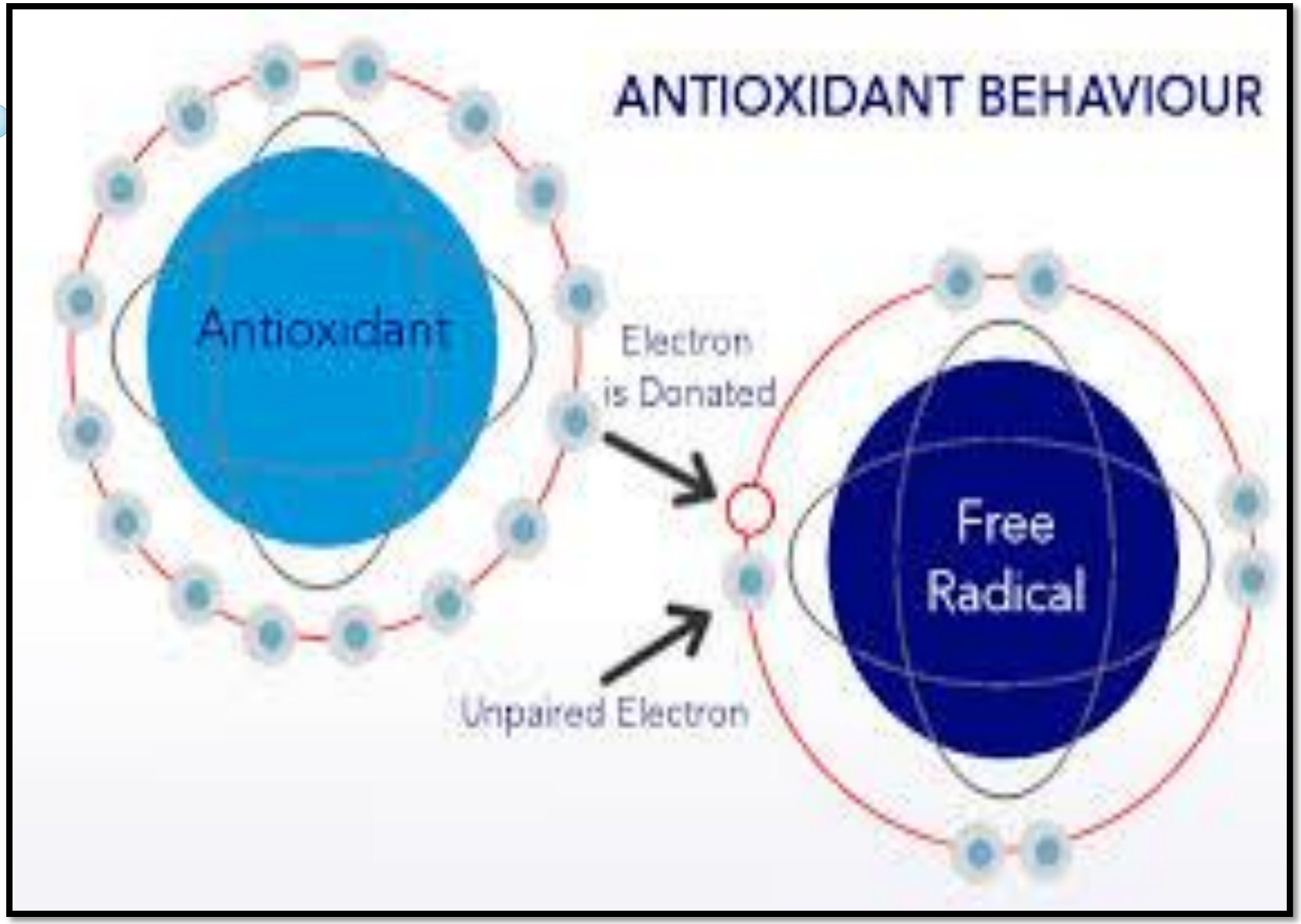
Mass spectral Fragmentation (JBF2)



PART – B

Pharmacological Screening of aerial parts of *Justicia bedommei*




ANTIOXIDANT BEHAVIOUR
Antioxidant
Electron
is Donated
Free
Radical
Unpaired Electron

Screening Methodology

Acute toxicity

The acute toxicity study was performed to know the acute toxic effects and to determine minimum lethal dose of the drug extract.

- The albino mice male and female weighing 25-30 g were used for the experiment.
- The extract was administered orally to the different groups (n= 6) of overnight fasted mice at the dose of 50, 100, 250, 500, 1000, and 2000mg/kg body weight.
- After administration of drug extract, all the animals were observed continuously for signs of toxicity and mortality during 24 h, 48 h, 72 h.
- Minimum lethal dose is 1000mg

Antioxidant activities of aerial parts of JB by DPPH method

Sl.No.	Drug Treatment	Absorbance at 517nm	% of Scavenging
1.	Blank	0.9645	0.00
Standard			
2.	20 µg/ml	0.0917	90.49
3.	40 µg/ml	0.0876	90.91
4.	80 µg/ml	0.0859	91.09
5.	160 µg/ml	0.0835	91.34
6.	200 µg/ml	0.0820	91.49
Ethanolic Extract			
7.	20 µg/ml	0.5001	48.14
8.	40 µg/ml	0.4773	51.51
9.	80 µg/ml	0.4385	54.53
10.	160 µg/ml	0.4073	57.77
11.	200 µg/ml	0.3805	60.54
Chloroform Extract			
12.	20 µg/ml	0.5201	46.07
13.	40 µg/ml	0.5024	47.91
14.	80 µg/ml	0.4837	49.84
15.	160 µg/ml	0.4656	51.72
16.	200 µg/ml	0.4522	53.11

Antioxidant activities of JBF1 and JBF2 by DPPH method

Sl.No.	Drug Treatment	Absorbance at 517nm	% of Scavenging
1.	Blank	0.9876	0.00
Standard			
2.	20 µg/ml	0.1254	87.30
3.	40 µg/ml	0.1123	88.62
4.	80 µg/ml	0.1076	89.10
5.	160 µg/ml	0.0951	90.37
6.	200 µg/ml	0.0811	91.78
JBF1 Ethylacetate:Ethanol (20:80)			
7.	20 µg/ml	0.5492	44.39
8.	40 µg/ml	0.5306	46.27
9.	80 µg/ml	0.5140	47.95
10.	160 µg/ml	0.4866	50.72
11.	200 µg/ml	0.4169	57.78
JBF2 Ethylacetate:Ehanol (50:50)			
12.	20 µg/ml	0.4869	50.69
13.	40 µg/ml	0.4762	51.78
14.	80 µg/ml	0.4559	53.83
15.	160 µg/ml	0.4304	56.41
16.	200 µg/ml	0.4036	59.13

Antioxidant activity of ethanolic extracts of leaves of *JB* by *In-vivo* method.

Sl.No.	Drug Treatment	Absorbance at 517nm	% of Scavenging
1.	Blank	0.0997	0.00
	Standard		
2.	Serial 1	0.0102	89.76
3.	Serial 2	0.0087	91.27
4.	Serial 3	0.0063	93.68
5.	Serial 4	0.0039	96.08
6.	Serial 5	0.0011	98.89
	Ethanolic Extract 200 mg		
7.	Serial 1	0.0467	53.15
8.	Serial 2	0.0375	62.38
9.	Serial 3	0.0267	73.21
10.	Serial 4	0.0196	80.34
11.	Serial 5	0.0143	85.65
	Ethanolic Extract 500 mg		
12.	Serial 1	0.0339	65.99
13.	Serial 2	0.0276	72.31
14.	Serial 3	0.0230	76.93
15.	Serial 4	0.0155	84.45
16.	Serial 5	0.0110	88.96

Antioxidant activity of *JB* by SOD method

Sl. No.	Drug Treatment	Absorbance at 560nm	% of Inhibition
1.	Blank	0.3624	0.00
	Standard		
2.	Serial 1	0.0412	88.63
3.	Serial 2	0.0393	89.15
4.	Serial 3	0.0371	89.76
5.	Serial 4	0.0348	90.39
6.	Serial 5	0.0320	91.16
	Ethanolic Extract		
7.	Serial 1	0.1643	54.66
8.	Serial 2	0.1545	57.36
9.	Serial 3	0.1416	60.92
10.	Serial 4	0.1299	64.15
11.	Serial 5	0.1222	66.28
	Chloroform Extract		
12.	Serial 1	0.1759	51.46
13.	Serial 2	0.1679	53.66
14.	Serial 3	0.1603	55.76
15.	Serial 4	0.1517	58.14
16.	Serial 5	0.1467	59.51

Antioxidant activity

Results and discussion

❖ The ethanolic extracts of JB at the concentration of 500 mg/ml showed excellent inhibition (88.96%) & 200mg/ml showed strong inhibition (85.65%) by DPPH method.

❖ Fraction: JBF2 showed significant inhibition of (59.13%) at 200µg/ml & JBF1 showed strong inhibition of (57.78%) at 200µg/ml by DPPH method.

❖ The ethenolic extract showed (66.28%) of inhibition by SOD method.

❖Antioxidant activity of triterpenoids/flavonoids depends as the structure and substitution patterns of –OH groups.

❖The essential requirements for effective radical scavenging is the 3, 4, -orthodihydroxy configuration in ring 3 and 4 carbonyl groups, giving a catechol-like structure in ring C, is also beneficial for antioxidant activity of triterpenoids/flavonoids.

❖ Probably, aerial parts of *JB* extracts and fractions may contain the above similar type of triterpenoids/flavonoids. So that which exhibits the excellent antioxidant activity.

Antioxidant activity

Conclusion

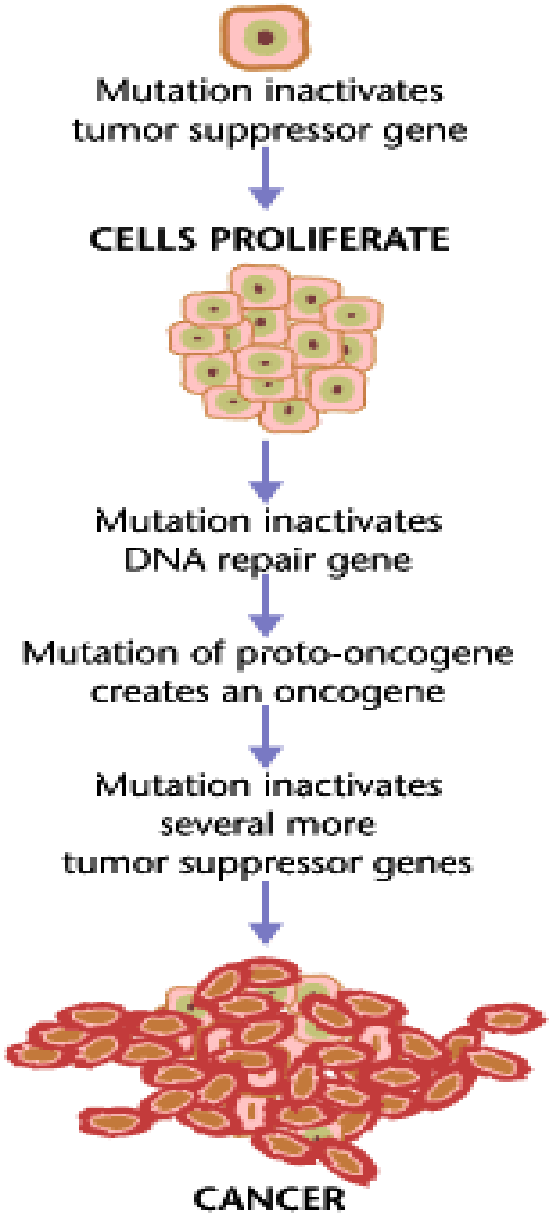
- By using DPPH assay percentage of scavenging was determined and IC50 was determined by SOD method.

- The ethenolic extract of *JB* showed an excellent antioxidant activity.

- The in vivo and in vitro studies showed considerable antioxidant activity, mainly based a scavenging of oxygen radicals.

- Hence leaves parts of *JB* are to be claimed as good Antioxidant properties.

Pathophisiology of Cancer



Antitumour activity of ethanolic extract of *JB* given po against the sc implanted EAC

Treatment schedule	MTD[a] (mg/kg^{-1})	TI[b]	Dose (mg/kg^{-1})	SGD[c]	ILS%	Survival
D1	> 2000	16.66	100 200	-0.08 0.04	14.28 19.04	3/6 6/6
D 1, 3, 5, 7, 9, 11	> 2000	16.66	100 200	-0.75 -0.85	23.80 33.33**	6/6 6/6

Antitumour activity of chloroform extract of *JB* given po against the sc implanted EAC

Treatment schedule	MTD[a] (mg/kg^{-1})	TI[b]	Dose (mg/kg^{-1})	SGD[c]	ILS%	Survival
D1	> 2000	16.66	100 200	0.29 0.20	9.52 14.28	4/6 6/6
D 1, 3, 5, 7, 9, 11	> 2000	16.66	100 200	-0.33 -0.64	76.19 5.71***	4/6 6/6

Antitumour activity of *JBF2* given po against the sc implanted EAC

Treatment schedule	MTD[a] (mg/kg^{-1})	TI[b]	Dose (mg/kg^{-1})	SGD[c]	ILS%	Survival
D1	> 2000	16.66	30 60	0.12 0.04	28.57 33.33	6/6 6/6
D 1, 3, 5, 7, 9, 11	> 2000	16.66	30 60	-0.54 -0.80	57.14 71.42**	6/6 6/6

Anticancer activity

RESULTS AND DISCUSSION

In recent years, relatively few new bioactive flavonoids/terpenoids have emerged with proved highly effective antiproliferation activity against EAC-induced in mice. The ethanolic and chloroform extracts of *JB* contains flavonoids/terpenoids etc. Most of flavonoids/terpenoids have been reported to inhibit proliferation in many kinds of cultured human cancer. Similarly the presence of flavonoids/terpenoids of *JB* was responsible to exhibit potent antitumour activity in EAC-induced mice.

Treatment efficacy was assessed with two parameters: SGD and ILS% Table summarizes the SGD of ethanolic, chloroform extract and isolated fractions JBF2 was -0.83, -0.64 and -0.80 respectively. Similarly the percentage of life span (ILS%) was 33.33%, 85.71% and 71.42% of ethanolic, chloroform and JBF2. Cancer prevention was generally associated with inhibition, reversion or retardation of cellular hyperproliferation. The presence of flavonoids/terpenoids of *JB* has been exhibited inhibition of proliferation.

The presence of flavonoids/terpenoids of *JB e*xhibits the above similar anticancer property, hence we got optimistic results in criteria to anticancer activity against Ehrlich's Ascites Carcinoma induced mice. These findings have ecological and economic significance for application of *JB* contain certain flavonoids/terpenoids could inhibit tumour initiation as well as tumor progression.

CONCLUSION

The *JB* leaves contained flavonoids/terpenoids are generally non-toxic and manifest a diverse range of beneficial biological activities like antioxidant property and antitumour activity. The tabulated results shown significant decrease in tumor volume and ILS% increase at the dose of 200 mg/kg body weight of chloroform extract and isolated fraction: JBF2 of 200 mg/kg of body weight. These findings suggested that the presence of flavonoids/terpenoids is *JB* possess carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation and antioxidation. These promising results will stimulate development of *JB* flavonoids/terpenoids for cancer-chemoprevention and chemotherapy.

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