


Evaluation of Antioxidative and Hepatoprotective Activity of Leaves of *Holarrhena pubescens* Wall ex.G.Don

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<p>*For Correspondence: Lachoo Memorial College of Science & Technology (Autonomous), Pharmacy Wing, Jodhpur (Raj.), India</p>	<p>ABSTRACT</p> <p>Objective: The objective of the study is to screen of various phytochemicals and to evaluation the antioxidative and hepatoprotective activity of leaves of holarrhena pubescens wall (holarrhena antidysenterica). Material and Method: The antioxidant activity was determined by DDPH (2, 2-diphenyl-1-1-picrylhydrazyl) radical scavenging activity where ascorbic acid was control. The antioxidant property was later exploited and the ethanolic extract of plant was tested for hepatoprotective activity in CCl₄ induced albino rats. The extract was tested for its hepatoprotective activity at three doses 100, 200 and 300 mg/kg respectively where silymarin 25 mg/kg was taken as standard reference drug. All results are presented as mean+SD (Standard Deviation). Significant differences between experimental groups were determined by tukey's test. Results: The ethanolic extract showed strong antioxidant activity with inhibition of more than 90% DDPH free radicals at concentration of 80µg/ml. The hepatoprotective activity of ethanolic extract on CCl₄ induced albino rats test were significant ($p \leq 0.05$) for effect of 300 mg/kg after 24hrs of treatment and ($p \leq 0.01$) for 100 mg/kg of extract after half hour of treatment compared to control. Conclusion: The presence of flavonoids, phenolic compound suggested that they may be partially responsible for antioxidant and hepatoprotective activity.</p> <p>Keywords Antioxidant, Hepatoprotective, DDPH, Holarrhena pubescens, Holarrhena antidysenterica..</p>
<p>Received: 14.07.2017 Accepted: 22.12.2017</p>	
<p>Access this article online</p>	
<p>Website: www.drugresearch.in</p>	
<p>Quick Response Code:</p> 	

INTRODUCTION

Hepatoprotective drugs mean the drugs that are prevent the liver disease. The liver is an organ that is located in the upper right side of the abdomen [1]. The *H. pubescens* traditionally useful in anemia, colic pain, diarrhea, asthma and colic pain, chronic bronchitis, Colitis, stomachic, tonic [2] and liver disorders [3]. Phytoconstituents of *Holarrhena pubescens* around 30 alkaloids have been isolated from the plant, mostly from the bark. These include conessine, kurchine, kurchicine, holarrhimine, conarrhimine, conaine, conessimine, iso-conessimine, conimine, holaceticin and conkurchin [4].



Material and Method

Collection of plant material

Holarrhena pubescens was collected from AFRI, Jodhpur. The plant and leaves were identified and authentication from Botanical Survey of India (BSI), Jodhpur. A voucher specimen of the plant was deposited in BSI, under Number BSI/AZRC/I.1214/Tech./2016-17-(Pl.Id.)/505.

Extract of plant material

Leaves of *Holarrhena pubescens* were washing with water and dried under shades after the complete drying, dried leaves were crushed for further procedure.

Cold maceration method

The 70 gm dried crushed leaves of *Holarrhena pubescens* were soaked in 450 ml of ethanol in conical flask with ethanol and crushed leaves extraction. The flask for ethanol was covered with aluminum foil & allowed to stand for 7 days for extraction with occasionally shaking. Then extract were filtered through muslin cloth. The filtered was allowed to evaporate ethanol till complete drying and then stored in close container.



Figure: Ethanolic extract of *H. pubescens* using cold maceration method

The filter is dried and extract was stored at room 4 gm and extraction yield was calculated.

Amount of crushed leaves used = 70 g

Amount of Extract = 4 g

% Extractive yield = (weight of extract/weight of crushed leaves) x 100

$$= (4/70) \times 100$$

$$= 5.7 \%$$

Table: Percentage yield of alcoholic extract

Extract	Appearance	Odour	Percentage yield
Ethanollic Extract	Brown	Characteristic	5.7 %

Result: The percentage yield of Ethanollic extract of *H. pubescens* of leaves was found to be 5.7 %.

Animal and treatment

Healthy albino rats (150-200 gm) of either sex received from animal house of Lachoo Memorial College of Science and Technology, Jodhpur (Autonomous). The animals were housed in conducive environmental situation i.e. temperature ($25 \pm 2^{\circ}$ c) Humidity (45-55%) and 12 hrs of dark and light cycle. The animals were fed ad libitum with normal laboratory chow standard pellet diet. The animals were allowed to acclimatize for 7 days before commencing the experiment. The study was conducted in accordance with the Institutional Animal Ethical Committee (No. 1719/PO/ERe/S/13/CPCSEA). The protocol was prepared and approved by IAEC of Lachoo Memorial College of Science and Technology, Jodhpur (Autonomous).

Pharmacological screening

Drugs, chemicals and devices required

- Silymarin (silybon)
- Carbon tetrachloride (CCl₄)
- Chloroform
- Olive oil
- Formalin
- Trinocular upright microscope
- DPPH (2, 2-diphenyl-1-picryldrazyl)
- DMSO
- Ascorbic acid
- Methanol

Evaluation of hepatoprotective activity

Pharmacological evaluation of acute and chronic hepatoprotective activity of ethanollic extract of leaves of *holarrhena pubescens* in CCl₄ induced hepatotoxicity in rats

Experimental design of hepatoprotective activity of extract for acute model

Rats were divided in 6 groups for acute models containing 3 animals in each group. Group I and II serve as positive control (vehicle treated) and negative control respectively and received 1 ml normal saline orally. Group III served as standard receiving silymarin (25 mg/kg, p.o.) suspended in 1ml normal saline ^[5]. Group IV, V and VI received 100, 200 and 300 mg/kg of *H. pubescens* ethanollic leaves extract suspended in 1ml normal saline respectively as shown in tables (a). All the treatment was given once a day for 7 days in acute models. Carbon tetrachloride (CCl₄) 1 ml/kg i.p. (1:1 in olive oil) was administered on the seventh days, after the administration of respective treatment to all the animals of group except group I (positive control group) ^[6]

Experimental design of hepatoprotective activity of extract for chronic model

For chronic study experimental design was same as acute models table (b). Rats were divided in 6 groups containing 3 animals in each group. All the treatment were given once a day for 14 days CCl₄ 1 ml/kg, i.p. (1:1 in olive oil) was administration on the seventh and fourteenth day for chronic model to all the groups of animals except group-I (positive control group)^[7]

Preparation of plant extracts and drug dilute solution

Extraction

Continuous cold maceration method was used for the extraction of dried leaves of *H. pubescens*

Drug dilution

Standard: silymarin (silbyon) 25 mg/kg which was suspended in 1ml of normal saline.

Test drug: Different doses (100, 200 and 300 mg/kg) which were suspended in 1 ml of 1% normal saline.

Table (a): Experimental design for hepatoprotective activity for acute model

Groups	Treatment
Group I	Positive control (Normal)
Group II	Negative control CCl ₄ 1 ml/kg in olive oil (1:1) i.p + vehicle
Group III	Silymarin (standard) 25 mg/kg) suspended in 1 ml of normal saline +CCl ₄
Group IV	HP 100 (100 mg/kg ethanolic extract of leaves of <i>H. pubescens</i> suspended in 1 ml of normal saline; p.o.) + CCl ₄ 1 ml/kg in olive oil (1:1) i.p
Group V	HP 200 (200 mg/kg ethanolic extract of leaves of <i>H. pubescens</i> suspended in 1 ml of normal saline; p.o.) + CCl ₄ 1 ml/kg in olive oil (1:1) i.p
Group VI	HP 300 (300 mg/kg ethanolic extract of leaves of <i>H. pubescens</i> suspended in 1 ml of normal saline; p.o.) + CCl ₄ 1 ml/kg in olive oil (1:1) i.p

Table (b): Experimental design for hepatoprotective activity for chronic model

Groups	Treatment
Group I	Positive control (Normal)
Group II	Negative control CCl ₄ 1 ml/kg in olive oil (1:1) i.p + vehicle
Group III	Silymarin (standard) 25 mg/kg) suspended in 1 ml of normal saline +CCl ₄
Group IV	HP 100 (100 mg/kg ethanolic extract of leaves of <i>H. pubescens</i> suspended in 1 ml of normal saline; p.o.) + CCl ₄ 1 ml/kg in olive oil (1:1) i.p
Group V	HP 200 (200 mg/kg ethanolic extract of leaves of <i>H. pubescens</i> suspended in 1 ml of normal saline; p.o.) + CCl ₄ 1 ml/kg in olive oil (1:1) i.p
Group VI	HP 300 (300 mg/kg ethanolic extract of leaves of <i>H. pubescens</i> suspended in 1ml of normal saline; p.o.) + CCl ₄ 1 ml/kg in olive oil (1:1) i.p

Collection of blood for acute and chronic model

After 24 hrs. of CCl₄ treatment at the 8th day of acute and 14th day of chronic models, animals were anaesthetized by ^[8] chloroform and blood samples were collected. Approximately 2 ml of blood was collected through the heart puncture. Blood samples were collected in vacuoners ^[9].

Estimation of biochemical parameters for acute and chronic models

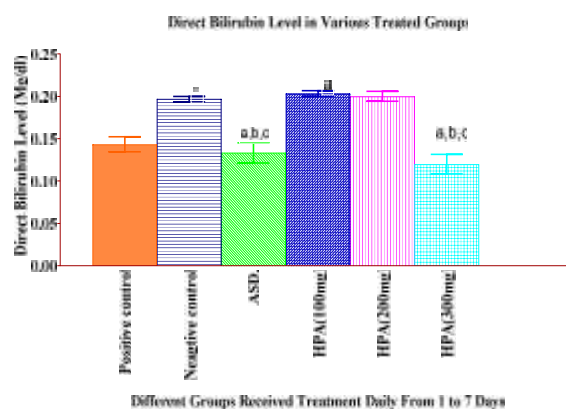
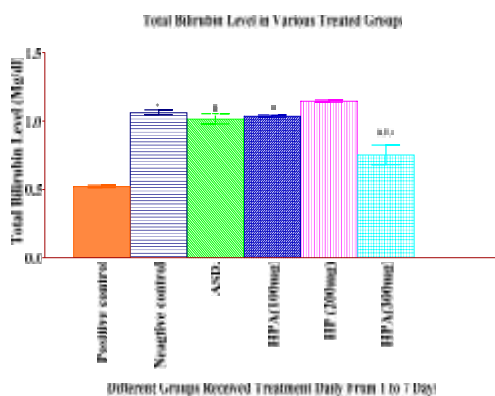
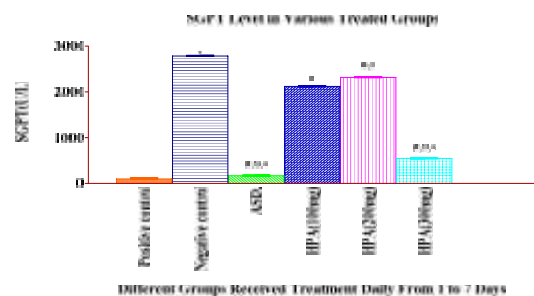
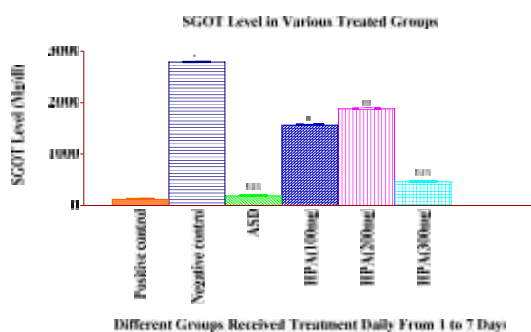
Collected blood samples were used for the analysis of biochemical parameters SGOT, SGPT, Bilirubin (total and direct) and for hepatoprotective activity of acute and chronic models.

Statistical analysis

Data are expressed as Mean \pm SEM and analyzed statistically by one-way analysis of variance (ANOVA), followed by Tukey's test at $p \geq 0.05$

Effect of ethanolic extract of leaves of *H. pubescens* on biochemical parameters in carbon tetrachloride induced hepatic injury in rats for acute models

Groups \ Parameters	Positive Control	Negative Control	Silymarin +CCl ₄	HP 100 +CCl ₄	HP 200 +CCl ₄	HP 300 +CCl ₄
SGOT (U/L)	124.6± 1.69	2792.6± 1.24*	191.3± 3.704 ^{a,b,c}	1569± 0.81 ^a	1883± 0.81 ^{a,b}	464.6± 1.02 ^{a,b,c}
SGPT(U/L)	108.6± 2.46	2788± 2.94*	174.3± 5.55 ^{a,b,c}	2124.6± 1.02 ^a	2323± 0.81 ^{a,b}	552± 0.81 ^{a,b,c}
Total bilirubin (mg/dl)	0.533± 0.06	1.06± 0.01*	1.01± 0.004 ^b	1.05± 0.05 ^a	1.14± 0.01	0.72± 0.05 ^{a,b,c}
Direct bilirubin (mg/dl)	0.14± 0.006	0.203± 0.006*	0.14± 0.014 ^{a,b,c}	0.20± 0.01 ^a	0.20± 0.01	0.13± 0.01 ^{a,b,c}



Data are expressed as Mean±SEM and analyzed statistically by one-way analysis of variance (ANOVA), followed by Tukey's test at P≤0.05. ASD= acute standard, HP=*H. pubescens*

*= Significant increase in levels by CCl₄ in comparison to control

a= Significant (P≤0.05) hepatoprotective activity in comparison to negative control group

b=Significant (P≤0.05) hepatoprotective activity in comparison to HP 100

c=Significant (P≤0.05) hepatoprotective activity in comparison to HP 200

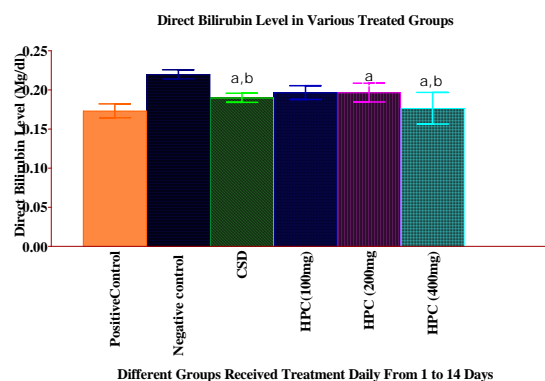
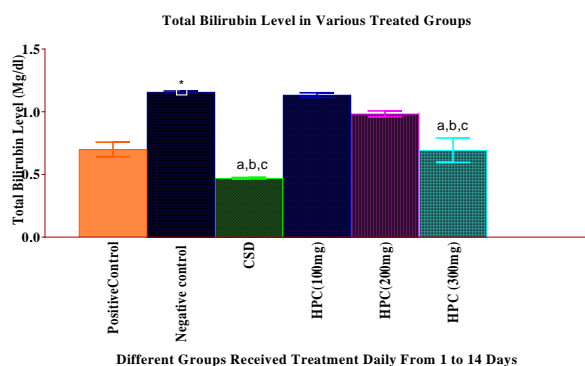
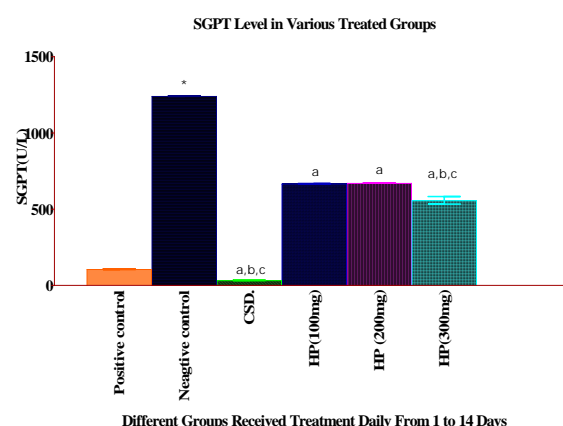
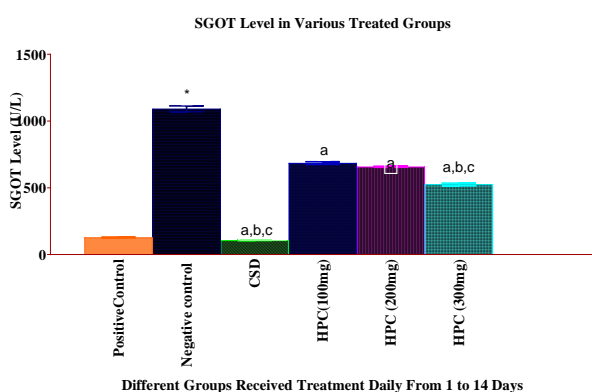
Results and discussion:

The results of our study on various biochemical parameters such as SGOT, SGPT, total bilirubin and direct bilirubin for acute model are depicted in table (a) and figure. In this study plant extract was found to have hepatoprotective activity against CCl₄ induced hepatotoxicity. CCl₄ is metabolized by

cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of CCl_3O^- , a reactive oxidative free radical, which initiates lipid peroxidation, leading to liver damage. ^[10, 11] Administration of a single dose of CCl_4 produces a centrilobular necrosis and fatty changes, within 24 hrs ^[12, 13]. Elevation in the biochemical parameters such as SGOT, SGPT and Bilirubin (total bilirubin and direct bilirubin) is conventional indicator of liver injury. ^[14, 15] In this study, it was seen that positive control group of rats showed normal serum level of SGOT (124.6 U/L) and SGPT (108.6 U/L). The serum level of SGOT (1883 U/L) and SGPT (2788 U/L) 24 hrs after administration of CCl_4 (1ml/kg, i. p.) were significantly increased as compared to positive control group. When compared with negative control group the serum SGOT and SGPT were significantly decreased in Standard SGOT (191.3 U/L) and SGPT (174.3 U/L), HP 100 mg/kg SGOT (1569 U/L) and SGPT (2124.6 U/L), HP 200 mg/kg SGOT (1883 U/L) and SGPT (2323 U/L) and HP 300 mg/kg SGOT (464.6 U/L) and SGPT (552 U/L). When compared to HP 100 mg/kg, significant decrease in SGOT and SGPT level was observed in standard and HP 300 mg/kg. When compared to HP 200 mg/kg, serum level of SGOT was found to be significantly decreased in standard, HP 300 mg/kg. Thus we can say that result was dose dependent and HP 300 shows better activity than HP 200 and HP 100 mg/kg while standard shows better effect. The study revealed that the exposure of CCl_4 causes release of SGOT and SGPT enzymes from the cells, thus significantly increases their level in blood, due to hepatocyte necrosis or abnormal membrane permeability ^[16]. Oral administration of HP at different doses (100, 200 and 300 mg/kg) attenuated the increased level of these serum enzymes produced by CCl_4 and caused a subsequent recovery towards the normal values. The result suggests that the extract protected the membrane integrity of the liver cells against CCl_4 induced leakage of serum enzymes into the circulation. When compared the total bilirubin level in all group of rats, it was found that positive control group of rats showed normal level of total bilirubin (0.53 mg/dl), Negative control group showed the significant increase in level of total bilirubin (1.06 mg/dl), when compared to positive control group. When compared with negative control group the level of total bilirubin was significantly decreased HP 300 mg/kg total bilirubin (0.72 mg/dl). When compared to HP 100 mg/kg, significant decrease in total bilirubin was observed and HP 300 mg/kg. When compared to HP 200 mg/kg, serum level of total bilirubin was found to be significantly decreased in standard, HP 300 mg/kg. Thus we can say that result was dose dependent and HP 300 shows better activity than HP 200 and HP 100 mg/kg while standard shows. HP 300 mg/kg shows significant decrease in total bilirubin level as compared to Negative Control and all other treatments. The CCl_4 induced elevation of Total bilirubin in the serum is lined up with high level of serum bilirubin. Administration of ethanolic extract of HP (100 and 300 mg/kg) decrease bilirubin level and stabilized normal functions of the liver ^[17]. When compared the direct bilirubin level in all group of rats, it was found that positive control group of rats showed normal level of direct bilirubin (0.14 mg/dl), Negative control group showed the significant increase in level of direct bilirubin (0.203 mg/dl), when compare to positive control group. When compared to negative control group the level of direct bilirubin was significantly decreased in Standard direct bilirubin (0.14 mg/dl) and HP 100, HP 300 mg/kg direct bilirubin (0.13 mg/dl). When compared to HP 100, significant decrease in direct bilirubin was observed in standard and HP 300 mg/kg. When compared to HP 200 mg/kg, serum level of direct bilirubin was found to be significantly decreased in standard, HP 300 mg/kg. HP 300 mg/kg shows better activity than HP 200 and HP 100 mg/kg. HP 300 mg/kg shows significant decrease in direct bilirubin level as compared to Negative Control and all other treatments. The CCl_4 induced elevation of direct bilirubin in the serum is lined up with high level of serum bilirubin. Administration of ethanolic extract of HP (100 and 300 mg/kg) decrease bilirubin level and stabilized normal functions of the liver ^[17].

Effect of ethanolic extract of leaves of *H. pubescens* on biochemical parameters in carbon tetrachloride induced hepatic injury in rats for chronic models

Groups	Positive Control	Negative Control	Silymarin +CCl ₄	HP 100 +CCl ₄	HP 200 +CCl ₄	HP 300 +CCl ₄
Parameters						
SGOT (U/L)	128 _± 0.81	1092 _± 15.43*	106.93 _± 1.26 ^{a,b,c}	685.3 _± 6.85 ^a	657.6 _± 2.77 ^a	525 _± 7.118 ^{a,b,c}
SGPT(U/L)	106.3 _± 1.64	1241.3 _± 0.62*	33 _± 0.408 ^{a,b,c}	667.3 _± 0.849 ^a	671.3 _± 0.623 ^a	558.3 _± 7.118 ^{a,b,c}
Total bilirubin (mg/dl)	0.7 _± 0.040	1.15 _± 0.006*	0.47 _± 0.004 ^{a,b,c}	1.13 _± 0.01	0.983 _± 0.68	0.693 _± 0.06 ^{a,b,c}
Direct bilirubin (mg/dl)	0.173 _± 0.119	0.22 _± 0.004	0.10 _± 0.006 ^{a,b}	0.196 _± 0.006	0.15 _± 0.01 ^a	0.116 _± 0.004 ^{a,b}



Data are expressed as Mean_±SEM and analyzed statistically by one-way analysis of variance (ANOVA), followed by Tukey's test at P≤0.05. ASD= acute standard, HP=*H. pubescens*

*= Significant increase in levels by CCl₄ in comparison to control

a= Significant (P≤0.05) hepatoprotective activity in comparison to negative control group

b=Significant (P≤0.05) hepatoprotective activity in comparison to HP 100

c=Significant (P≤0.05) hepatoprotective activity in comparison to HP 200

Results and discussion:

Administration of CCl₄ to albino rats on 7th and 14th day of experiment resulted in chronic hepatotoxicity as revealed from level of biochemical parameters shown in Table (b) and figure. In this study, it was seen that positive control group of rats showed normal serum level of SGOT (128 U/L) and SGPT (106.3 U/L). The serum level of SGOT (1092 U/L) and SGPT (1241 U/L) in negative control group, 24 hrs after administration of CCl₄ (1ml/kg, i. p.) were significantly increased as compared to positive control group. When compared to negative control group the serum level of SGOT and SGPT was significantly decreased in Standard SGOT (106.93 U/L) and SGPT (33 U/L), HP 100 mg/kg SGOT (685.3 U/L) and SGPT (667.3 U/L), HP 200 mg/kg SGOT (657.6 U/L) and SGPT (671.3 U/L) and HP 300 mg/kg SGOT (525 U/L) and SGPT (558.3 U/L). When compared to HP 100 mg/kg, significant decrease in SGOT and SGPT level was observed in standard and HP 300 mg/kg. When compared to HP 200 mg/kg, serum level of SGOT and SGPT was found to be significantly decreased in standard and HP 300 mg/kg. Thus we can say that result was dose dependent and HP 300 mg/kg shows better activity than HP 200 mg/kg and HP 100 mg/kg. The study revealed that the exposure of CCl₄ causes release of SGOT and SGPT enzymes from the cells, thus significantly increases their level in blood, due to hepatocyte necrosis or abnormal membrane permeability^[16]. Oral administration of HP at different doses (100, 200 and 300 mg/kg) attenuated the increased level of these serum enzymes produced by CCl₄ and caused a subsequent recovery towards to positive control group of rats. The result suggests that the extract protected the membrane integrity of the liver cells against CCl₄ induced leakage of serum enzymes into the circulation. When compared the total bilirubin level in all group of rats, it was found that positive control group of rats showed normal level of total bilirubin (0.7 mg/dl). Negative control group showed the significant increase in level of total bilirubin (1.15 mg/dl) when compare to positive control group. When compared to negative control group the level of total bilirubin was significantly decreased in Standard total bilirubin (0.47 mg/dl) and HP 300 mg/kg total bilirubin (0.693 mg/dl). When compared to HP 100 mg/kg, significant decrease in total bilirubin was observed in HP 300 mg/kg and standard. When compared to HP 200 mg/kg, serum level of total bilirubin was found to be significantly decreased in HP 300 mg/kg. Thus we can say that result was dose dependent and HP 300 mg/kg shows better activity than HP 200 and HP 100 mg/kg. When compared to the direct bilirubin level in all group of rats, it was found that positive control group of rats showed normal level of direct bilirubin (0.173 mg/dl),. Negative control group showed the significant increase in level of direct bilirubin (0.22 mg/dl), when compare to positive control group. When compared to negative control group the level of direct bilirubin was significantly decreased in Standard direct bilirubin HP 200 mg/kg (0.10 mg/dl), HP 300 mg/kg (0.116 mg/dl). When compared to HP 100 mg/kg, significant decrease in direct bilirubin was observed in HP 300 mg/kg and standard. When compared to HP 200 mg/kg, serum level of direct bilirubin was found to be similar as in HP 300 mg/kg. Thus we can say that result was and HP 300 shows better activity than HP 100 mg/kg while standard shows. HP 300 mg/kg shows significant decrease in direct bilirubin level as compared to Negative Control and all other treatments. Administration of ethanolic extract of HP (100 and 300 mg/kg) decrease bilirubin level and stabilized normal functions of the liver^[17].

Analysis of histopathology of livers were investigation in both acute and chronic model

After collection of the blood samples (8th day for acute and 15th day for chronic model), livers were excised from the animals of each group and washed with normal saline, then preserved in 10% formalin solution. The different section was examined microscopically for the evaluation of histopathological change by using trinocular upright microscope

Effect of ethanolic extract of leaves of *H. pubesens* on histopathology of liver for acute model

Histopathological section of positive control, negative control, standard and different doses of ethanolic extract of leaves of *H. pubescens* (100, 200 and 300 mg/kg) treated group of rats for acute models show in figure.

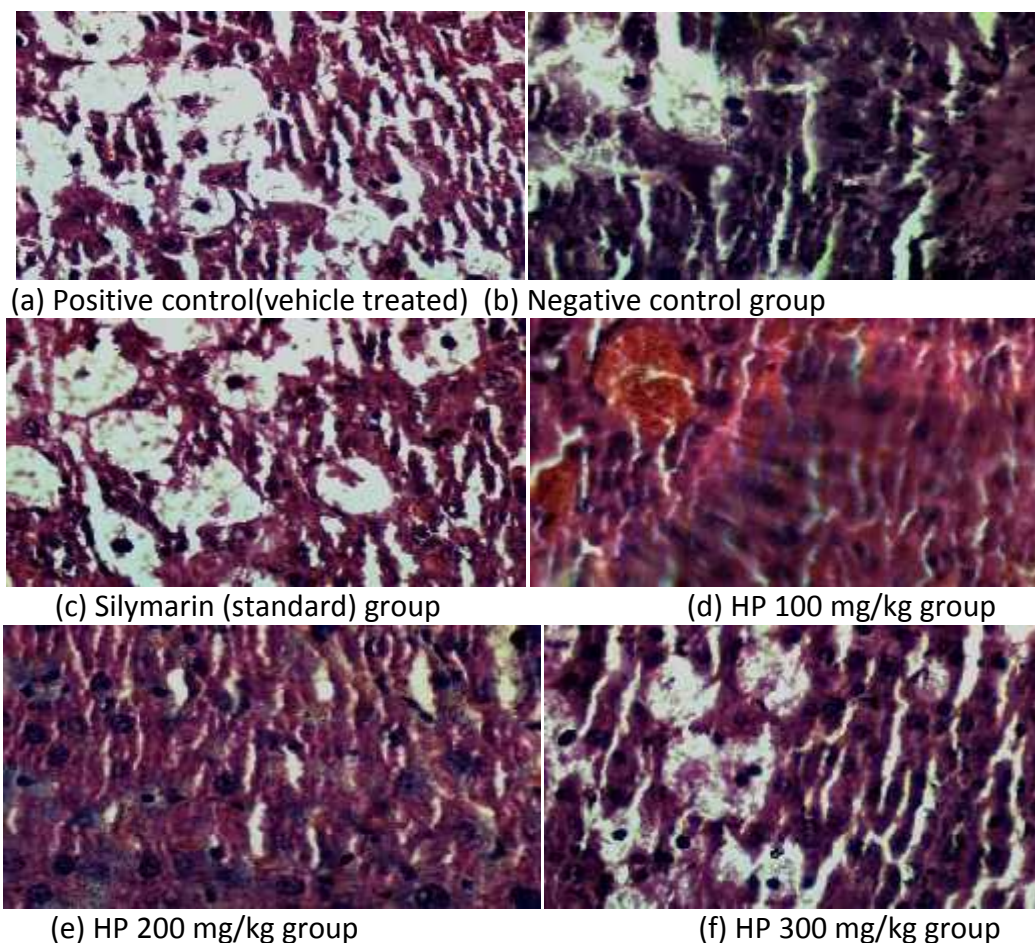


Figure: Histopathological section of the liver for acute model, magnification (HEX40)

Result and discussion:

Effect of ethanolic extract of leaves of *H. pubescens* on portal triad structure of rat liver for acute study

(a) Positive control: Positive control group of rats has shown the normal architecture of hepatocytes on portal triad structure of histopathological section of the liver cells and appeared as normally. However, very mild dilation of central vein appearance has been seen in histopathological section of the liver of this group, which did not reveal any significant lesion of pathological importance.

(b) Negative control: Negative control group of rats for acute study has conversely shown fatty vacuoles (appearance of losing cell nucleus and collection of glycogen) centrilobular congestion of sinusoids and kupffer cell hyperplasia with fibrosis, which indicates the development of early necrosis due to CCl_4 induced hepatic injury.

(c) Silymarin (25 mg/kg standard): Histological investigation of standard treated group of rats for acute study has shown a little fibrosis at central vein and sinusoidal space. Moreover, a normal arrangement of kupffer cells and hepatocytes cells showed protection against CCl_4 induced hepatic injury.

(d) HP 100 mg/kg group: The section of liver of dose HP 100 mg/kg group of rats for acute study showed kupffer cell and sinusoids space hyperplasia and central lobular necrosis with fibrosis.

Disturbed hepatocytes and sinusoids indicate the development of early necrosis and poor protection of hepatocyte cell of CCl₄ induced hepatic injury.

(e) HP 200 mg/kg group: Histological section of the dose HP 200 mg/kg group of rats for acute study showed the little dilation of central vein and sinusoidal space with reduction in necrosis area and inflammatory infiltrates. Fibrosis is also seen which indicate the slight protection against CCl₄ induced hepatic injury.

(f) HP 300 mg/kg group: The histopathological investigation of liver animal treated of dose HP 300 mg/kg for acute study shows that architecture of hepatocytes and kupffer cells is normal but mild fibrosis and central vein enlargement is seen. It indicates significant protection against CCl₄ induced hepatic injury. This effect is comparable with standard drug silymarin.

Effect of ethanolic extract of leaves of *H. pubescens* on histopathology of liver for chronic model

Histopathological section of positive control, negative control, standard and different doses of ethanolic extract of leaves of *H. pubescens* (100, 200 and 300 mg/kg) treated group of rats for chronic models show in figure.

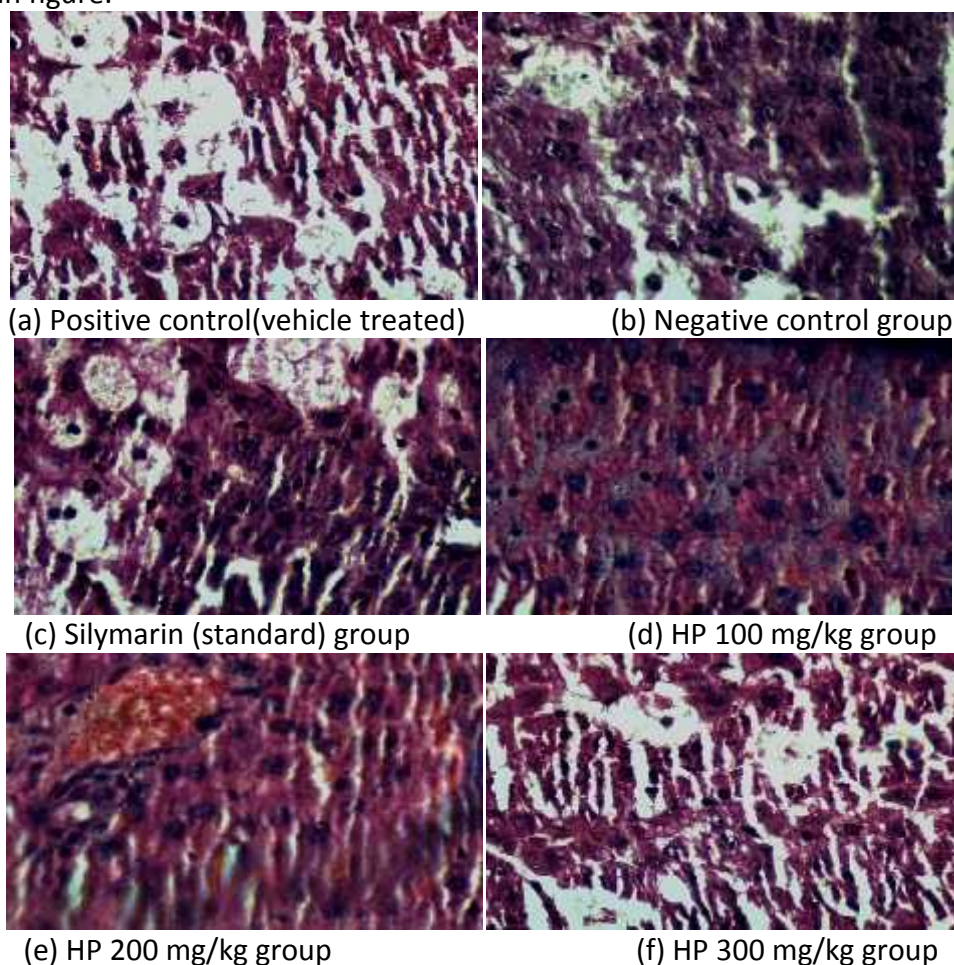


Figure: Histopathological section of the liver for chronic model, magnification (HEx40)

Results and discussion:

Effect of ethanolic extract of leaves of *H. pubescens* on portal triad structure of rat liver for chronic study

(a) Positive control: Positive control group of rats has shown the normal architecture of hepatocytes on portal triad structure of histopathological section of the liver cells and appeared as normally.

However, very mild dilation of central vein appearance has been seen in histopathological section of the liver of this group, which did not reveal any significant lesion of pathological importance

(b) Negative control: Negative control group of rats for acute study has conversely shown fatty vacuoles (appearance of losing cell nucleus and collection of glycogen) centrilobular congestion of sinusoids and kupffer cell hyperplasia with fibrosis, which indicates the development of early necrosis due to CCl₄, induced hepatic injury

(c) Silymarin (25 mg/kg standard): Histological investigation of standard treated group of rats for acute study has shown a little fibrosis at central vein and sinusoidal space. Moreover, a normal arrangement of kupffer cells and hepatocytes cells showed protection against CCl₄ induced hepatic injury.

(d) HP 100 mg/kg group: The section of liver of HP 100 mg/kg treated group of rats for chronic study shown the dilation of sinusoidal space, marked centrilobular necrosis of hepatocytes, severe fibrosis near central vein and hepatocytes with degenerative change in hepatocytes and irregular architecture of kupffer cell which reveal poor protection of hepatocytes from CCl₄ in chronic model.

(e) HP 200 mg/kg group: The histopathological investigation of liver of dose HP 200 mg/kg group of rats shown greater reduction in necrosis area and sparse inflammatory cell around the central vein with normal architecture of hepatocytes and better arrangement of kupffer cell. It clearly indicates the protection of HP 100 mg/kg against CCl₄ induced hepatic injury.

(f) HP 300 mg/kg group: The histopathological investigation of liver of animal received dose HP 300 showed normal architecture of hepatocytes with better arrangement of kupffer cell. No marked central vacuole formation and fibrosis is seen. Section of liver of HP 300 mg/kg showed normal hepatocytes with well- preserved cytoplasm, nucleus and central vein. It clearly indicates the protection offered by HP 300 mg/kg against CCl₄ induced hepatic injury. The histopathological examination clearly reveal that the hepatic cells, central vein and sinusoid are almost normal in HP 300 mg/kg and silymarin treated group of rats in contrast to negative control group of rats which only received CCl₄. The study showed that HP and silymarin showed significant protective effect against CCl₄ induced liver injury in both animal models which was evident from their histopathological examination and biochemical parameters. Thus, HP 300 mg/kg can be considered to be an effective hepatoprotective agent as it ameliorates the damage cause by CCl₄ to hepatic function which appears almost too normal.

Evaluation of antioxidant activity by DDPH method

Free radical scavenging test

Free radical scavenging potentials was tested against methanolic solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). DPPH (control) solution was prepared to 80 µM. Ascorbic acid (standard) and ethanolic extract of leaves of *H. pubescens* were dissolved in DMSO and prepared in different concentration of ranging from 10 to 100 µg/ml separately^[18]. Solution of different concentration of both ascorbic acid and extract were taken in different test tubes. Then the volume was adjusted to 1000 µl with DMSO. To this 3 ml of methanolic solution of DPPH was added, shake well and mixture was allowed to stand at room temperature for 30 minutes. Change in the absorbance was observed at 517 nm to measure the antioxidant activity^[19]. Scavenging activity was expressed as the percentage inhibition calculated using the formula^[20]. (Abs=absorbance)

$$\% \text{ inhibition} = \frac{\text{Abs control} - \text{Abs test drug}}{\text{Abs control}} \times 100$$

Absorbance of DPPH=0.9123

Antioxidant DDPH free radical

Free radical scavenging activity of ethanolic extract of leaves of *H. pubescens* and ascorbic acid.

Concentration µg/ml	<i>H. pubescens</i>		Ascorbic acid	
	Absorbance	% inhibition	Absorbance	% inhibition
10	0.861	5.623	0.616	32.47
20	0.765	16.14	0.587	35.65
30	0.601	34.12	0.523	42.67
40	0.505	44.64	0.466	48.92
50	0.461	49.46	0.413	54.72
60	0.423	53.63	0.356	60.97
70	0.377	58.67	0.289	68.32
80	0.289	68.32	0.196	78.51
90	0.256	71.93	0.169	81.47
100	0.213	76.65	0.090	90.13

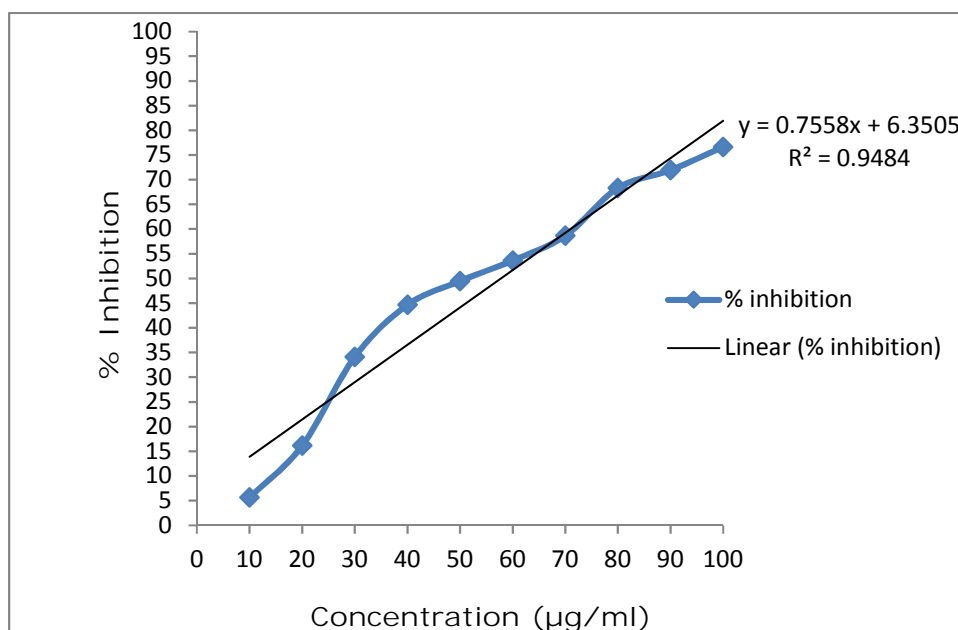


Figure: DPPH radical scavenging activity of *H. pubescens*

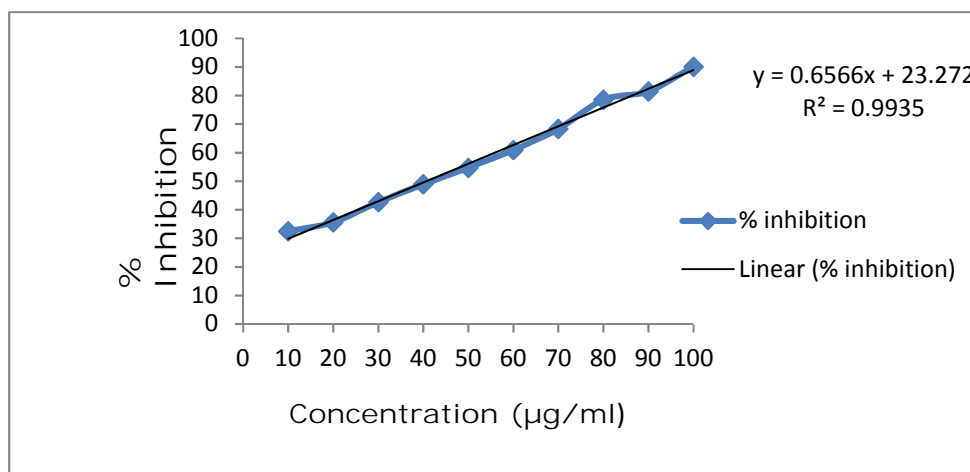


Figure: DPPH radical scavenging activity of Ascorbic acid

IC₅₀ value of ethanolic extract of leaves of *H. pubescens* and ascorbic acid

Compounds	IC ₅₀
Ethanolic extract of <i>H. pubescens</i>	57.81
Ascorbic acid	41.12

Result and discussion

Both Figures shows the scavenging effect increased with the increasing concentration of test compound and standard compound respectively. The IC₅₀ value for ethanolic leaves extract (HP) 57.81 was µg/ml which was comparatively higher than the IC₅₀ (41.12 µg/ml) of ascorbic acid. Free radical scavenging test, which is based on the ability of DPPH (a stable free radical) to be decolorized in the presence of anti-oxidants, is direct and reliable method for determining radical scavenging action. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple colour. When DPPH accepts an electron donated by an anti-oxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Scavenging of DPPH radical was found to rise with the increase in concentration of the extract. Anti-oxidant effect of plant products is mainly due to radical scavenging activity of phenolic compound such as flavonoids, tannins, polyphenols and phenolic terpenes^[21]. The anti-oxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxide^[22].

The free radical disrupts the structure and function of lipid and protein macromolecules in the cell organelles^[23]. Lipid per oxidation caused by free radicals alters membrane permeability and in turn damages tissue^[18]. Thus, anti-oxidant (free radical generation inhibition) is important. Percentage inhibition and IC₅₀ value indicated that *H. pubescens* is having anti-oxidant activity which is helpful to inhibit the oxidant and prevent the free radicals to damage the hepatic cells. The anti-oxidant activity of this plant extract may also be playing important role in hepatoprotective activity.

CONCLUSION

In the evaluation of hepatoprotective activity of the extract of the leaves of *H. pubescens* was found changes in biochemical parameters (SGOT, SGPT, total bilirubin and direct bilirubin) in both acute and chronic model which are affected by the CCl₄. The CCl₄ was given as 7th day as for acute & chronic model was carried out for 7 and 14 days respectively. *H. pubescens* extract was found to produce dose dependent effect. High dose used (HP 300 mg/kg). Various abstract was studied for hepatoprotective studies in acute model result shows that extract significantly reduces various biochemical parameters (SGOT, SGPT and Bilirubin (total and direct) in acute as well as chronic model. The histopathological examination of liver clearly reveals that the hepatic cells, central vein and sinusoids are almost normal in *H. pubescens* treated group. Form the results of all biochemical parameters and histopathology it was found that the plant extract in acute modal shows significant hepatoprotective activity at the doses 200 and 300 mg/kg/day while in chronic model it showed significant hepatoprotective activity at all the doses (100, 200 and 300 mg/kg/day). From the study it can be concluded that pre-treatment with *H. pubescens* have potential hepatoprotective effect on both the models and attenuates the hepatotoxic effects of CCl₄.

Free radicals have been recognized as playing a prominent role in the causation of several diseases. Therefore, free radical scavenging activity may have great relevance in the prevention of free radical mediated diseases. In this study free radical scavenging activity was seen and percent inhibition and IC50 value indicated that *H. pubescens* is having anti-oxidant activity which is helpful to inhibit the oxidation and prevent the free radical to damage the hepatic cells. The antioxidant activity of this leaves extract may also be playing important role in hepatoprotective activity. Future prospects From this study it is proved that ethanolic extract of leaves of *H. pubescens* possesses potent hepatoprotective activity. Thus this hepatoprotective activity of the extract of leaves *H. pubescens* warrants further investigation involving components of possible development of new class of hepatoprotective drugs. From the result of phytochemical study of the plant there is scope for the individual refinement of isolation process to confirm the presence of phytoconstituents which might be responsible to produce these pharmacological activities and its molecular way of action. The structure of the isolated compounds can be confirmed by IR, ¹H NMR, ¹³C NMR and Mass spectral data.

ACKNOWLEDGMENT

The authors were grateful to Lachoo Memorial College of Science and Technology (Autonomous), Jodhpur for providing lab facilities for carrying out the required research work.

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