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Research Article

ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITE FROM *HABENARIA INTERMEDIA* D.DON FOR EVALUATION OF HEPATOPROTECITVE ACTIVITY AGAINST CARBON TETRACHLORIDE INDUCED LIVER DAMAGE IN ALBINO RATS

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ABSTRACT

Objective: Isolation and characterization of secondary metabolite from *Habenaria intermedia* D Don for assessment of hepatoprotecitve activity against carbon tetrachloride (CCl_4) induced liver damage in albino rats.

Methods: The phenolic constituents present in ethanolic fraction of tubers of *H. intermedia* was isolated by column chromatography using gradient elution technique. The isolated phenolic compound was characterized by infrared, ^1H nuclear magnetic resonance and mass spectral analysis. The isolated compound was screened for hepatoprotecitve activity against liver toxicity induced in Albino rats by intraperitoneal injection of CCl_4. Albino rats weighing 150-200 g were randomly divided in to four groups of six rats each. Group I served as normal control and received only 1% tween in distilled water. Group II served as a negative control and received CCl_4 in liquid paraffin at the dose of 0.7 ml/kg.p.o. CCl_4 on alternate days. Group III and IV were intoxicated with CCl_4 0.7 ml/kg.p.o. before the administration of silymarin 100 mg/kg.p.o. and isolated phenolic constituent (gallic acid) in polyethylene glycol at the dose of 25 mg/kg.p.o. respectively. Various liver function biochemical parameters such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT); serum bilirubin and total protein were assessed before and after treatment to investigate the hepatoprotecitve activity. Histopathology of liver sections of rats treated with isolated phenolic constituents was also studied.

Results: It was observed that in CCl_4 intoxicated group SGPT, SGOT, serum bilirubin levels were elevated, and the total protein content was decreased when compared to the control group. Administration of isolated phenolic constituent at the dose of 25 mg/kg.p.o. reduced these pathological damages caused by CCl_4 intoxication compare to normal, and silymarin treated groups. The results were further supported by histopathology of isolated phenolic constituent treated rat liver, which showed the presence of normal hepatic cords, absence of necrosis and fatty infiltration.

Conclusion: The present study has justified that the isolated phenolic constituent (gallic acid) exhibited significant hepatoprotecitve potential against CCl_4 induced toxicity in Albino rats, thus enabling to expand the spectrum of novel hepatoprotecitve formulations.

Keywords: *Habenaria intermedia*, Gallic acid, Hepatoprotecitve, Carbon tetrachloride toxicity, Silymarin.

INTRODUCTION

Hepatic damage is a global metabolic and epidemic disease affecting essential biochemical activities in almost every age group [1]. Therapies developed along the principles of western medicine are sometimes limited in efficacy, have adverse effects, expensive especially for the developing world. Drug-related hepatotoxicity is the leading cause of acute liver failure among patients referred for liver transplantation due to intentional or unintentional overdose of drugs in the United States [2]. Plants are a natural source of biologically active compounds known as phytonutrients. Phytonutrients are secondary metabolites that have either defensive or disease preventive properties [3]. In spite of the tremendous advances made, no significant and safe hepatoprotecitve assets are available in modern therapeutics. Therefore, there is a great demand for the development of new effective drugs and due importance has been given globally to develop plant based hepatoprotecitve drugs effective against various liver disorders. Liver protective plants contain a variety of chemical constituents such as phenol, coumarins, lignans, essential oil, carotinoids, glycosides, flavanoids, organic acids, lipids, xanthenes [4]. Recent experience has revealed that drugs are comparatively non-toxic, safe and even free from side-effects [5]. There are reports about hepatoprotecitve effects and of *Silybum marianum* and *Cichorium intybus* extract on liver cells due to the presence of polyphenols and their antioxidant extracts [6].

In our previous study, the phytochemical investigations of ethanolic extract of tuber of *Habenaria intermedia* showed the presence of phenolic constituent, by preliminary test and thin layer chromatography (TLC) studies, which exhibited hepatoprotecitve potential [7]. Therefore, the present protocol is designed for isolation and characterization of phenolic component from ethanolic and action of *H. intermedia* and evaluated for hepatoprotecitve activity.

METHODS

Preparation of crude extract

About 4 kg of tubers of *H. intermedia* was extracted with 90% ethanol. The crude residue obtained was evaporated and dried. The total yield was 10.5 g. The residue was used for the separation of phenolic constituent by column chromatography [8].

Isolation of phenolic component by column chromatography

About 5 g of ethanolic fraction was subjected to acid hydrolysis with 2M HCl for 0.5 hrs. Extraction was carried out with ether (10 ml aliquots, 3 times), evaporated to get light yellow colored residue.

Adsorbent	Silica gel (60-120 mesh size)
Activation	110°C for 1 hr
Length of the column	45 cm
Diameter	Outer - 4.2 cm, inner - 3.8 cm
Length of the adsorbent	30 cm
Rate of elution	12-18 drops/minute
Volume of elute collected	10 ml each
Type of elution	Gradient elution

Preparation of sample for column

A total of 2 g of residue from ethanolic fraction was dissolved in 10 ml of methanol and mixed with 2 g of silicagel (60-120 mesh size) and dried in a vacuum oven at 45°C. The adsorbed material was then transferred to the column and elution was carried out by gradient method.

Gradient elution

Gradient elution was carried out by using ethyl acetate, ethyl acetate: benzene in different proportion. The elution rate was adjusted to 10 ml/minutes. Different fractions like 1-10, 11-20, 21-50, 51-75, 76-100, 101-150, 151- 200, 201-225, 226-250, 251-275, 276-300, 301-325 were eluted. TLC studies carried out using ethyl acetate:benzene (90:10) as mobile phase and Folin-ciocalteu/ammonia as spreading agent for all the fractions. The fractions 1-200 did not exhibit any spots. However in between 201 and 225 fraction exhibited single spot with Rf value (0.4), which turned blue with Folin reagent and ammonia. Fractions with similar spots were pooled together and concentrated at reduced pressure and temperature. The concentrated component after evaporation resulted in bust white colored powder represented in Table 1.

Phytochemical test for isolated phenolic compound

Ferric chloride test

To the test solution, freshly prepared ferric chloride solution was added-bluish black coloration was observed.

Characterization of phenolic compound

Structure of an isolated compound MG-2 was established based on infrared (IR), ^{1}H nuclear magnetic resonance (NMR) and mass spectral studies.

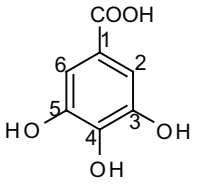
Spectral data of MG-2

Buff white colored powder (fluorescent under ultraviolet), 105 mg, Rf 0.40, MP 249°C, IR spectrum exhibited a characteristic peak at 1612/cm which was due to carbonyl peak of COOH group. A broad peak at 3387/cm was due to hydroxyl groups.

^{1}HNMR spectrum displayed a singlet at δ 6.91 (2-H) was assigned to two aromatic protons at C_2 and C_6. Two hydroxyl groups comes to resonate at δ 9.18, broad singlet corresponds to two −OH groups at C_3 and C_5. The other hydroxyl group comes to resonate at δ 8.83 broad singlet corresponds to this OH group at C_4. The carboxylic acid −OH group also appeared as broad singlet at δ 12.23 respectively (Fig. 1).

The mass spectrum of MG-2 showed a molecular ion peak at m/z 169.0, which was due to its molecular formula ($C_7 H_6 O_5$) and molecular weight (Fig. 2).

Hence, by comparing the spectral data obtained and earlier reported data the structure assigned to new phenolic compound (MG-2) found in *H. intermedia* was in good agreement with phenolic acid, which is confirmed as gallic acid.



MG-2

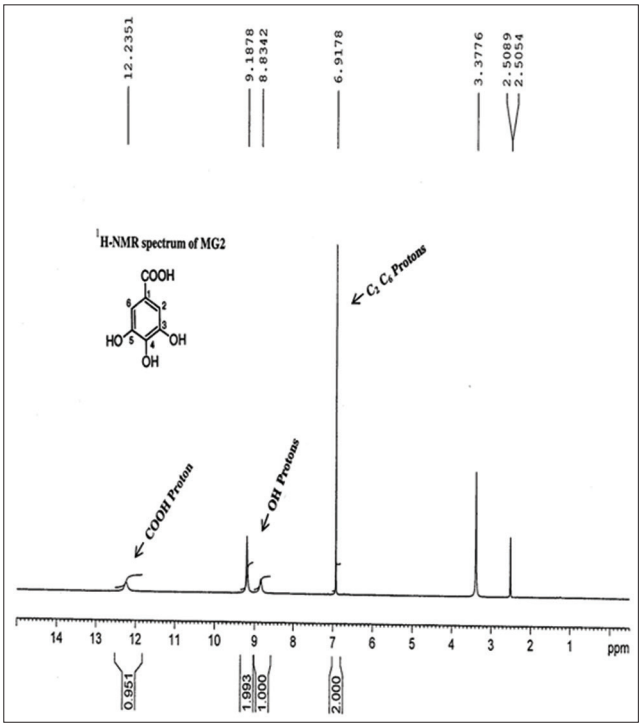


Fig. 1: 1H nuclear magnetic resonance spectrum of MG_2

Table 1: Elution of phenolic acid from *H. intermedia* D. Don.

Fraction no	Composition and proportion of the solvent system (%)	Color of the elute	TLC studies solvent system	Number of spots and Rf values	Yield
1-10	Ethyl acetate (100)	No color	Ethyl acetate:benzene (9:11)	No spot	_
11-20	Ethyl acetate:benzene (99-1)	No color	Ethyl acetate:benzene (9:11)	No spot	_
21-50	Ethyl acetate:benzene (98-2)	No color	Ethyl acetate:benzene (9:11)	No spot	_
51-75	Ethyl acetate:benzene (96-4)	No color	Ethyl acetate:benzene (9:11)	No spot	_
76-100	Ethyl acetate:benzene (96-4)	No color	Ethyl acetate:benzene (9:11)	No spot	_
101-150	Ethyl acetate:benzene (90-10)	Light brown	Ethyl acetate:benzene (9:11)	No spot	_
151-200	Ethyl acetate:benzene (85-15)	No color	Ethyl acetate:benzene (9:11)	No spot	_
201-225	Ethyl acetate:benzene (80-20)	Pale yellow	Ethyl acetate:benzene (9:11)	Single spot (0.4) blue spot with Folin reagent and ammonia	105 mg
226-250	Ethyl acetate:benzene (75-25)	Light yellow	Ethyl acetate:benzene (9:11)	No spot	_
276-300	Ethyl acetate:benzene (50-50)	No color	Ethyl acetate:benzene (9:11)	No spot	-
301-325	Ethyl acetate:benzene (25-75)	Light green	Ethyl acetate:benzene (9:11)	No spot	

H. intermedia: Habenaria intermedia, TLC: Thin layer chromatography

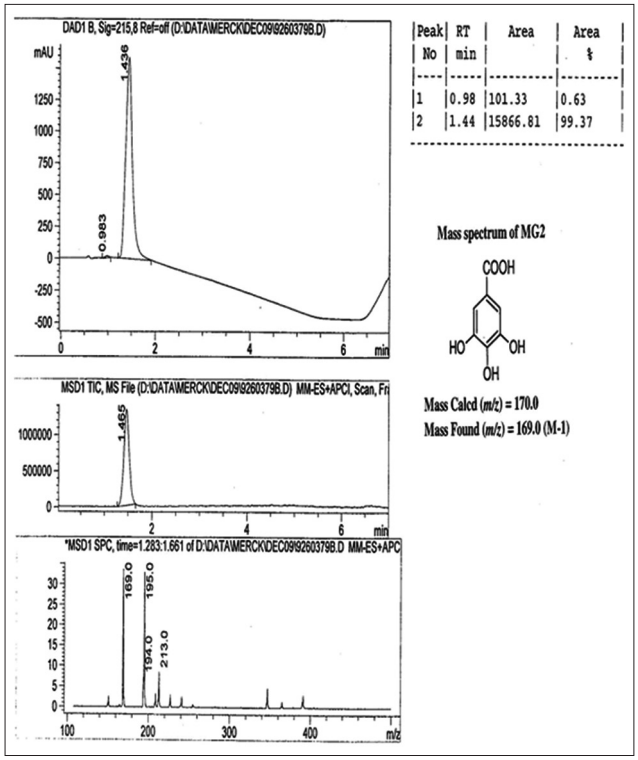


Fig. 2: Mass spectrum of MG_2

Though gallic acid is reported to be present in various medicinal plants, this is the first report of its presence in the tubers of *H. intermedia*.

Experimental protocol

Rats were divided into four groups comprising of six animals in each group. Group I served as normal control and received normal saline (5 ml/kg.p.o.) for 7 days. Group II was administered with carbon tetrachloride (CCl_4) in liquid paraffin (0.7 ml/kg.p.o, 1:1, v/v, i.p, on alternate days) [9,10]. Group III was administered with silymarin (100 mg/kg.p.o.) simultaneous with toxicant [11]. Group IV was treated with gallic acid (25 mg/kg.p.o.). Suspension of gallic acid was prepared by tween-80 and distilled water (2:8).

Assessment of hepatoprotecitve activity

On the 7th day after administration of the last dose of gallic acid, the rats were anesthetized by light ether anesthesia and blood was collected from the retro-orbital plexus. It was allowed to coagulate for 30 minutes, and serum was separated by the cold centrifugation at 2500 rpm for 15 minutes. The centrifugate was used to estimate the serum glutamic-pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT) [12], serum bilirubin [13] and total protein content [14]. Finally, the rat liver were isolated and subjected to histopathological observations.

RESULTS

Statistical analysis

The data were expressed as mean standard error mean (n=6). The data were analyzed using one-way ANOVA, followed by multiple comparison tests. p<0.01 were considered statistically significant [15].

Rats treated with CCl_4 (0.7 ml/kg body weight) suffered from hepatotoxicity. The serum levels of SGPT, SGOT and bilirubin level were significantly elevated and protein level was significantly decreased as shown in Table 2 and Figs. 3 and 4. Pretreatment with scopoletin (25 mg/kg.p.o) for 7 days significantly decreased enzyme levels and bilirubin levels. Meanwhile it showed the increase in protein content in the blood, when compared to control and CCl_4 treated group (p<0.01) and (p<0.001). Results were also comparable with standard drug Silymarin (100 mg/kg.p.o).

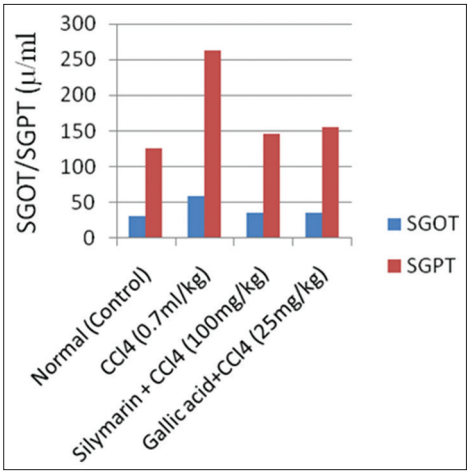


Fig. 3: Effect of gallic acid on biochemical parameters in carbon tetrachloride induced hepatotoxicity, effect on serum glutamic oxaloacetic transaminase/serum glutamic-pyruvic transaminase

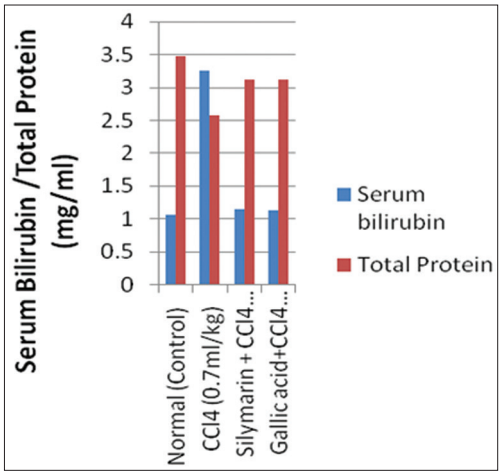


Fig. 4: Effect of gallic acid on biochemical parameters in carbon tetrachloride induced hepatotoxicity, effect on serum bilirubin/ total protein

Table 2: Effect of isolated compound of *H. intermedia* on CCl_4 induced hepatotoxicity

Groups	SGPT (µ/ml)	SGOT (µ/ml)	Serum bilirubin (mg/ml)	Total protein (mg/ml)
Control	125.65±1.25	30.68±4.48	1.06±0.18	3.475±0.28
CCl4 (0.7 ml/kg)	252.3±3.12*	59.62±3.64*	3.25±0.13*	2.580±0.21*
Silymarin+CCl_4 (100 mg/kg.p.o)	145.52±2.08[a]	35.56±0.89[a]	1.16±0.08[b]	3.115±0.23[a]
Gallicacid+CCl_4 (25 mg/kg)	156.10±1.48[b]	36.26±1.56[a]	1.14±0.21[b]	3.115±0.28[b]

p<0.01 against normal control, [a]p<0.01 against normal control, [b]p<0.001 against hepatotoxic control, *H. intermedia: Habenaria intermedia*, SGPT: Serum glutamic-pyruvic transaminase, SGOT: Serum glutamic oxaloacetic transaminase, CCl4: Carbon tetrachloride

Histopathological observations

Histopathology of normal rat liver revealed prominent central view, the normal arrangement of hepatic cells (Fig. 5). Microscopic examination of

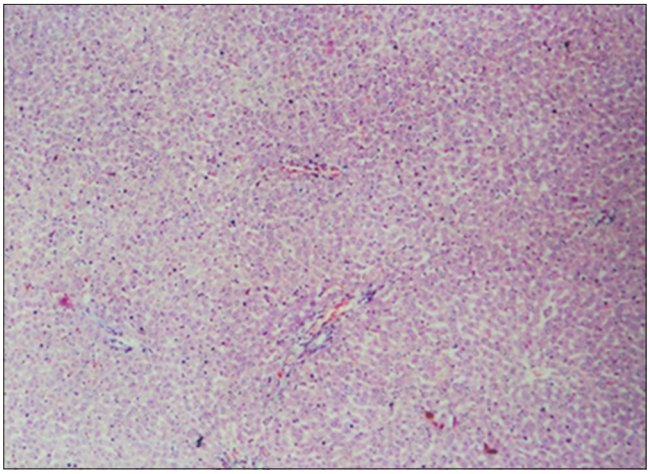


Fig. 5: Liver tissues of different groups of Albino rats, normal group

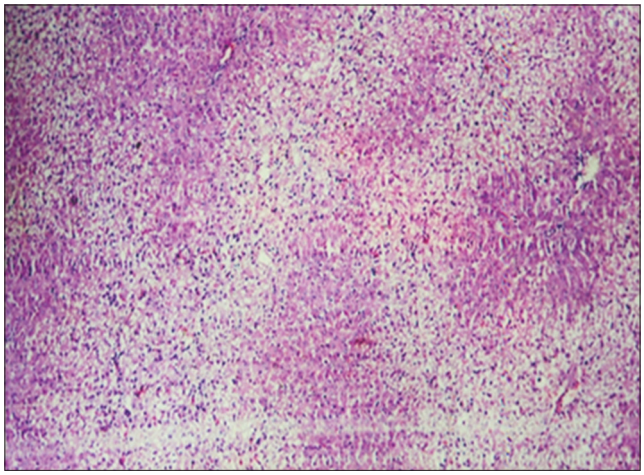


Fig. 6: Liver tissues of different groups of Albino rats, carbon tetrachloride treated group

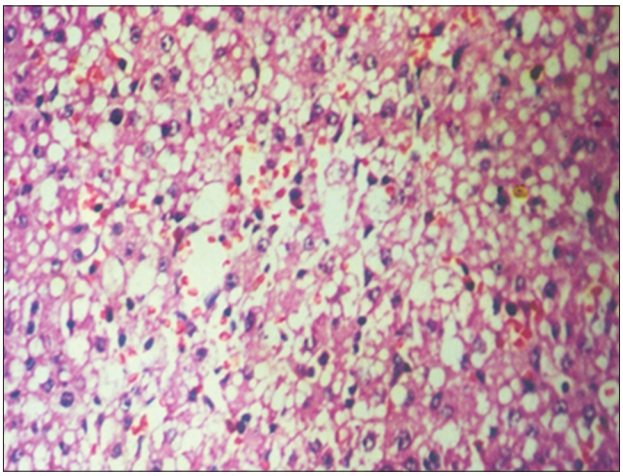


Fig. 7: Liver tissues of different groups of Albino rats, silymarin treated group

CCl_4 treated liver section shows necrosis and fatty degeneration (Fig. 6). Liver section treated from silymarin protected the structural integrity of hepatocyte cell membrane and recovery of hepatocyte cells (Fig. 7). Gallic acid treated group showed maximum recovery of hepatocytes, no fatty degeneration, and necrosis and exhibited significant protection against CCl_4 induced liver toxicity in rats (Fig. 8).

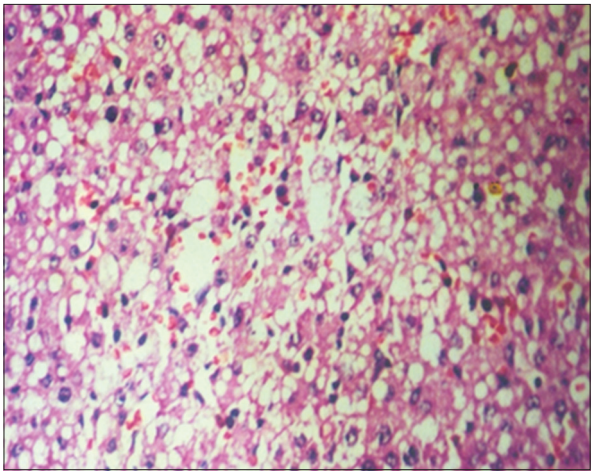


Fig. 8: Liver tissues of different groups of Albino rats, gallic acid treated group

DISCUSSION

CCl_4 may cause liver damage due to the accumulation of fat (fatty liver), inflammation and centrilobular necrosis [16]. The liver damage is caused due to a variety of reasons such as drugs, toxic chemicals alcohol, and viruses. To induce hepatotoxicity, a convenient agent should be chosen amongst various toxicants, especially known to cause hepatic damage. The chemical agent of recurring incidences to cause hepatotoxicity is CCl_4 [17].

In the present study, CCl_4 treated group exhibited significant rise in the enzyme levels of SGOT and SGPT and in bilirubin level and decrease in protein level. The liver damage may be due to necrosis or fatty accumulation in the liver. The enzymes are sensitive to hepatic dysfunction after liver damage protein levels are decreased due to deficiency [18,19]. However, gallic acid treated group showed significant protection against CCl_4 induced liver toxicity by decreasing the elevated enzyme levels and in bilirubin level and increase in the protein content to normal value in the blood.

CONCLUSION

The overall results indicate that isolated compound gallic acid from *H. intermedia* plays a significant role in restoring the disturbed liver function in CCl_4 induced hepatitis. Results were further supported by histopathology of rat liver. The present liver-protective effect of gallic acid could be through preventing the accumulation of excessive free radicals and glutathione-mediated detoxification.

ACKNOWLEDGMENT

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Ethical clearance

The research work was approved by Institutional Animal Ethical Committee (NCP/IAEC/CLEAR/25/02/2009-10, dated 09/03/2010).

REFERENCES

1. Divya B, Praneetha P, Swaroopa RV, Ravi Kumar B. Hepatoprotective effect of whole plant extract fractions of *Marsilea minuta* Linn. Asian J Pharm Clin Res 2013;6(3):100-7.
2. Lee MM. Drug induced acute liver failure in the United States results from the US Acute Liver Failure Study Group-FDA-PhRMA-AASLD. Hepatotoxicity Steering Committee Meeting; 2005.
3. Marimuthu A, Arumugum G, Mariappan A, Padmanabhan M. Determination of secondary metabolites, LD50 value and antioxidant

of seed extract of *Cucurbita pepo* Linn. Asian J Pharm Clin Res 2014;7(3):173-7.
4. Sharma SK, Ali M, Gupta JE. Plants having hepatoprotective activity. Phytochem Pharmacol 2002;2:253-70.
5. Momin A. Role of Indigenous Medicines in Primary Health Care. New Delhi, India: International Seminar on Unani Medicine; 1987. p. 54.
6. Madani H, Talebolhosseini M, Asgary S, Nader GH. Hepatoprotective activity *of Silbum marianum* and *Cichorium intybus* against thi acetamide in rats. Pak J Nutr 2008;7(1):172-6.
7. Goudar MA, Jayadevappa H, Mahadevan KM, Shasthry RA, Sayeswara HA. Hepatoprotecitve potential of tubers *Habenaria intermedia* D. Don against carbon tetrachloride induced hepatic damage in rats. Int J Curr Res 2014;6(11):10090-7.
8. Lee KJ, Wodo R, Choic CR. Protective effects of acetoside on CCl_4 induced hepatotoxicity. Life Sci 2004;74:1051-6.
9. Majumdar AB, Anuradha S, Pradhan U. Effect of *Azdirachta india* leaf extract on CCl_4 induced hepatic damage in Albino rats. J Indian Pharm Sci 1998;60:363-8.
10. Dahanukar RN. Hepatoprotecitve effects of *Tinispora*. Indian Drugs 1984;21:544-8.
11. Rietman. Colorimetric method for determination of SGOT and SGPT. Am J Clin Path 28;56-63.
12. King EJ. A method for determination of tartarate labile prostatic acid. Am J Clin Pathol 1959;12:85.
13. Jendrassik FL, Grof P. Manual of medical lab techniques. Biochemistry 1938;81:297-301.
14. Galigher AE. Querctive treated ups showed significant protection against CCl_4 induced liver toxicity. India Drugs Phila 1971;18:77-80.
15. Dunnet CW. New tablets for multiple comparisons with a control. Biometrics 1964;20:482-91.
16. Ambay RY, Vanmala SJ. Overview of hepatitis. Indian Drugs 2002;1:39-45.
17. Turner RA. Screening Methods in Pharmacology. New York: Academic Press; 1965. p. 103-14.
18. Farber JL, El-Mofty SK. The biochemical pathology of liver cell necrosis. Am J Pathol 1975;81(1):237-50.
19. Chaudan CK, Nanewadekar SA, Billimoria FR. Effect of herbal hepatoprotecitve product on drug metabolism in patients of cirrhosis and hepatic enzyme function in experimental liver damage. Indian J Pharmacol 1992;24(2):107-10.