International Journal of Phytomedicine 5 (2013) 452-459
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Evaluation of hepatoprotectve activity and isolation of 2-(3, 4-dihydroxy phenyl)-7-hydroxy-3-(2-hydroxy ethoxy) 4-H-chromen-4one from column fractions of leaves of the extract of *Crataeva magna*.

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Abstract

Nature is only source of hepatoprotective drugs in modern medicine to prevent and treat drug-induced liver damage. The alcoholic extract of fresh leaves of the plant *Crataeva magna*, previously reported for its hepatoprotective activity was fractionated into three parts to chemically identify the most potent bioactive fraction. The current study was designed to evaluate the hepatoprotective activity, isolation and characterization property of effective column fractions of the leaves of the plant *Crataeva magna*. The hepatopreventive effect of the column fractions (F1, F2 and F3) of the leaves of the plant were evaluated by measuring the levels of serum liver damage marker enzyme such as alanine transaminase, aspartate transaminase, alkaline phosphatase, total and direct bilurubin. As per the result, since the F3 fraction of the extract showing out the significant reduction in the elevated serum level which can be compared with the standard drug silymarin therefore, phytochemical investigation of the F3 fractions led to the identify the structure of the flavonoid compound which was established by spectroscopic methods (UV, IR, ^{1}H NMR, ^{13}C NMR and EI-MS) as 2-(3, 4-Dihydroxy Phenyl)-7-Hydroxy-3-(2-Hydroxy Ethoxy) 4-H-Chromen-4one.

Keywords: *Crataeva magna,* hepatopreventive effect, column fractions, flavonoid compound.

Introduction

In the light of recent scientific development throughout the world the medicinal properties of plants have been investigated, due to their potent pharmacological activities, low toxicity and economical viability. Flavonoids are widely distributed phenolic compounds in the plant kingdom and they occur in all parts of plants as complex mixtures of different components [1, 2]. Flavonoid consists of different subgroups where flavones, flavonols and flavanones are considered to be important sub-groups of flavonoid [3].

Liver diseases have become one of the major causes of morbidity and mortality all over world. From among, drug induced liver injury (DILI) is one of the most common causative factor that possess a major clinical and regulatory challenge. In spite of tremendous advances in modem medicine, there are hardly any reliable drugs that protect the liver from damage and help in regeneration of hepatic cell [4-7]. Many active plant extracts are frequently utilized to treat a wide variety of clinical diseases including liver disease. Therefore, searching for effective and safe drugs for liver disorders are continues to be an area of interest.

Crataeva magna, Varuna, is well known traditional plant used to treat various ailments in particular to urolithiasis [8], hepatoprotective [9], cardio protective [10], anti-arthritic [11] [12] and rubifacient [13]. Folkloric uses suggest its potentiality as oxytosic, diuretic, laxative, anti-periodic, and bitter tonic [14].

Leaves of the plants of this species have been reported to contain several compounds such as flavonoids, phytosterols and triterpenoids [15].

The protective effect of methanoic extract obtained from sequential process of C. magna against the hepatotoxicity induced by carbon tetrachloride is already known [16]. Review of the scientific literature showed the absence of any experimental data to justify the presence of the phytoconstituents responsible for the protective role of the leaves of this plant in hepatotoxicity. Herein, the hepatoprotective effect of the column fractions of the methanolic extract on paracetamol induced hepatic damage was evaluated and isolation and characterization of the effective fractions are reported. This is particularly important in view of the fact that the frequent use of N-acetyl-cysteine limited the treatments of acute human intoxications with paracetamol [17].Thus, the aim of the present paper, therefore was to develop an efficient method to separate out through column fractions of the methanolic extract of the leaves of the plant and validate for its hepatoprotective activity against paracetamol induced hepatic damage in rats and further isolate and characterize the flavonoid composition of the effective fraction by using different types spectral techniques.

Material and methods

Plant material

The botanically identified plant material was collected in the month of December 2011 from Balesore district, Odisha, India. A voucher specimen no. SPS 04 has been preserved in the herbarium of the Pharmacognosy department of School of Pharmaceutical Sciences, Siksha O Anusandhan University, India.

Preparation of extraction method

Air dried *C. magna* leaves powered into a soxhelt apparatus and was extracted sequentially with petroleum ether, chloroform and methanol [18]. The solvent were evaporated under reduced pressure and the extracts were then placed in a vacuum oven at 35 c for about 24 hr to remove any residual solvent. Further the methanolic crude extract was fractionated by means of column chromatography using silica gel (60-120 mesh) and eluted with n-hexane, chloroform followed with a solvent combination n-hexane: ethyl acetate: methanol in a ratio of 7: 2: 1. Fractions were collected and monitored by thin layered chromatography (TLC), and fractions with similar TLC profiles were combined and tested for activity.

Experimental animals

Adult albino Wister rats (150-200gm) of either sex (OUAT, Bhubaneswar) used in the experiment were allowed to acclimatize to the laboratory conditions for 7 days in acrylic cages prior to commencement of the experiment with 12hr day & night schedule at a temperature of 26±4 c. The animals were maintained with standard pellet diet & water ad libitum.

Acute toxic study

Thirty five Wister albino rats were divided in seven groups for five animals each. Group 1 received distilled water 10ml/kg orally and animal from groups 2 to 4 were given methanolic extract obtained from sequential process of *C. magna* at dose 200, 400, 500, 1000, 2000 mg/kg orally, respectively. Symptoms of toxicity and mortality were observed for 24hr, the behavioral and CNS profiles such as spontaneous rearing and grooming evidence of calmness and sedation and loss of writhing reflex were also observed.

In vivo hepatoprotective activity

In vivo hepatoprotective activity was evaluated on the basis of the model described by Priscilla D' Mello and Milan Rana, 2010 [19] was employed with some modification [20]. The rats were divided into six groups experimental of six animals (table1). Group I- was kept on normal diet and sever as normal control and received distilled water (10ml/kg) daily for 14 days and group II- serve as toxic control group and received distilled water (10ml/kg) daily for fourteen days and then received paracetamol (1g/kg, p.o.) diluted with sucrose solution (40% w/v) on day 14, 30 min after administration of distilled water. Group III received standard drug Silymarin 100mg/kg p.o., fourteen consecutive days; group IV, V and VI were treated with F1, F2 and F3 extracts obtained by column separation process of the leaves of the study plant at a dose of 200mg/kg body weight daily for 14 days respectively. Paracetamol was administered to the animal group of III, IV, V and VI in a single dose of 1g/kg p.o. diluted with sucrose solution as described previously. Twenty four hours after administration of paracetamol administration. The rats were sacrificed on day 15th day under light ether anesthesia. The blood was collected from all groups by cardiac puncture and serum was separated by centrifugation at 3500 rpm (Eppendorf 5403) at 4 c for 15 min and analyzes for various biochemical parameters. The serum parameters for liver function test such as Asparate aminotransferase (AST) [21], alanine amino transferase (ALT) [22], alkaline phosphate (ALP) [23] and total and direct bilurubin were estimated. The liver tissue was immediately transferred into 10% formalin for histopathological investigation.

Characterization of the compound from F3 column fraction

The third fractions (F3) of the column fractionate was subjected for characterization by UV-Visible spectroscopy, IR spectroscopy, nuclear magnetic spectroscopy (1H NMR and ^{13}C NMR) and mass spectroscopy.

Statistical analysis

All the experiments were carried out in triplicates and the results are reported as mean ± standard error. The data were analyzed by one–way analysis of variance (ANOVA) and Tukey post test. P-values < 0.05 were considered significant.

Results and Discussion

In vivo hepatoprotective activity

The results of acute oral toxicity studies in rats indicated that no visible toxic effect up to 1000 mg/kg for the methanolic extract obtained from sequential process. A significant elevation in the levels of ALT, AST, ALP and total and direct bilurubin were observed with the administration of the paracetamol at a dose of (1g/kg) which depicted in the table no1. The group III, IV, V and VI stands for standard Silymarin and treatment groups (F1, F2 and F3) respectively with 50 mg/ kg and 100 mg/ kg respectively demonstrated that reductions in the level of the enzymes. The group III and VI showed significant reduction in the level of enzymes suggest out that the F3 fraction having potent hepatoprotective effect which can be compared with that of standard drug silymarin.

The screening of Hepatoprotective drugs [24, 25] tested on paracetamol induced liver injuries and the extent of liver damage is assessed by the level of released biochemical enzymes marker (ALT, AST, ALP and total and direct Bilurubin). The result of the

hepatoprotective study reveals that the fractionation process improved the hepato protection which can be correlated well with the previous reports about the efficacy of the sequential extraction than the extract of maceration method which is involved in removing most of the interfering substances [16, 18]. This study also encourages to the identification of the active principles, which was depicted further.

Table 1 - Effect of silymarin and *Crataeva magna* leaf extract on serum AL T, AST, ALP and bilurubin levels in paracetamol induced acute liver injury in rats.

Group	Serum enzyme activities of (U/L)(Mean ± SEM)				Bilurubin mg/dl	
	Dose	ALT	AST	ALP	Total	Direct
Group I Control	Solvent	72.75±1.7*	186.21±4.3*	398.18±6.21**	0.8±0.08	0.21±0.05
Group II Toxicant control	(1000 mg/Kg)	385.00±8.1	641.60±38.1**	749.54±15.4	2.71±0.22*	0.48±0.01
Group III Standard Silymarin	50mg/kg	75.31±12.5	167.64±9.8*	322.3±10.5	0.94±0.02	0.23±0.02**
Group IV Column fraction F1	100mg/kg	281.00±13.4**	345.21±17.1	624.51±11.2 **	2.71±0.06*	0.41±0.02*
Group V Column fraction F2	200mg/kg	219.4±6.2	453.82.4±6.7*	547.32±17.2	2.01±0.034	0.40±0.07**
Group VI Column fraction F3	200mg/kg	86.25±11.8**	178.92±8.1*	339.21±11.6*	1.78±0.014	0.35±0.02

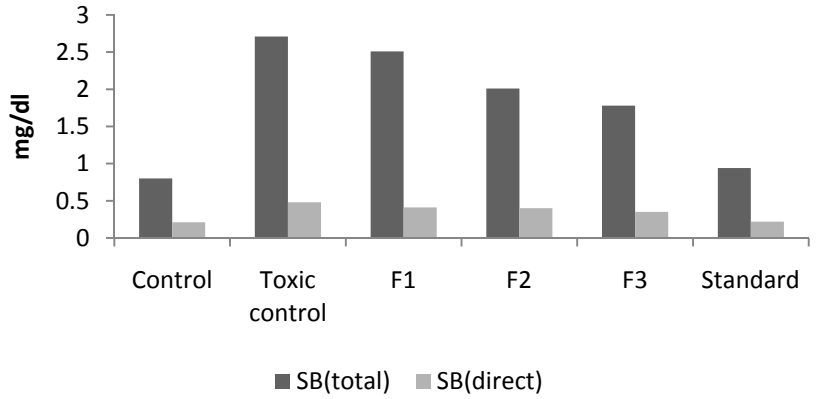

Figure 1. Hepatoprotective action of *C. magna* in rats: serum enzyme activity of ALT, AST and ALP among different treatment groups. Values are mean± S.E.M (n = 4)

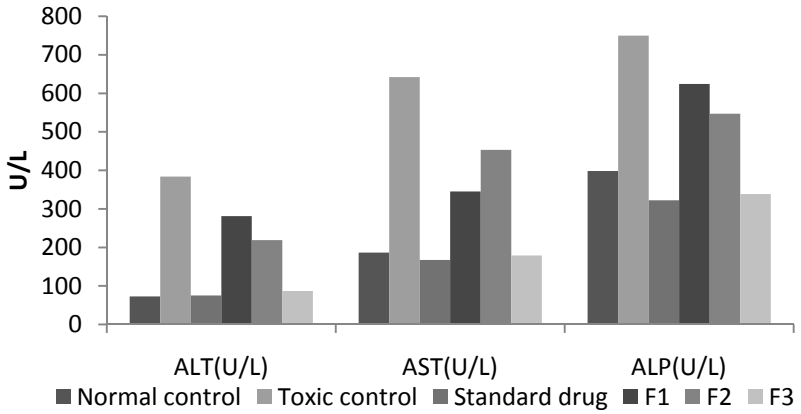

Figure 2. Hepatoprotective action of *Crataeva magna* in rats: Serum bilurubin total and Serum bilurubin direct among different treatment groups. Values are mean± S.E.M (n = 4)

Histopathological condition of liver

Figure (3) shows liver section of normal control rats showed normal hepatic cell with well preserved cytoplasm, prominent nuclei & nucleoli and well brought out central vein. The histoarchitecture of paracetamol treated rat liver sections showed cloudy swelling and fat cells, degeneration of hepatocytes and heavy hemorrhage. Necrosis of cells was also observed with complete degeneration of liver cells, broken cell pieces, irregular appearance due to oozing of cell materials and cell death were observed in figure (4). Though the extent of cellular necrosis was moderately pronounced compared to the paracetamol treated group were observed in animals of administrating F1 and F2 fractions of the extracts were observed in figure (5) and figure (6) respectively. As is apparent in fig (7), the severe hepatic lesions induced by paracetamol were remarkable lowered by the administration of F3 fractions which is good agreement with the results of the biochemical analysis which is comparable to standard Silymarin treated liver section in figire (8).

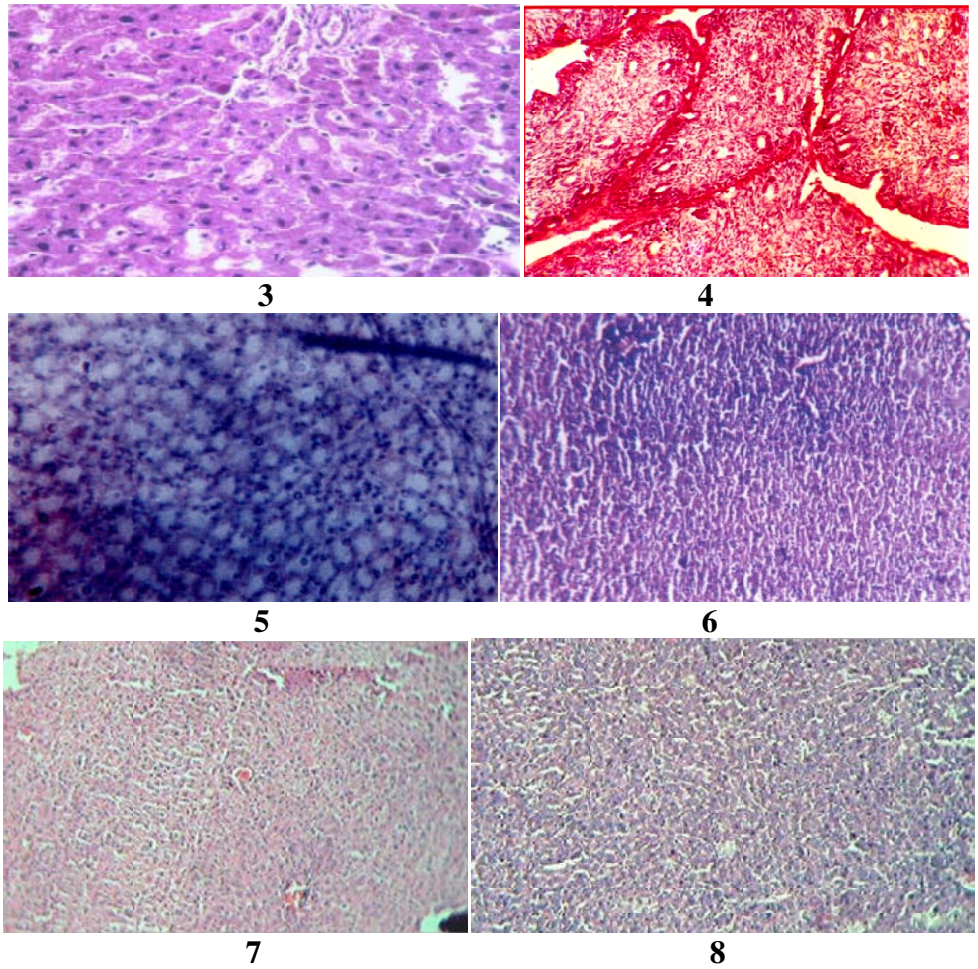

Figure 3-8. Representative microphotographs of H&E (10)-stained histological sections of liver (3) normal control; (4) paracetamol overdose treated rat liver; (5) treated with F1 fraction of extract (100 mg/kg) and paracetamol overdose treated rat liver; (6) F2 fraction of extract (100 mg/kg) and paracetamol overdose treated rat liver; (7) F3 fraction of extract (100 mg/kg) and paracetamol overdose treated rat liver; (8) mice treated with Silymarin 50 mg/kg, p.o. and paracetamol overdose treated rat liver.

Identifications of the isolated compound from F3 column fraction

The compound isolated from F3 column fraction developed a yellow color and exhibited UV absorption bands of flavonoids. The UV spectrum of the compound in MeOH showed two major absorption at 254nm (band II) and 370nm (band I) which are typical for flavonols. Analysis of the UV spectrum utilizing shift reagents indicated the presence of hydroxyl groups at C-5, C-7, C-3′, and C-4′.

The IR spectral data of the compound F3 fraction showed peaks at 3369.03 cm-1, 3648.6 cm-1, 3749.9 cm-1 and 3837.8 cm-1 suggesting OH groups and 1653.66 cm^{-1} conforms the presence of C O and 1598.7 cm^{-1}, 1507.1 cm^{-1} value represent the presence of aromatic structure.

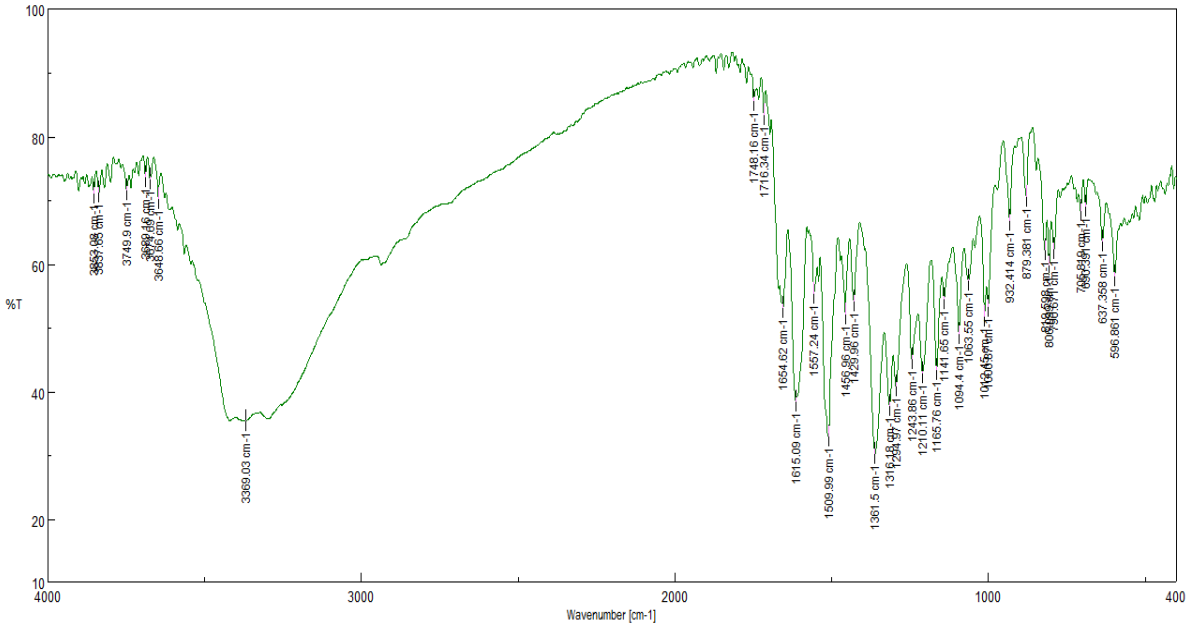

Figure 9. IR spectra of F3 column fraction of the leaves of the plant *C. magna*.

^{1}H NMR 500 (MHz) spectra of the compound F3 fraction was obtained by using DMSO as a solvent and the main data are reported here: 12.48 (1H, 5-OH); 10.83 (1H, 7-OH); 9.67 (1H, 3-OH); 9.39 (1H, 4′-OH); 9.29 (1H, 3′-OH); 7.67 (1H, H-2′); 7.54 (1H, H-6′); 6.87 (1H, H-5′); 6.40 (2H, H-8); 6.19 (1H, H-6).

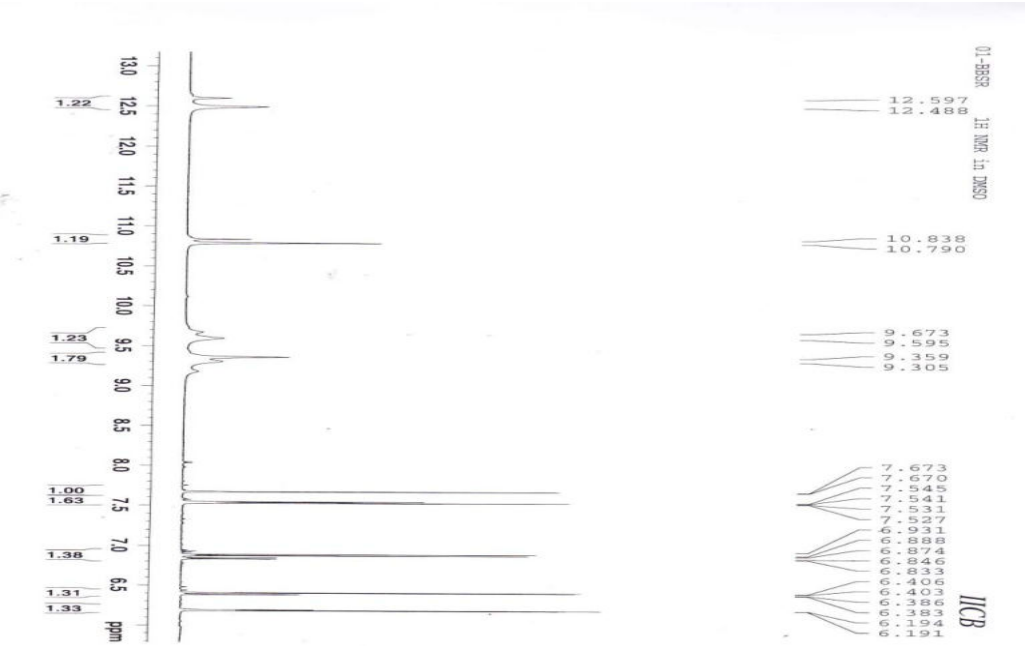

Figure 10. ^{1}H NMR spectra of F3 column fraction of the leaves of the plant *C. magna*.

^{13}C NMR (125 MHz) spectra of the compound F3 fraction was obtained by using DMSO as a solvent and the main data are reported here: 177.38 (C-4), 164.06 (C-7), 148.4 (C-4′), 144.7 (C-3′), 133.3 (C-3), 121.9 (C-6′), 121.1 (C-1′), 116.2 (C-5′).

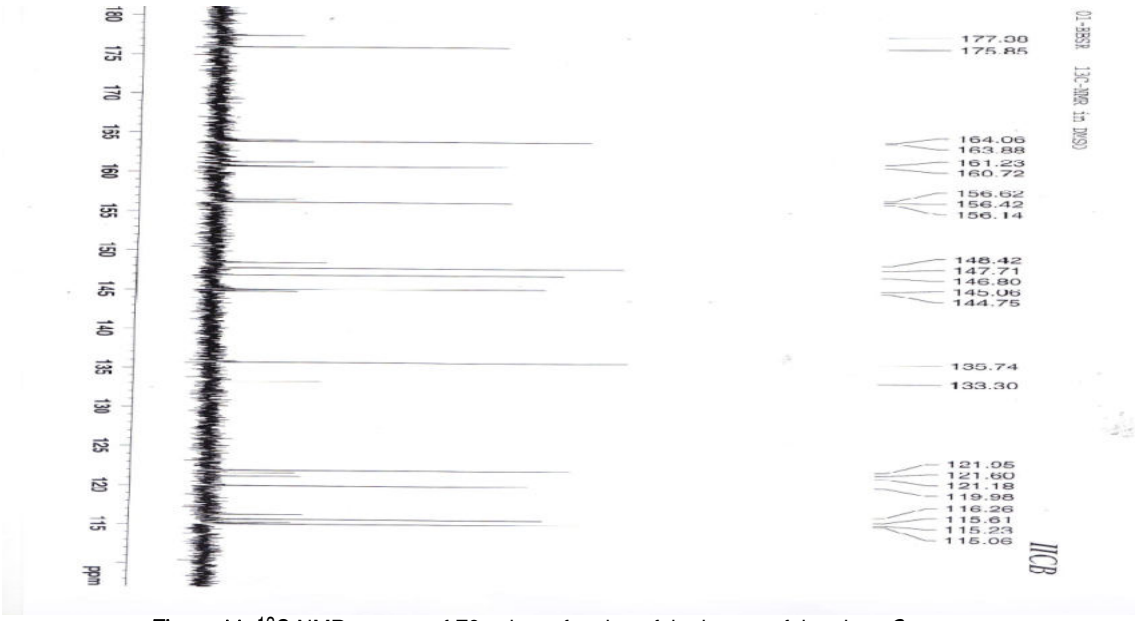

Figure 11. ^{13}C NMR spectra of F3 column fraction of the leaves of the plant *C. magna*.

The EI-MS spectrum of the compound F3 fraction was found to be 331.1. The EI-MS spectrum of the F3 fractions compound was in agreement with the assigned structure. It revealed the empirical formula $C_{17}H_{14}O_{7.}$

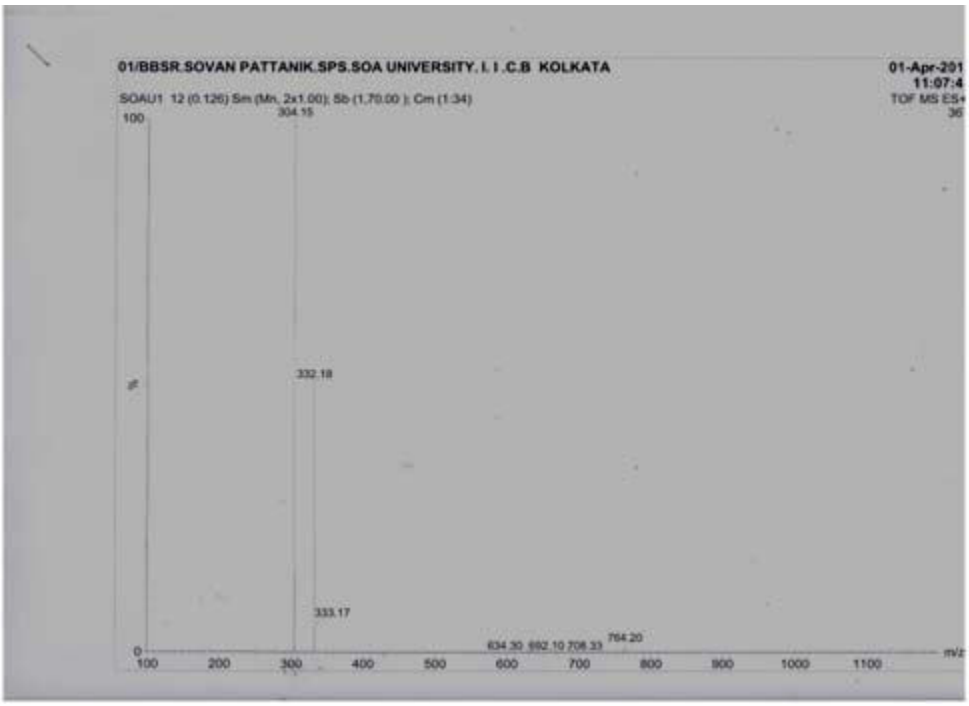

Figure 12. Mass spectra of F3 column fraction of the leaves of the plant *C. magna*.

The UV, IR, H-NMR, ^{13}C-NMR and EI-MS data led to the identification of the F3 fraction compound as 2-(3, 4-Dihydroxy Phenyl)-7-Hydroxy-3-(2-Hydroxy Ethoxy) 4-H-Chromen-4one.

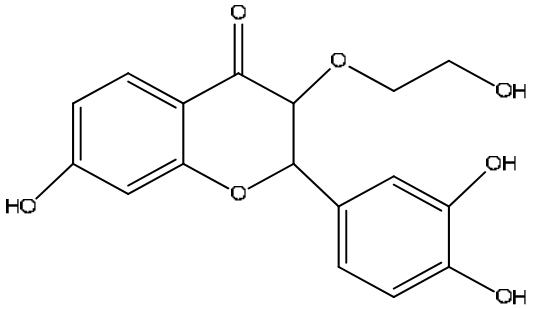

Figure 13. 2-(3, 4-Dihydroxy Phenyl)-7-Hydroxy-3-(2-Hydroxy Ethoxy) 4-H-Chromen-4one.

Conclusion

In conclusions, the column fraction (F3) obtained from methanolic extract of the plant *Crataeva magna* containing the active constituent 2-(3, 4-Dihydroxy Phenyl)-7-Hydroxy-3-(2-Hydroxy Ethoxy) 4-H-Chromen-4one showed protective effect against paracetamol-induced hepatotoxicity.

Authors' contributions

SP: Prepare the extract , carried out the study and drafted the manuscript.
SCS: Conceived of the study, and participated in its design.
AP: Participated in the design of the study and performed the statistical analysis.
SSN: Participated in its design and coordination and helped to draft the manuscript.

Acknowledgements

The authors are grateful to the authorities of School of Pharmaceutical Sciences, Siksha 'O'Anusandhan University, Bhubaneswar Orissa, India and I.I.C.B Kolkata for providing the necessary facility to carry out this research work.

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