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Study to Evaluate the Antioxidant and Hepatoprotective Activities of Roots Extracts of *Doronicum hookeri* in CCl_4 Treated Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author SNS designed the study and did statistical analysis. Authors WR and AK helped in writing the protocol and analysis of the study. While author AAK conducted histological examination, authors SM and PAK did the biochemical analysis. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To screen the hepatoprotective and antioxidant activity of ethanol (EDH) and aqueous (ADH) extracts of roots of *Doronicum hookeri* Hook. f.(Asteraceae).
Study design: Animal study
Place and Duration of Study: Department of Pharmacology, Biochemistry and Anatomy (Histology section), J N Medical College, AMU, Aligarh, India, between July 2010- July 2012.
Methodology: The extracts were subjected to antioxidant tests (Total reducing power and Total phenolic content) and preliminary phytochemical screening. The rats were divided into 7 groups. The Control groups comprising of normal control (Saline 1ml/kg), negative control group (CCl_4) and positive control group (Silymarin 50mg/kg). The Test drugs were given in a dose of 300mg/kg and 500 mg/kg for both EDH and ADH extract. Blood was collected for assaying biochemical parameters (AST, ALT, ALP, Total Bilirubin). The liver tissue was used for histopathological examination and *in vivo* antioxidant tests [Catalase (CAT), Glutathione Reductase (GSH) and Malonlydialdehyde

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(MDA)].
Results: The phytochemical study showed the presence of flavanoids, alkaloids, saponins, cardiac glycosides. EDH 500mg/kg showed a significant ($p<0.01$) increased in levels of AST, ALT and ALP as compared to negative while EDH 300 mg/kg ($p<.05$) and ADH group showed minimal activity. The GSH ($p<0.001$) and CAT ($p<0.05$) in EDH 500 mg/kg were significantly increased while MDA levels were decreased ($P< 0.01$) as compared negative control. The findings were confirmed histopathological examination.
Conclusion: The ethanol extract of *Doronicum hookeri* showed dose dependent partial hepatoprotection against CCl4 toxicity.

Keywords: Doronicum; hookeri; hepatoprotective; antioxidant; aqueous; ethanol.

1. INTRODUCTION

Liver in an important organ involved in many biochemical processes in the body. It is a vital site for the metabolism of carbohydrates, proteins and lipids. Not only the liver synthesizes many regulatory enzymes and hormones, it also stores many nutrients necessary for the daily housekeeping of the body. In addition, it acts as a detoxifier that helps to get rid of harmful endogenous and exogenous chemicals, thus liver diseases have severe metabolic and functional consequences for the body. Liver diseases are 5th leading cause of death in England and 12th in United States of America [1].

Plants are an important source of medicines since time immemorial. Many plants have shown to have protective effect on liver against diverse injuries, one such plant is *Doronicum hookeri* Hook. f.(Asteraceae). It is commonly known as leopard's bane and belongs to family Asteraceae. It is native to the foothills of Himalayas in India, Nepal, Bhutan and Tibet [2,3]. *Doronicum hookeri* has shown to possess antibacterial,[4] antifungal [5] and antioxidant activity [6]. *Doronicumh ookeri* is used in Unani medicine for the treatment of liver diseases (as liver tonic) but no scientific study has ever been performed in this respect [7]. In this study we intend to confirm the hepatoprotective activity of the ethanol and aqueous extracts of roots of *Doronicum hookeri*.

2. MATERIALS AND METHODS

2.1 Plant Material

Roots of *Doronicum hookeri* were collected from local Unani medicine plant dealer in September 2009. The taxonomical identity of the plant was confirmed by a botanist Dr. Athar Ali Khan, Assistant professor, Department of Botany, AMU, Aligarh, India and a specimen sample of the same was deposited in the herbarium of the department of wildlife (Specimen no. 1632. s. HWD). The Ethanol (EDH) and aqueous (ADH) extracts of powdered roots of *Doronicum hookeri*, was prepared by using soxhlet's apparatus.

2.2 Phytochemical Screening

Both the ethanol and aqueous extracts were screened for phytochemicals [8]. The phytochemical tests used were for flavonoids, terpenoids, glycosides, saponins, alkaloids, sugars and steroids.

2.3 *In vitro* Antioxidant Tests

For doing *in vitro* antioxidant tests the test solution of concentration 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 mg/ml were made by serial dilution.

Total Reducing Power was performed by the method described by Oyaizu et al. [9] 1% potassium ferrocyanide (2.5 ml) and 2.5 ml of phosphate buffer (0.2 M, pH 6.6) was mixed with 1 ml of the extract and incubated at 50ºC for 30 min. To this solution 2.5 ml of trichloroacetic acid (10%) was added and centrifuged at 3000 RPM for 10 min. 2.5 ml of the supernatant was collected and mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). Absorbance was measured at 700 nm using a spectrophotometer. Serial dilutions of Ascorbic acid were used to make a control curve.

Total Phenolic content was done as described by Saucier et al.[10] 100 µl of Folin Ciocalteau's (FC) reagent was added to a mixture of 20 µl of extract or standard (gallic acid) in 1.58 ml of distilled water. 300 µl of 20% of sodium bicarbonate solution was added between 30 seconds and 8 min and shaken. The solution was incubated for 2 h at 24ºC. Gallic acid solution at concentration of 0, 50,100,150, 250 and 500 mg/l served as control. Absorbance was measured at 765 nm using spectrophotometer.

2.4 Experimental Animals

The ethical clearance was obtained from Institutional Animal Ethical Committee, J.N.M.C., A.M.U., Aligarh and all the experiments were performed as per the guidelines described by CPCSEA. Experimental animals used were Charles Foster albino rats of either sex weighing 200-250 g. The animals were housed under standard conditions and were given a standard pellet diet and water *ad libitum*.

2.5 Acute Toxicity Study

The study was done according to OECD guidelines 423. 3 non-pregnant female rats were given the drug in a dose of 300 mg/kg and 2 g/kg b.w. and observed for 14 days for any mortality. The same test then repeated in 2 g/kg b.w. group to confirm the findings. As no mortality was observed at both 300 mg/kg and 2 g/kg b.w doses, therefore the LD_{50} was found out to be 2 g/kg b.w. Hence, two lower doses of 300mg/kg and 500mg/kg were then chosen for the study.

2.6 Hepatoprotective Activity

In this study, the hepatotoxicity was induced by per orum (p.o.) administration of Carbon tetrachloride (CCl_4) [11]. The animals were divided into 7 groups of 6 animals each. Group one was control group given distilled water (1ml/kg ,p.o.), second group was of negative control group given only CCl_4 orally in a dose of 1ml/kg diluted in olive oil (1:1 v/v), Third group was a positive control group in which silymarin was given with CCl_4 (50mg/kg b.w., p.o.) while in groups four to seven respective test extracts (EDH 300mg, EDH 500mg, ADH 300mg and ADH 500mg) were given along with CCl_4. All the extracts and drugs were given every day for 7 days. The animals were sacrificed one day after the last dose to collect the blood and liver tissue. The blood was centrifuged and the serum was separated. The serum was used to perform liver function test (AST, ALT, ALP and Total Bilirubin) [12-14]. The liver tissue was homogenized (1 g of liver was homogenized with 10 ml of buffer) and the

homogenate was used for *performing in vivo* antioxidant tests (GSH, Catalase and MDA). A part of liver tissue was also stored in 10% formalin for histopathological examination.

2.7 *In vivo* Antioxidant Tests

For doing GSH, [15] 320 µl of distilled water and 80 µl of 50% w/v of trichloroacetic acid, 400 µl of tissue homogenate was added in a test-tube and was centrifuged at 1800 g for 15 min. Supernatant was separated and 400 µl of it was supplemented by 2200 µl of tris buffer (0.4 M, PH 8.9) and 400 µl of 5, 5 dithiobis (0.01 M) and was mixed by shaking. Optical density was measured at 412 nm within 5 min and expressed as µmol/ mg of protein.

For performing MDA and Catalase assay, the tissue homogenate was centrifuged at 6000 RPM for 10 min and the supernatant was used for experiments.

Catalase was done according to Beers and Sizer [16] in brief, 20 µl of tissue extract was added to a solution of 2 ml of phosphate buffer (0.2M, pH-6.6) and 0.5 ml of 30 mM H_2O_2. The absorbance of the sample was measured at 0 and 60 sec at a wavelength of 240 nm and expressed as units of H_2O_2 consumed/ min/ mg of protein.

MDA was in accordance to the method described by Niehans and Samuelsson [17]. In short, 8.1% of sodium dodecyl sulphate (0.2 ml), 20% of acetic acid (1.5 ml) and 0.8% of thiobarbituric acid (1.5 ml) were added to 0.2 ml of tissue homogenate and the volume was made up to 4 ml by adding distilled water. The samples were then heated at 95ºC for 60 min in water. After cooling, a 5 ml mixture of n-Butanol and pyridine (15:1) was added and centrifuge at 4000 RPM for 10 min. Absorbance of organic layer (upper layer) was measured at 532 nm using a UV spectrophotometer and expressed as µmol/mg of tissue.

2.8 Percentage of Hepatoprotection

The percentage of hepatic protection shown by the extract was calculated according to the formula described by Dsagupta et al. [18].

$$H = 1 - ((\mathrm{T} - \mathrm{V})/(\mathrm{C} - \mathrm{V})) \times 100$$

Where, H = Percentage of Hepatoprotection, T = Mean value of the group treated with test drug + CCl_4, C = Mean value of the group treated with CCl_4 and V = Mean value for the control group animals.

2.9 Statistical Analysis

The results were presented as Mean ± Standard Error of Mean (SEM). The groups were compared by one way analysis of variance (ANOVA) followed by post hoc “Dunnett’s Multiple comparison test” to analyze statistical significance. A value of $P < 0.05$ was considered significant.

3 RESULTS AND DISCUSSION

After 72 hour of Soxhlet’s extraction, the yield of ethanol (EDH) and aqueous (ADH) extracts of roots of *Doronicum hookeri* was found to be 8.1% and 7.5%, respectively.

3.1 Phytochemical Analysis

The EDH was found to possess phytochemicals like flavonoids, alkaloids, saponins, cardiac glycosides and sterols while ADH contains fewer phytochemicals (flavonoids and sugars).

3.2 *In vitro* Antioxidant Analysis

The free radical scavenging potential of the extracts was evaluated by using two tests (total reducing power and total phenolic content). The tests showed that the antioxidant activity of ethanol extract was higher than aqueous extract. The reducing power depends on the redox potentials of the compounds present in EDH and ADH. Both the extracts showed dose dependent increase in the antioxidant potential in total reducing power tests as shown in Fig. 1.

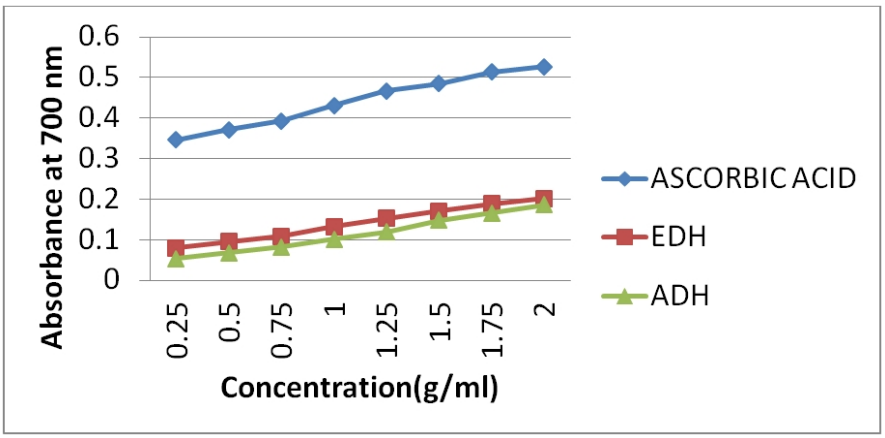

Fig. 1. Total reducing power of ethanol and aqueous extracts of *Doronicum hookeri*

Phenols are antioxidants present in plant extracts and acts by free radical-scavenging and chelating metal ions. The concentration of total phenolic compounds in different extracts was expressed as mg of gallic acid equivalents (GAE)/ g of dried extract, using a standard curve of gallic acid. The Gallic acid equivalent was 39.8 and 12.3 mg GAE/g extract for EDH and ADH respectively.

3.3 Hepatoprotection

Hepatic injury was induced in the animals by administering carbon tetrachloride. CCl_4 causes oxidative stress inside the cells especially hepatocytes by converting to CCl_3* free radicals by the action of cytochrome P450 system (CYP2E1 and CYP2B1/B2) [19,20]. These free radicals are highly reactive species which attack organic components of the cells by oxidising them, thus causing widespread damage. The liver damage is reflected by the hepatocyte enzymes. These hepatocyte specific enzymes like transaminases (AST and ALT) and alkaline phosphatase are present in cytoplasm, any damage to cytostructures especially cell membrane leading to their release into the circulation. So in the negative control group, in which only CCl_4 was given, the liver enzymes were raised significantly ($p<0.001$) implying severe damage to the hepatocytes

Table 1. Results of liver function test and percentage hepatoprotection after CCl_4 induced liver toxicity

Biochemical test (Mean ± SEM)	Groups (Percentage hepatoprotection %)						
	Normal contorl (Water)	Negative control (CCl_4)	Positive control (SILYMARIN)	*D. hookeri* Ethanolic (300mg/kg)	*D. hookeri* Ethanolic (500mg/kg)	*D. hookeri* Aqueous (300mg/kg)	*D. hookeri* Aqueous (500mg/kg)
AST (IU/ml)	39.1 ± 1.4	154.0 ± 6.1***	60.5± 6.4***	133.6 ± 5.4* (17.7%)	120 ± 9.1** (32.2%)	152.8 ± 4.5 (1.0%)	130 ± 3.0* (18.3%)
ALT (IU/ml)	38.8 ± 1.2	161.3 ± 2.2***	55 ± 2.6***	140.1 ± 3.7** (17.0%)	128.7 ± 3.9** (36.5%)	159.5 ± 5.0 (1.2%)	138.3 ± 3.6** (18.5%)
ALP (KAU/dl)	42.8 ± 2.3	78.6 ± 4.2***	50.2 ± 4.7**	58.1 ± 4.2*	55.2± 5.9**	72.3 ± 4.5	65.3 ± 4.2
BILIRUBIN (mg/ml)	0.7 ± 0.026	0.83 ± 0.049	0.7 ± 0.026	0.78 ± 0.031	0.76± 0.033	0.83 ± 0.042	0.78 ± 0.031

*Negative control group was compared with Normal control group and all other groups were compared with negative control group, * p< 0.05, **p<0.01 and ***p<0.001 were considered significant*

Table 2. Results of *In vivo* antioxidant tests after CCl_4 induced liver toxicity

Biochemical Test (Mean ± SEM)	Groups						
	Normal contorl (Water)	Negative control (CCl_4)	Positive control (SILYMARIN)	*D. hookeri* Ethanolic (300mg/kg)	*D. hookeri* Ethanolic (500mg/kg)	*D. hookeri* Aqueous (300mg/kg)	*D. hookeri* Aqueous (500mg/kg)
Catalase (U/ min/mg)	82.2 ± 3.7	47.4 ± 1.6***	67.7 ± 1.0***	52.0 ± 2.5	54.1 ± 2.4*	47.9 ± 0.6	48.4 ± 0.7
GSH (µmol /mg)	5.25 ± .21	2.15 ± 0.25***	4.44 ± 0.11***	3.15 ± 0.11***	3.33 ±0.16***	2.23 ± .012	2.52 ± 0.17
MDA (nmol/mg)	203.9 ± 8.9	445.9 ± 17.8***	255.8 ± 7.7***	382.6 ± 10.6	371.1 ± 16.5**	428.7 ± 23.2	417.1 ± 24.3

*Negative control group was compared with Normal control group and all other groups were compared with Negative control group, * p< 0.05, **p<0.01 and ***p<0.001 were considered significant*

The damage to a great extent was neutralized by silymarin which served as the positive control group. The percentage protection as a function of ALT shown by the EDH extract in a dose of 300 mg/kg and 500 mg/kg was 17% and 36.2% ($p<0.01$) respectively, while 300 mg/kg of ADH extract showed 1.2% protection ($p<0.01$) respectively, while 500 mg/kg of ADH extract showed 18% protection ($p<0.01$). Alkaline Phosphatase also showed similar results in control and test groups. There were no significant changes in the values of Total Bilirubin which may be due to the short duration of the present study (Table 1).

The plant extracts in the study might be acting by neutralizing the free radical CCl_3* and thereby protecting the cellular structures from the harmful effects of reactive oxidizing species. The levels of oxidizing damage can be indirectly checked by measuring the levels of antioxidant enzymes GSH and Catalase in the hepatocytes. The antioxidant enzymes accept electrons to convert free radicals into harmless species. A high level of these enzymes in the test groups as compared to negative control indicate more antioxidative potential inside the cells, which is an indirect measure of the free radical scavenging potential of the test compound. In our experiment, the levels of the GSH ($p<0.001$) and Catalase ($p<0.001$) were decreased significantly by CCl_4

The levels of GSH ($p<0.001$) and Catalase ($p<0.05$) were significantly increased in EDH 500 mg/kg group while no significant difference was observed in other groups (EDH 500 mg/kg, ADH 300 mg/kg and ADH 500 mg/kg). These findings were further substantiated by the MDA levels. MDA is a byproduct of lipid oxidation in the cells. Free radicals are responsible for the cascade of reactions which leads to oxidation of lipids present in the membrane. In the study, the MDA levels are significantly lower ($p<0.01$) in EDH 500 mg/kg extract than the negative control suggesting a decrease in oxidative stress inside the cell. In general, the ethanol extracts fared better than aqueous extracts and each extract showed a dose dependent increase in hepatoprotective and antioxidant activity (Table 2).

3.4 Histopathological Examination.

Figs. 2, 3 shows the histopathological features of the different groups. Complete loss of hepatic architecture, loss of normal contour of cells and presence of large amount of inflammatory infiltrates define the negative control group. EDH 300mg showed some inflammatory infiltrates, the architecture was maintained in most part of the field while EDH 500mg demonstrated superior results than 300mg, apart from a few local areas of necrosis and coagulative changes rest of the architecture was well maintained.

Hepatic toxicity is a complex process comprising multipronged assault on hepatocyte. Hepatic injury, as a result of exogenous of endogenous noxious stimuli, leads to dysfunction of mitochondria. The mitochondria help regulate energy generation and oxidative stress inside the hepatocytes. Therefore, a mitochondrial injury leads to increase of oxidative elements especially reactive oxygen species inside the cells [21]. In addition, the induction of CYP2E1, NADPH oxidase released by macrophages and iron overload also promotes oxidative injury [19,22]. The injury leads to activation of defense mechanism resulting in the recruitment of Kuffer cells and neutorphils which cause further oxidative stress [23]. The fate of the cells then depends on the amount of oxidative stress. If there is a massive stress built up then the cell may directly go into necrosis, otherwise the cell may upregulate genes like NfKB, p53, JNK etc. triggering apoptosis [24]. Regardless of the process the hepatocyte dies, causing a further compromise in the hepatic function. It is thus noted, that the oxidative damage and cellular injury go hand in hand in hepatic toxicity. Hence, the drugs having antioxidant and free radical scavenging action also found to be effective in hepatic toxicity.

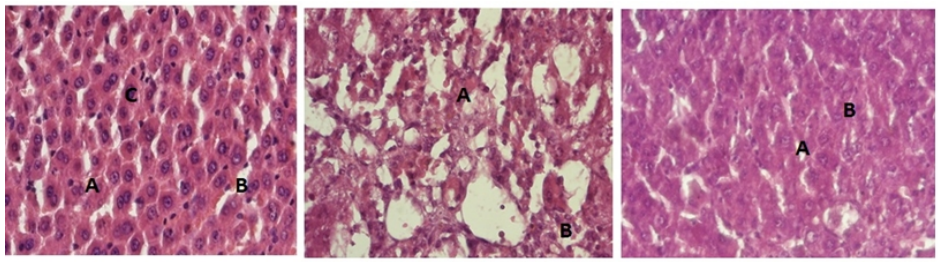

Figure 2A: Normal Control Figure 2B: Negative Control Figure 2C: Positive Control

Fig. 2. Histology of Control Group

Fig. 2A. *Photomicrograph of rat liver from* ***Normal Control Group*** *showing normal liver microstructure with intact hepatic cords (A) and sinusoids (B). Hepatocytes (C) show normal contour. There is neither obvious congestion nor inflammatory cell infiltration. X400. H & E stain*

Fig. 2B. *Photomicrograph of rat liver from* ***Negative control group*** *shows almost complete disorganization of hepatic microstructure. Most of the hepatocytes show either lytic or coagulative changes (A) and there is inflammatory cell infiltration (B).*

Fig. 2C. *Photomicrograph of rat liver from* ***Positive control group*** *showing maintained hepatic microstructure. The hepatic cords (A) are intact and the contour of hepatocytes appear very akin to normal (B). There is no obvious inflammatory cell infiltration.*

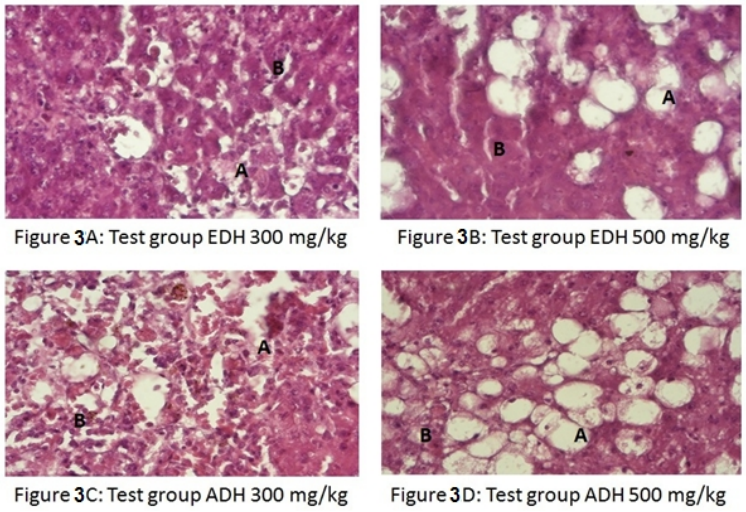

Figure 3A: Test group EDH 300 mg/kg Figure 3B: Test group EDH 500 mg/kg

Figure 3C: Test group ADH 300 mg/kg Figure 3D: Test group ADH 500 mg/kg

Fig. 3. Histology of Test group (EDH & ADH)

Fig. 3A. *Photomicrograph of rat liver from* ***Test group EDH 300 mg/kg*** *showing obvious degenerative changes mainly in the right lower quadrant of the field (A). The hepatic cords and sinusoids are partially maintained in some regions(B). Some hepatocytes show vesicular changes but notobvious ballooning.*

Fig. 3B. *Photomicrograph of rat liver from* ***Test group EDH 500 mg/kg*** *showing ballooning of some hepatocytes (A) while others though spared appear to be partially affected by coagulative changes. The sinusoids appear compressed (B). There is no obvious congestion or inflammatory cell infiltration.*

Fig. 3C. *Photomicrograph of rat liver from* ***Test group ADH 300 mg/kg*** *showing marked degeneration affecting almost all hepatocytes affected by both lytic and coagulative changes (A). The hepatic microstructure is completely lost. There is also sign of congestion and marked inflammatory cell infiltration (B).*

Fig. 3D. *Photomicrograph of rat liver from* ***Test group ADH 500 mg/kg*** *showing a mixed picture of degeneration and protection. The spared hepatocytes also do not appear normal when compared with the control while the affected hepatocytes show ballooning (A).*

The extracts of roots of *Doronicum hookeri* showed a dose dependent hepatoprotective activity in CCl4 treated rats. The hepatoprotection of ethanol extracts were better than their aqueous counterparts. The superior activity of ethanol extract was due to the presence of phytochemicals like flavonoids, alkaloids, glycosides and sterols which were lacking in the aqueous extract. Deepika et al. [6] showed the antioxidant potential of methanol and dichloromethane extract of *Doronicum hookeri* roots using a battery of in vitro antioxidant tests [6]. They also concluded that the higher concentration of flavonoids in the dichloromethane extract was responsible for the better DPPH & the superoxide ion scavenging activity, nitric oxide neutralization and metal chelating activity. The antioxidant flavonoids, like apigenin, rutin and querectin, which were isolated in the ethanol extract of various doronicum species, might have played central role in free radical scavenging and thus hepatoprotection shown by the ethanol extract of the plant [25].

4. CONCLUSION

This is the first study demonstrating the effectiveness of *Doronicum hookeri* in hepatotoxicity. The study concluded that the ethanol extract in a dose of 500 mg/kg offered a partial hepatoprotection (36%) against toxicity induced by carbon tetrachloride in rats ($p<0.01$). The study also showed good antioxidant activity of 500mg/kg ethanol extract in *in vivo* (GSH $p<0.001$) and *in vitro* tests. While the ethanol extract of 300 mg/kg was less effective, the aqueous extracts (300and 500 mg/kg) showed statistically not significant hepatoprotective and antioxidant activity. Further studies are required for isolation and characterization of the active principle, which may serve as a lead for the development of new antioxidant-hepatoprotective agent.

CONSENT

Not applicable.

ETHICAL APPROVAL

The ethical clearance was taken from Institutional Animal ethical committee, JNMC, AMU, India (401/CPCSEA – 23/3/2012).

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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