Chapter Five

Isolation, Purification and Characterization of Hepatoprotective Principle from Coscinium Fenestratum

5.1 Introduction

Wide prevalence of liver diseases has necessitated to develop satisfactory hepatoprotectants. Virtually not a single curative therapeutic agent is available so far except herbal preparations which support or promote the process of healing or regeneration of liver cells. The use of traditional medicine is widespread and plants still present a large source of structurally novel compounds that might serve as leads for the development of novel drugs.

Most of the commercially available anti-hepatotoxic natural products are derived from folk medicine and not discovered by systematic scientific screening. The remedies available in modern medicine for the treatment of hepatic ailments are corticosteroids and immunosuppressants which provide only symptomatic relief mostly without influencing the disease process, and their use is associated with the risk of relapse and danger of side effects. [4]

It is worth to have a better understanding on the traditional plant-based knowledge in curative therapies. [91] The rapidly growing scenario in the current status of medicinal plants warrants an imminent need for purification and characterization of the active principles offering therapeutic potential.

Herbal drugs are prescribed widely even when their biologically active compounds are unknown because of their effectiveness, minimal side effects in clinical experience and relatively low cost. It is high time that India should launch a

programme on drugs from plants, based on clues from traditional knowledge and harnessing modern technologies for the development of new chemical entities.

Discovery of new therapeutic agents for hepatic dysfunctions is genuine and urgent. More than 600 claimed commercial herbal preparations world wide available as hepatoprotectants in which about forty indigenous preparations are in clinical use. These preparations contain about 100 indigenous medicinal plants belonging to 52 plant families.[4] Various categories of compounds offering hepatoprotective efficacy have been isolated. A list of such compounds having liver-protecting potential is given below.[4]

Name of compound	Type of compound	Plant from which the compound was isolated
Apocyanine	phenol derivative	*Picrorhiza kurroa*
Valerolactam	cyclic amide	*Clusenia lansium*
Glycolic acid & Glyceric acid	carboxylic acids	*Cynara scolymus*
Chlorogenic acid	"	*Coffea sp*
Arachidonic acid	"	*Linum usitatissimum*
Desmethylricinine	pyridine derivative	*Ricinus communis*
Caffeine	purine derivative	*Coffea sp*
Boldine	alkaloid	*Peumus boldus*
Protopyne	"	*Fumaria indica*
Berbamine	"	*Berberis vulgaris*
Resperine	"	*Rauwolfia sp*
Isofraxidin and umbelliferon	coumarin derivatives	*Artemisia abrotanum*
Esculin and herniarin	"	*Artemisia messerschmidiana*
Armillarisin A	"	*Armillariella tabescens*
Catechin	flavonoids	*Acacia catechin*
Flamin	"	*Helichrysum arenarium*
Eupatolin	"	*Artemisia cappillaris*
Patuletin	"	*Tagetus patula*

Myricitroside	flavonoids	*Ceris siliquastrum*
Luteolin	"	*Reseda luteola*
Cappillartemisin	"	*Artemisia cappillaris*
Garcinokolin	lignans	*Garcinia kola*
Silymarin	"	*Silybum marianum*
Schisandrins		*Schizandra chinensis*
Schizantherins	"	*Schizandra sphenanthera*
Desoxypodophyllotoxin	"	*Thujopsis dolabrata*
Phyllanthin	glycosides	*Phyllanthus niruri*
Picroside and picroliv	"	*Picrorhiza kurroa*
Aglycone	"	*Gardenia jasminoids*
Sweroside aglycone	"	*Swertia japonica*
Aglycone of loganin	"	*Patrinia villosa*
Syringiopicroside	saponins	*Syringa oblata*
Saponins	"	*Glycine max*
Salkosaponins	"	*Bypleureum falcatum*
Ginseng saponins	terpenoids	*Panax ginseng*
(+)-borneol	"	*Dryobalanops aromatica*
Lindstrene	"	*Lindera strychinifolia*
Andrographolide	"	*Andrographis paniculata*
Papyriogenin	"	*Tetrapanax papyriferum*
Zygophillin	"	*Zygophyllum coccineum*
Glycyrrhizin	carotenoids	*Glycyrrhiza glabra*
Crocin and crocetin	miscellaneous	*Gardenia florida*
Capsaicin	"	*Capsicum sp*
Naphtho-r-Pyrone	"	*Cassia tora*
Cochloxanthine	"	*Cochlospermum tinctorium*
Furangerminone	"	*Zedoriae schzoma*
Tritoqualine		*Piper nigrum*

Our previous study revealed the anti-hepatotoxic [264] as well as antioxidant efficacy of *C. fenestratum* stem in rat model of hepatotoxicity. This study was undertaken to isolate, purify and characterize the active hepatoprotective principle of this plant in an endeavour to formulate some effective drugs from it.

5.2 Materials and Methods

Stem portions of *C. fenestratum* formed the plant materials. Stem powder of this plant was prepared as per the procedure given in 2.1.a. Stem powder was defatted in petroleum ether (60 - 80^{0}C) (yield 0.81 %) and then extracted with methanol for 36 h, using a Soxhlet extractor. (yield 2.6%) The methanol extract was made to powder with the help of rotary evaporator under reduced pressure. Powdered methanol extract (MECF) was prepared in sufficient quantity and it was used for further purification.

MECF (40 g) was then successively fractionated with petroleum ether (60 - 80^{0}C), chloroform, acetone, methanol, ethanol-water (1:1, v/v) and distilled water. The residue left over after fractionation yielded light yellow coloured flakes on repeated treatment with activated charcoal. These were then subjected to recrystallisation from glacial acetic acid so that yellow needle shaped crystals (2.20 g) were obtained. (The yield of the purified compound in respect of crude stem powder was 0.143 % w/w)

The purity of the compound was checked by thin layer chromatography on silica gel G, using ethyl acetate-methanol (5 : 1, v / v) as mobile phase and iodine as the detecting reagent. As the compound emerged as a single spot on the TLC plate, its purity was confirmed. The compound was found to be soluble in methanol and water, and insoluble in ethyl acetate. It decomposed at 196 - 200^{0}C. The purified compound was then subjected to spectroscopic (^{1}H NMR, ^{13}C NMR, IR and UV) studies.

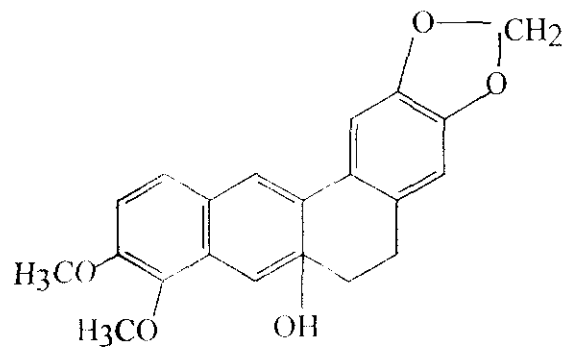
After its characterization, the compound was tested for anti-hepatotoxic as well as antioxidant activity in CCl_4-intoxicated rats. The experimental procedure was the same as mentioned in 2.5.6 and 2.5.8 , with the exception that the purified compound at doses 5, 10 and 15 mg / kg bw was administered daily, orally, by intubation to rats to evaluate its efficacy. 6 animals were used in each group. The duration of the experiment was 30 days.

5.3 Results and Discussion

5.3.1 Spectroscopic study

The structure of the compound was elucidated by spectroscopic methods. The IR (infra red) spectrum (Fig 5.1) showed absorbance at 3482 cm^{-1}, indicating a hydroxyl (OH) group. The mass spectrum (Fig 5.2) showed a peak at m/z 353 and a base peak at m/z 178. UV absorption spectrum (fig 5.3) of the compound showed absorption values λ_{max} 266 and 350. ^{1}HNMR spectrum (Fig 5.4) showed a singlet at δ 9.7 assigned to C-8 proton. The other aromatic protons appeared at δ 8.7 (singlet), 8.1 (doublet), 7.99 (doublet), 7.66 (singlet) and 6.96 (singlet). The singlet appeared at δ 6.10 was assigned to the methylenedioxy protons (C-2 protons). The two methoxy protons appeared at δ 4.20 and 4.10 as singlets. One methylene proton at C-5 appeared as triplets centred at C-6 came as a merged peak along with the H-O-D peak at δ 4.87. All the carbon atoms were accounted in the ^{13}C NMR spectrum (Fig 5.5).

IR and NMR spectra match with the standard spectra of berberine contained in Aldrich Library of NMR [265] and IR [266] spectra. UV spectrum too showed almost near absorption values for the Merck Index [267] for the literature values for berberine. Hence, the compound was tentatively characterized as **BERBERINE**. Emperical formula of the compound is $\mathbf{C^{20}H^{19}O^5N}$.



Structure of Berberine

NB : * IR spectrum was recorded in Nicolet Magna 560 FTIR spectrometer.

* NMR spectra were recorded in Bruker Avance DPX 300 FT NMR spectrometer in methanol d-4.
* Mass spectrum was recorded in Shimadzu GCMS 5050 A spectrometer.
* UV absorption spectrum was recorded in Shimadzu UV-3101 PC NIR scanning spectrophotometer in methanol.
* The chemical shifts were reported in δ (ppm) relative to Me_4Si as internal standards.

(Spectroscopic study courtesy of Regional Research Laboratory (CSIR), Thiruvananthapuram.)

5.3.2 Berberine

Berberine is the most important member of isoquinoline alkaloids which form an important class of natural products endowed with biological application. [268] & [269]. It is a quaternary alkaloid from many *Berberis* and *Mahonia* spp (Berberidaceae) and very many other species of several different families. This alkaloid shows a wide variety of pharmacological effects including respiratory stimulation, transient hypotension and convulsion. It is an inhibitor of cholinesterase, tyrosine decarboxylase and tryptophanase. It can serve as an anti-anaemic agent, and

also shows anti-bacterial, anti-fungal, anti-neoplastic and cytotoxic activity. Berberine is used for the treatment of gastro-intestinal disorders, cholera and infantile diarrhoea. [270 - 273]

5.3.3 Hepatoprotective and antioxidant activity of berberine from *C. fenestratum*

The activities of liver marker enzymes, such as AST, ALT, ALP and GGT in the serum of CCl_4-treated rats (group-II) were found to increase significantly as compared to the pairfed controls. (group-I). (Table 5.1). But in the case of rats co-administered with berberine (groups-III, IV and V, administered berberine at the dose of 5, 10 and 15 mg / kg bw respectively), all these enzymes showed significantly decreased activity, thus elucidating the hepatoprotective potential of berberine. It was found that berberine, when administered at the dose of 10 mg / kg bw evoked the maximum percentage of hepatoprotection, and accordingly this is deemed as the effective dose of the drug. (Table 5.2)

The antioxidant enzymes, such as SOD, CAT and GPX showed significantly diminished activity in CCl_4 treated rats as compared to normal controls, thus revealing the oxidative stress prevalent in these rats due to CCl_4 exposure. Though in the group-III, IV and V rats (berberine co-administered), the activities of the antioxidant enzymes improved further, group-IV animals exhibited the maximum percentage of hepatoprotection. (Table 5.3 and 5.4). These findings further corroborate the effective dose of berberine administered.

5.1 Effect of the purified compound from *C. fenestratum* on the activities of liver marker enzymes.

Parameter	Group I	Group II	Group III	Group IV	Group V
AST (IU / L)	24.98 ± 1.31	32.69 ± 1.46*	28.02 ± 1.56♣	25.31 ± 1.28♣	27.43 ± 2.61♣
ALT (IU/ L)	28.24 ± 1.64	57.98 ± 3.96*	42.71 ± 3.62♣	29.43 ± 1.76♣	40.89 ± 4.64♣
ALP (IU/ L)	82.37 ± 4.02	118.64 ± 5.02*	98.49 ± 4.62♣	86.08 ± 4.84♣	100.41 ± 5.91♣
GGT (IU/ L)	3.08 ± 0.28	16.97 ± 1.28*	10.24 ± 1.61♣	4.46 ± 0.48♣	8.06 ± 2.23♣

Values are mean ± SEM of 6 animals in each group
*$p < 0.01$ as compared to group I , ♣ $p < 0.01$ as compared to group II.
Group I : pairfed control, Group II : CCl_4 only, Group III : CCl_4 + purified sample 5 mg/ kg, Group IV : CCl_4 + purified sample 10 mg / kg, Group V : CCl_4 + purified sample 15 mg / kg.

5.2 Percentage of hepatoprotection offered by the purified sample in respect of marker enzymes

Dose of Compound	Percentage of hepatoprotection in respect of			
	AST	ALT	ALP	GGT
5mg / kg bw	60.6	51.3	67.8	48.5
10mg / kg bw	95.7	95.99	89.8	90.1
15mg / kg bw	68.2	57.46	50.3	64.2

5.3 Effect of the purified compound from *C. fenestratum* on the activities of antioxidant enzymes.

Parameter	Group I	Group II	Group III	Group IV	Group V
SOD (U / mg protein)	12.46 ± 0.48	7.68 ± 0.04*	10.94 ± 0.81♣	11.88 ± 0.68♣	10.14± 0.89♣
CAT (U / mg protein)	9.08± 0.42	4.31 ± 0.38*	6.78 ± 0.71♣	8.78 ± 1.76♣	6.39 ± 0.48♣
GPX (U / mg protein)	0.89 ± 0.04	0.54 ± 0.06*	0.72 ± 0.06♣	0.86 ± 0.07♣	0.74 ± 0.07♣

Values are mean ± SEM of 6 animals in each group

*$p < 0.01$ as compared to group I , ♣ $p < 0.01$ as compared to group II,

Group I : pairfed control, Group II : CCl_4 only, Group III : CCl_4 + purified sample 5 mg/ kg,

Group IV : CCl_4 + purified sample 10 mg / kg, Group V : CCl_4 + purified sample 15 mg / kg.

5.4 Percentage of hepatoprotection offered by the purified sample in respect of antioxidant enzymes

Dose of Compound	Percentage of hepatoprotection in respect of		
	SOD	CAT	GPX
5mg / kg bw	68.2	48.72	51.43
10mg / kg bw	87.9	88.16	91.43
15mg / kg bw	51.5	41.03	57.14

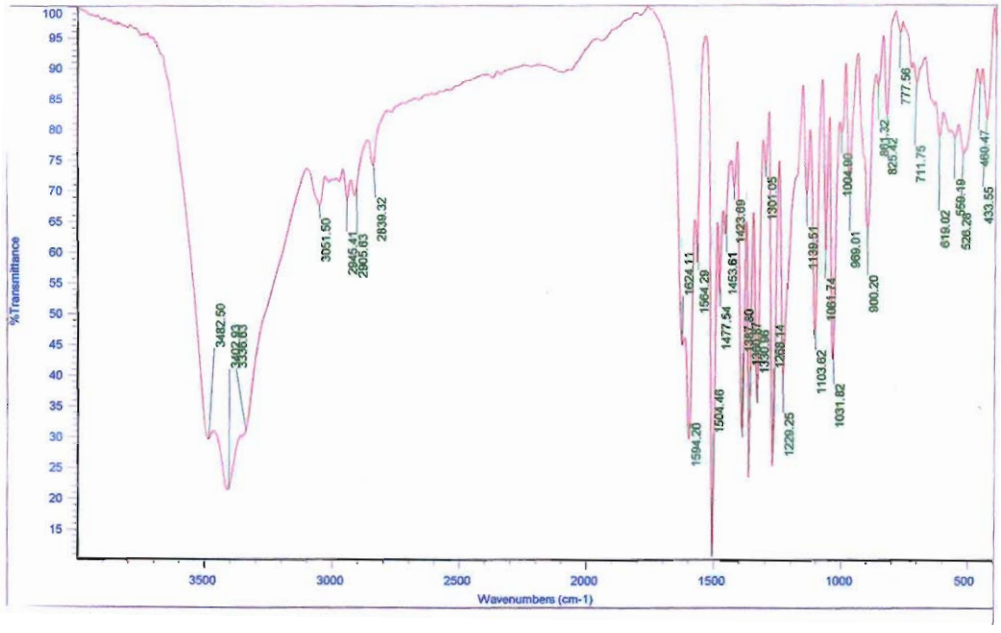


Fig. 5.1 IR spectrum of the purified compound

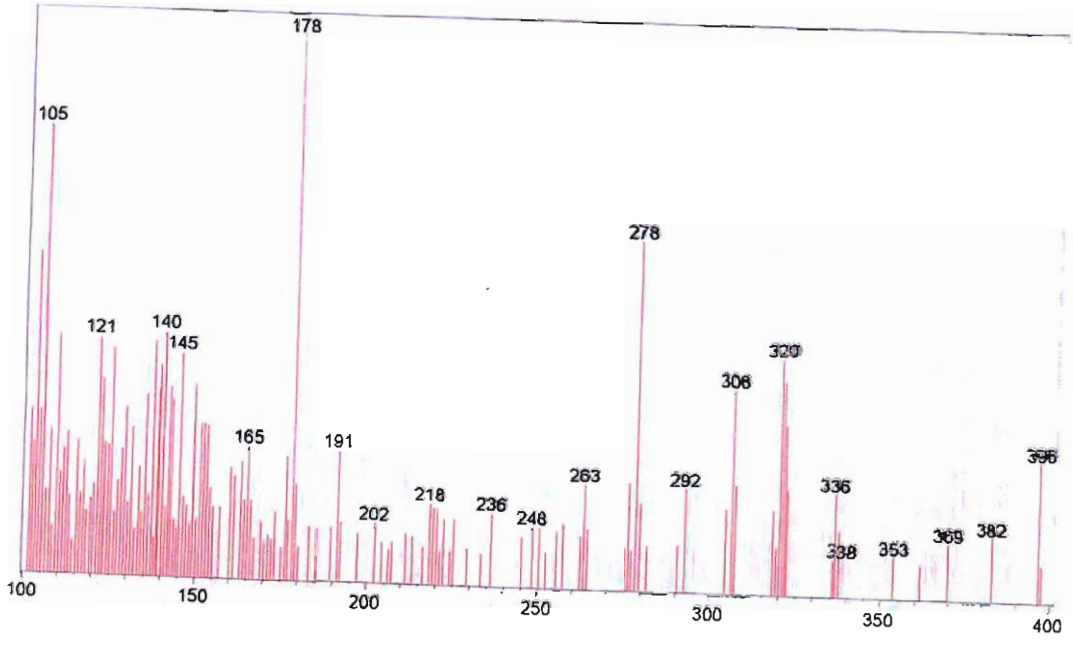


Fig. 5.2 Mass spectrum of the purified compound

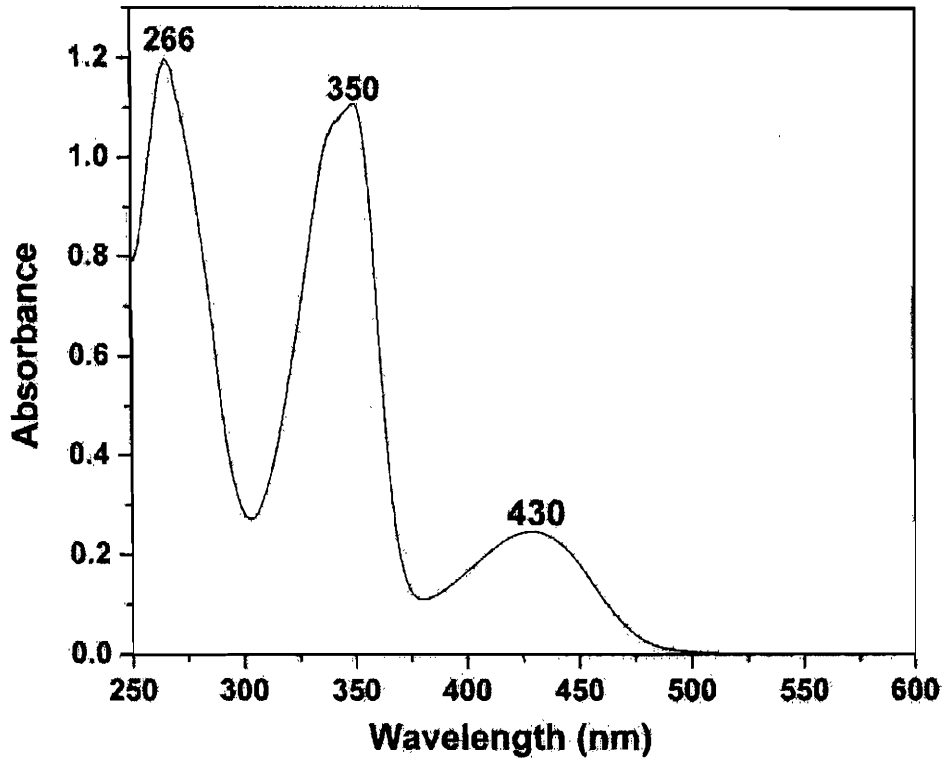


Fig. 5.3 UV spectrum of the purified compound

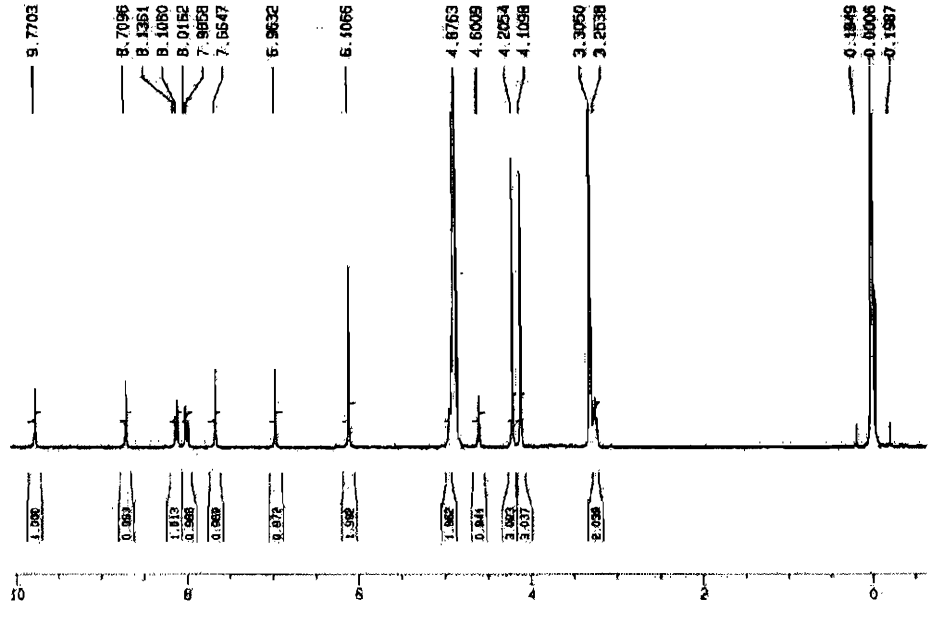


Fig. 5.4 ^{1}H NMR spectrum of the purified compound

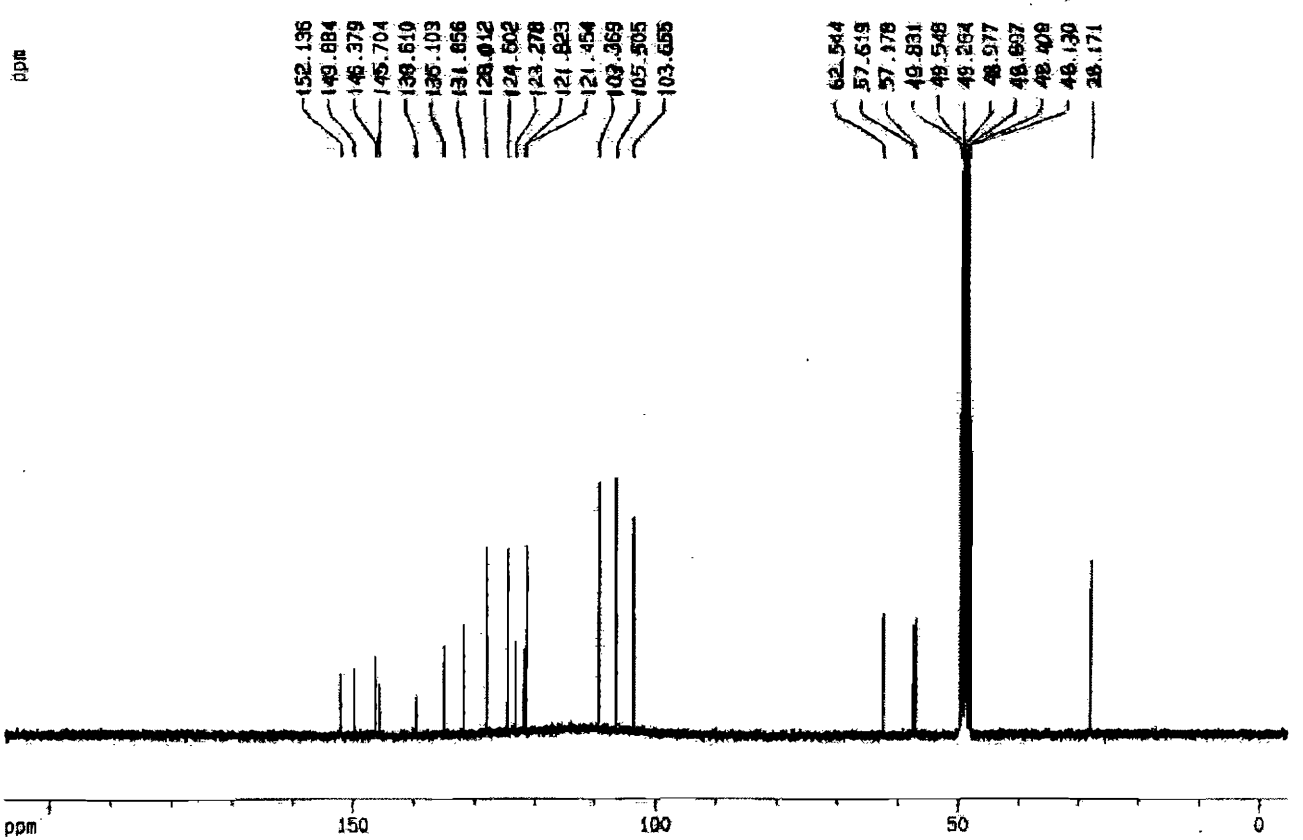


Fig. 5. 5 ^{13}C NMR spectrum of the purified compound

Hepatoprotective activity of berberine has been reported earlier [4] Clinical studies of berberine (as hydrochloride or sulphate) in patients with chronic cholestasis showed its effectiveness in eliminating clinical symptoms and in changing pathological indices such as decreased bile-bilirubin level and increased gall bladder bile volume. Berberine also had some positive effect in patients with toxic hepatitis and appeared to be most prominent with i.v administration, as it enhanced the concentration of bilirubin in the bile of experimental animals. [4]

It can be concluded that the hepatoprotective and antioxidant efficacy of *C. fenestratum* stem is due to the biologically active compound isolated from it (berberine), and that this compound can elicit hepatoprotective and antioxidant activity at as low a dose as 10 mg / kg bw.