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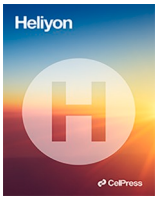

Research article

Attenuation of paracetamol-induced hepatotoxicity in *Ajuga bracteosa* extract treated mice

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ABSTRACT

Ajuga bracteosa (*Ab*) has tremendous medicinal value with long-established disease curing potential. The present study aimed to assess the hepatoprotective potential of *Ab* extracts in paracetamol-induced hepatotoxicity in mice. Group I (normal control) were treated with saline 1 ml/kg BW orally for 7 days while Group II (toxicant control) received saline 1 ml/kg BW for 6 days and Paracetamol (1000 mg/kg BW) on day7of the treatment. Group III received Standard drug silymarin (100 mg/kg BW) for 6 days and Paracetamol (1000 mg/kg BW) on day 7of treatment. Groups IV andV were administered with methanol extract (ME) 200 mg/kg BW and aqueous extract (AE) 1000 mg/kg BW for 6 days and Paracetamol (1000 mg/kg BW) on day 7th of the study. Both extracts showed hepatoprotective potential against the toxic effects of paracetamol, evidenced by serum analysis of biomarkers involved in liver injury and histopathological findings. Hepatotoxic mice pretreated with *Ab* plant extract or silymarin exhibited significant decrease in ALP, AST, and ALT enzyme level while GSH levels were markedly increased. According to histological observations, groups treated with PCM (toxicant control) showed significant necrosis and lymphocyte infiltration, while groups treated with silymarin and *Ajuga bracteosa* plant extract showed preservation of the normal liver structural features. The phytochemical analysis of ME and AE of *Ab* showed the presence of glycosides, phenolic compounds, tannins, fats, saponins, flavonoids, terpenes, oils, and fats. The antioxidant activity of these two extracts was determined by nitric oxide assay, DPPH assay, and ferric reducing power assay. The methanolic extract exhibited the highest antioxidant potential (78.09 ± 0.0806). The antioxidant potential of aqueous extract was 73.08 ± 0.248. The reducing power for methanolic extract and ascorbic acid (standard) 500 μg/ml was 0.933 and 0.987 respectively. The anti-inflammatory activity of both extracts was demonstrated by *in vitro* methods, namely albumin denaturation, proteinase inhibition, and membrane stabilization assays. The study suggests that *Ab* extracts have competence for attenuating inflammation, oxidants, and hepatotoxicity.

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1. Introduction

Plant-derived medicines play an essential role in basic health care, particularly in developing countries, due to a variety of characteristics including cultural acceptability, environment friendly or compatibility with the human body, and minimum adverse effects [1]. The body's defence system in the form of inflammation is triggered in response to heat, bacterial infection, mechanical injuries, and other potentially harmful substances [2]. Production of inflammatory mediators like prostaglandins (PGs), prostacyclin (PC), nitric oxide, cytokines, leukotrienes, and vasoactive amines trigger a spontaneous reaction [3]. Acute inflammatory diseases like rheumatoid arthritis are treated by both non-steroidal anti-inflammatory and steroidal drugs but these traditionally used drugs have failed to cure these chronic inflammatory illnesses. Additionally, several conventional painkillers have been linked to unfavourable side effects [4,5]. The body's most crucial organ is the liver, as it is responsible for the removal of hazardous substances [6]. Drugs are one potential cause of liver disease, which causes around 2 million deaths every year worldwide [7]. Irreversible effects on the liver are caused due to overdosing of some medications, resulting in hepatotoxicity [8,9]. Drug-induced liver toxicity (DILT) represents more than half of all acute cases of liver failure, with paracetamol representing 80 % of all recorded drug-induced acute liver failure cases [10–12]. Despite significant breakthroughs in contemporary medicine, the liver illness remains a global health problem, necessitating the continual quest for innovative treatments. As hepatocytes are involved in a wide range of metabolic processes, the development of hepato-protective drugs is critical for preventing liver injury. Plant extracts high in antioxidants have consistently been linked to hepatoprotective benefits [13–22].

Ajuga bracteosa belongs to the family *Labiatae.* It comprises about 3000 species and 170 genera growing in a great variety of soils and climates. Its distribution extends from Bhutan in the eastern Himalayas to Kashmir, China, and Pakistan which lie in the Western Himalayas, Tropical regions of Malaysia, and Indian sub-tropical regions. Its use in ethno-medicine is reported as antibacterial, antifungal, astringent, antihelmintic, and hypoglycaemic. It is used as a folk medicine in Asian countries for inflammation of the liver, rheumatism, gout, neuroinflammatory diseases, and pneumonia [23–25]. *Ajuga bracteosa* has many ethnopharmacological uses. Its leaves are used to manufacture herbal medicines that treat malaria, diabetes, and digestive issues. The whole plant has been used in Taiwan traditional medicinal system to cure various inflammatory problems, as pneumonia, bone diseases, and hepatitis [26]. Furthermore, leaf extracts have long been used to treat a variety of conditions, including constipation, jaundice, measles, acne, headaches, stomach hyperacidity, ear infections, hypertension and sore throats [27]. *Ab* is used to treat malaria in traditional Indian medicine [8] and its extracts are also used to treat jaundice, gout, and joint discomfort [28]. In India, the decoction obtained from bark, flowers, and leaves is employed in treating inflammation, malaria, diabetes, cancer, etc [29]. Various Extracts of the plant have been evaluated worldwide to assess its pharmacological applications [30]. The reported pharmacological properties of *Ab* are anti-inflammatory [31], anti-arthiritic [32], analgesic [33], antimicrobial [34], antinociceptive [35], antidepressant [30], anticoagulant [30], antioxidant [30], antimutagenic [36], antihypertensive [37], antihemolytic [38] and anticancer [39].

The present study was conducted to assess the hepatoprotective properties of aqueous and methanolic extracts of *Ajuga bracteosa* in paracetamol-induced damage to liver. The rationale for using *plant* extract to evaluate hepatoprotective activity is that the crude extracts when subjected to preliminary phytochemical examination, showed a range of secondary metabolites having hepatoprotective potential. Furthermore, in our previous study *Ab* plant extract showed good antioxidant potential due to presence of phytochemicals [34]. The phytochemicals present in crude extract such as flavonoids, alkaloids and phenols may exert hepatoprotective action by scavenging free radicals and prevent peroxidation of lipids [40].

Groups II, III, IV and V were pretreated respectively with saline 1 ml/kg per day, silymarin 100 mg/kg per day, methanol extract (ME) 200 mg/kg per day and aqueous extract (AE) 1000 mg/kg per day for 6 days and the toxicity was produced with a single dose of paracetamol 1000 mg/kg on day 7.Both the extracts were able to protect the liver from the toxic effects of paracetamol which was evidenced by serum analysis of biomarkers involved in liver injury and histopathological findings. The purpose of the study was to evaluate and confirm the anti-inflammatory, antioxidant and hepatoprotective properties of *Ajuga bracteosa*. The study advocates the identification and isolation of more specific phytochemicals with anti-inflammatory, antioxidant and hepatoprotective properties from *Ajuga bracteosa* extracts to be further trialled in clinical studies for the cure of various diseases including inflammation, oxidative stress and hepatotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

Absolute alcohol, Bovine serum albumin, copper sulphate, EDTA, Folins reagent, Formaldehyde, Glycine, Glucose-6-phosphate dehydrogenase, γ-glutamyl-*p*-nitroanalide, HCl, H2O2, α ketoglutarate, KCL, MgCL2, NADPH, NaOH, Na2CO3, Na–K-Tartarte, P-nitro phenyl phosphate, Perchloric acid, Silymarin (Micro Labs Limited, Goa, India) Paracetamol (Medley pharmaceuticals, Jammu) Pyrogallol, Phosphate buffer, Paraffin, assay kits for liver chemistry and other chemicals and reagents for phytochemical tests, reduced glutathione, Sulphosalicylic acid, Trichloro acetic acid, Thiobarbituric acid, Tris Buffer, Xanthine, 5,5-dithio bis-2-nitro benzene, 1-Chloro 2,4 dinitro benzene, Chloroform, Petroleum Ether, Methanol, Thioacetamide. All chemicals used were of analytical grade purchased from Sigma Aldrich and CDH.

2.2. Collection of Ajuga bracteosa

Ajuga bracteosa was collected from the Drang area of Kashmir Valley, where it grows abundantly. It was authenticated in the

Department of Taxonomy, University of Kashmir Srinagar by the concerned taxonomist, and a specimen sample under voucher No. 2425-(KASH) was preserved in the Herbarium, University of Kashmir, for future reference. The aerial parts were dried for 20 days at 30 °C in the shade.

2.3. Preparation of extracts

The aerial parts were macerated to fine powder after 20 days of shade drying. For successive extraction, petroleum ether, methanol and water were used as solvents. Hot extraction using the Soxhlet apparatus was carried out. First Petroleum ether was used as solvent and500 g of powder extracted for 18–20 h. Under reduced pressure using Rota vapor concentrated extract was obtained. After that, the marc was soaked in methanol (18–20 h) for extraction. And same process carried out for the concentration of the extracted material. For 6–8 h extraction of marc was then done with water. The yield of the extracts obtained from methanol, petroleum ether, and water was 28.72, 10.5, and 15.2 g respectively. all the extracts were kept at 4 °C in airtight glass vials for the experimental work.

2.4. Assessment of anti-inflammatory activity in-vitro

2.4.1. Inhibition of protein (albumin) denaturation

In the protein denaturation method for assessing the *in-vitro* anti-inflammatory activity of *Ajuga bracteosa*, bovine serum albumin (BSA) solution (0.2 % w/v) was made in Tris Buffer Saline using glacial acetic acid to adjust pH to 6.8. Stock solutions (10 mg/ml) of the aqueous and methanol extracts were prepared in water and methanol respectively. Five different concentrations of methanol and aqueous extracts (100–500 μg/ml) were prepared. To 50 μl (0.05 ml) of each extract in test tube, 5 ml of BSA (0.2%w/v) was added. Instead of extract, 50 μl of ethanol was added to control. Ibuprofen 100 μg/ml in ethanol with 5 ml of BSA (0.2%w/v) acted as a standard. After heating for 5 min at 72 °C the test tubes were allowed to cool for 10 min. At 660 nm, the absorbance of test samples was measured using UV spectrophotometer. Three duplicates of the experiment were run, and the average absorbance was calculated [41]. The percentage inhibition of denaturation was measured on a percentage basis in relation to control using the formula:

$$\text{Percentage of inhibition of denaturation } (\%) = \frac{(\text{Abs.control} - \text{Abs.sample})}{(\text{Abs.control})} \times 100$$

2.4.2. Proteinase inhibitory action

To 1 ml of test samples of aqueous and methanol extracts employing different concentrations (100–500 μg/ml), 1 ml 20 mM Tris HCl buffer (pH 7.4) and 0.06 mg trypsin was added. Before adding 1 ml of casein (0.8 % w/v), incubation at 37 °C for 5 min was done. Then incubation was again done for 20 min. The process was ultimately terminated by the addition of 2 ml of 70 % perchloric acid. Centrifugation of reaction mixture was caried out and the supernatant's absorbance was measured at 210 nm against a buffer blank [42]. The experiment was done in triplicate and average absorbance calculated. The percentage inhibition of proteinase inhibitory activity was estimated as:

$$\textit{Percentage inhibition} = \frac{(\textit{Absorbance of control} - \textit{Absorbance of sample}) \times 100}{\textit{Absorbance of control}}$$

2.5. Preparation of red blood cells (RBCs) suspension

RBC suspension was prepared. Blood was drawn from batch members who hadn't taken any NSAIDS 14 days prior to start of the experiment and transferred to centrifuge tubes. Tubes were then centrifuged for 10 min at 3000 rotations per minute. The supernatant was removed and the packed RBCs were rinsed three times with saline water. The 2 % v/v erythrocytes were prepared with saline water (0.85 % w/v NaCl) [43]. The membrane stabilization was assessed by the ability of extracts to offer protection from heat induced haemolysis.

2.6. Heat induced haemolysis

The control contained only saline in place of the test sample, whereas the reaction mixture (2 ml) contained 1 ml of the test sample at various concentrations (100–500 mg/ml) of aqueous and methanol extracts as well as 1 ml of a 10 % RBC suspension. Aspirin acted as a standard.

Centrifuge tubes containing reaction mixture were kept in a water bath at 56 °C for 30 min. After the incubation period, tubes were cooled with cold water. Centrifugation for 5 min at 2500 rpm was done, and absorbance of supernatant was observed at 560 nm [44]. Experiment was done in triplicate. Percentage inhibition of haemolysis was calculated as follows:

$$\text{Percentage inhibition} = \frac{(\textit{Abs control} - \textit{Abs sample}) \times 100}{(\textit{Abs control})}$$

2.7. Assessment of antioxidant activity

2.7.1. Determination of DPPH (2, 2-diphenyl-1-Picrylhydrazyl) radical scavenging

The radical scavenging property of *Ab* extracts was evaluated using DPPH assay. DPPH is a persistent free radical that has a deep violet colour with a maximal absorptive capacity of 517 nm. Antioxidants scavenge the unpaired electrons in DPPH, leaving it colourless. 0.2 mM stock solution of DPPH was prepared in 95 % ethanol. The reaction mixture containing 400 μL ascorbic acid standard solution, control or aqueous and methanolic extract at different concentrations (100–500 μg/ml) and 400 μL DPPH solution were incubated in a water bath for 30 min at 37 °C, and the absorbance was measured at 517 nm. Ascorbic acid was used as a standard material [45].

The following equation was used to calculate the percentage DPPH radical scavenging activity.

$$\text{Percentage DPPH radical scavenging activity} = (\mathbf{A0} - \mathbf{A1})/\mathbf{A0\ X100}$$

where A1 is absorbance of standard/extracts, and A0 is the absorbance of control.

2.7.2. Determination of reducing power

The reducing power capacity of plant extracts to form ferrous-ferry cyanide complex by reducing ferric-ferry cyanide [46]. The reaction mixture was prepared by mixing 2.5 ml of phosphate buffer 2.5 ml of 1 % potassium ferry cyanide with different concentrations of aqueous and methanolic extract. The incubation was done for 20 min at 50 °C. After 50 min of incubation 2.5 ml of 10% trichloro acetic acid was added to above mixture and centrifuged at 1000 g for 10 min. The upper layer of solution (2.5 ml) was added to 500 μl of 0.1 percent ferric chloride and 2.5 ml of distilled water. Absorbance was measured at 700 nm in a UV spectrophotometer. Increase in reducing power was indicated by increase in absorbance of reaction mixture. Similar method was adopted for Ascorbic acid which was used as a positive control.

2.7.3. Nitric oxide radical inhibition assay

In this assay, 0.5 ml of varied concentrations of extracts (100–500 mg/ml) were added to two ml sodium nitroprusside (SNP; 10 mM) in 0.5 ml phosphate buffer saline (PBS, pH = 7.4) and incubated in light for 60 min at 37 °C. Half of the aliquots were combined with Griess reagent, and incubated for 30 min in the dark. The absorbance of pink colored chromophore produced was observed at 546 nm in comparison to blank [47]. The experiments were conducted in triplicate. The reference substance was ascorbic acid, which was used in the same concentration range.

2.8. Assessment of hepatoprotective activity

2.8.1. Experimental animals

Healthy *Swiss albino mice* of either sex aged 6–8 weeks and weighing about 29–35 g were procured from the Council of Scientific & Industrial Research – Indian Institute of Integrative Medicine (CSIR-IIIM) Jammu. The mice were inbred in the animal house of Department of zoology, University of Kashmir. The mice were placed in polypropylene cages and were maintained under standard environmental conditions. Animals were given standard water and feed. Ethical clearance was obtained from the Ethical Committee, Department of Pharmaceutical Sciences, University of Kashmir (Approval No: F(IAEC-Approval) KU/2017/18.

2.8.2. Grouping and dosing of animals

Swiss albino mice were randomly divided into 5 groups (comprising six animals per group) for the hepatoprotective activity test. The animals in normal control (Group I) were treated with saline 1 ml/kg BW orally for 7 days. Toxicant control animals (Group II) were given saline 1 ml/kg BW for 6 days and paracetamol orally (1000 mg/kg BW) on day 7 of the experiment. Positive control animals (Group III) were administered with standard drug, silymarin 100 mg/kg BW orally for 6 days and paracetamol (1000 mg/kg BW) on the 7th day. Among the test groups, animals in Group IV (methanolic extract group) were administered ME (200 mg/kg BW) and animals in Group V (aqueous extract group) with AE (1000 mg/kg BW) for 6 days and paracetamol (1000 mg/kg BW) on day 7.

2.8.3. Hepatoprotective activity

Mice were weighed and slaughtered under light ether anaesthesia 24 h after the dose of paracetamol. Blood was extracted through the retro-orbital vein and collected in sterile heparinized centrifuge tubes. The serum was separated from the clot by centrifuging the blood at 3000 rpm for 10 min. Standard techniques were used to examine serum for biochemical parameters such as ALT, ALP, AST, oxidative serum markers, etc. After the animals were killed, the liver tissue was removed and preserved for future experiments at −80 °C after being rinsed in ice-cold saline. Hepatic tissue was homogenized and fractionated into sub cellular units for histopathological analysis.

2.8.4. Enzyme assay

2,4dinitrophenylhydrazine (DNPH) method of Reitman and Frankel [48]was used to check the enzymes, alanine transaminase (ALT), aspartate aminotransferase (AST), and serum alkaline phosphatase (ALP).

2.8.5. Oxidative stress markers

The oxidative stress markers namely catalase, superoxide dismutase, glutathione reductase and reduced glutathione were analysed by respective methods [49–51]. Lipid peroxidation in terms of the content of malondialdehyde in liver tissue was assessed by the method of Ohkawa et al. [52].

2.8.6. Biochemical estimation

The effect of extracts on biochemical parameters like Glucose-6 phosphate dehydrogenase (G6PD) [53] and Xanthine oxidase (XO) [54] was also estimated.

2.9. Histopathological studies

After sacrificing the mice, the livers were taken out for histological analysis. After being cleaned with saline buffer, livers were fixed in 10 % formalin. The livers were washed in tap water after fixing followed by dehydration in serial ethanol and finally cleared in xylene. The tissue was embedded in paraffin wax. Haematoxylin-eosin was then used to stain the liver. The slices were examined under a microscope for changes in histopathological architecture [55].

2.10. Statistical analysis

The statistical analysis was conducted by GraphPad Prism (Version, 8.4). The results are shown as Mean ± SEM. One-way ANOVA was done to compare the differences between the groups. Multiple comparisons were performed with Tukey's test. P value less than 0.01 was used as the cutoff for significance.

Table 1
Preliminary phytochemicals of *Ajuga bracteosa* extracts.

S. No	Phytochemical Tests	Extract of aerial parts of *Ajuga bracteosa*		
		Methanolic	Aqueous	Petroleum ether
1	**Test for Alkaloids**			
	Wagners test	++	+	–
	Dragendroffs test	++	+	–
	Hagers test	++	+	–
2	**Test for triterpenoids**			
	Salkowski test	++	+	+
3	**Test for phyto sterols**			
	Salkowski test	++	+	+
	Libermann Buchard's test	++	++	+
4	**Flavonoids**			
	Lead acetate test	+	+	+
	Shinoda test	++	++	+
	Alkaline reagent test	++	+	+
5	**Test for saponins**			
	Olive oil stain test	+	+	+
	Foam test	+	+	+
6	**Test for cardiac glycosides**			
	Keller Killani test	++	–	–
7	**Anthraquinone glycosides**			
	Borntrager's test - + +	–	+	–
8	**Phenolic compounds**			
	Ferric chloride test	++	+	+
9	**Proteins**			
	Biuret test	+	–	–
10	**Amino acids**			
	Paulys test Millons test	++	+	–
	Xanthoproteic test	++	+	–
	Ninhydrin test	–	+	–
	Millons test Paulys test	+	–	–
11	**Carbohydrates**			
	Seliwonoffs test	++	+	–
	Molish test	++	+	+
	Barfoeds test	+	+	+

+ Present; − Absent.

3. Results

3.1. Preliminary phytochemical assessment

Official herbal pharmacopeial identification tests were carried out to ascertain presence of various phytochemicals including alkaloids, triterpenoids, phyto sterols, flavonoids, saponins, cardiac glycosides, anthraquinone glycosides, phenolic compounds, proteins, amino acids, and carbohydrates in methanolic, aqueous, and petroleum ether extracts of aerial parts of *Ajuga bracteosa* (Table 1).

3.2. Anti-inflammatory activity

3.2.1. Albumin denaturation inhibition

Maximum inhibition of 72.98 % at 500 μg/ml was shown by methanol extract while aqueous extract at same concentration showed 67.09 % inhibition**.** Ibuprofen used as a standard anti-inflammatory agent showed an inhibition of 63.09 % at 100 μg/ml. The IC50 of methanolic and aqueous extracts of *Ab* was calculated as 235.34 and 316.62 μg/mL, respectively. Results are displayed in Table 2.

3.2.2. Proteinase inhibitory action

Aerial part extracts *of Ajuga bracteosa* exhibited strong anti-inflammatory potential by showing anti-proteinase inhibition at various concentrations (Table 3). Methanol extract demonstrated maximal inhibition of 78.10 % at 500 μg/ml. Similarly, at 200 μg/ml, aspirin had a maximal inhibitory effect of 64.80 %. The IC50 values of methanolic and aqueous extracts of *Ab* were found to be 303.65 and 350.18 μg/mL, respectively.

3.3. Heat induced haemolysis

Haemolysis was induced by using heat and was significantly inhibited by extracts in a concentration dependent manner. The extracts protected the erythrocyte membrane from heat-induced lysis in a concentration dependent manner (Table 4). At 500 μg/ml, methanol extract exhibited a maximal inhibition of 72.40 %. At 200 μg/ml, aspirin displayed a maximal inhibition of 65.03.The IC50 of methanolic and aqueous extracts of *Ab* was 240.09 and 339.30 μg/mL, respectively.

3.4. Anti-oxidant activity

3.4.1. DPPH radical scavenging assay

The ability of aqueous and methanolic extracts of *Ajuga bracteosa* to scavenge free DPPH radicals was measured as percentage inhibition. The percentage inhibition for methanol and aqueous extract at 500 μg/ml was 78.9 % and 69.55 % respectively whereas ascorbic acid showed an inhibition of 87.06 % at the same concentration. The results of DPPH radical scavenging activity are displayed in Fig. 1 with reference to standard ascorbic acid. Methanol extract showed IC_{50}value of 308 μg/ml value than aqueous extract having IC_{50} of 337 μg/ml.

3.4.2. Reducing power activity

Methanolic extract had greater reducing power than the aqueous extract. The reducing power shown by methanol extract at 500 μg/ml was 0.933 while ascorbic acid at the same concentration showed a reducing power equivalent to 0.987.(Table 5).

3.4.3. Nitric oxide radical inhibition assay

The capacity of extracts of aerial parts of *Ajuga bracteosa* to scavenge Nitric oxide radical was checked in terms of percentage inhibition which was found to be 73.08 % at 500 μg/ml for methanolic extract and 68.10%for aqueous extract at the same concentration, whereas standard ascorbic acid at 500 μg/ml showed an inhibition of 88.03 % as shown in Fig. 2. This assay shows that the

Table 2
Ajuga bracteosa mediated inhibition of albumin denaturation activity.

S. No	Extract/standard.	Concentration μg/ml	Percentage Inhibition
1	Methanol	100	36.16 ± 0.509
		200	47.29 ± 0.203
		300	56.77 ± 0.485
		400	66.93 ± 0.331
		500	72.98 ± 0.183
2	Aqueous	100	20.05 ± 0.417
		200	36.5 ± 0.188
		300	53.02 ± 0.317
		400	63.36 ± 0.485
		500	67.09 ± 0.398
3	Standard (Ibuprofen)	100	63.09 ± 0.352

Values are represented as Mean ± SEM; (N = 3).

Table 3
Antiproteinase activity of methanol and aqueous extracts of *Ajuga bracteosa*.

S. No	Extract/standard	Concentrationμg/ml	Percentage Inhibition
1	Methanol	100	22.62 ± 0.348
		200	35.34 ± 0.489
		300	48.30 ± 0.568
		400	63.03 ± 0.528
		500	78.10 ± 0.426
2	Aqueous	100	20.28 ± 0.280
		200	32.04 ± 0.348
		300	42.58 ± 0.351
		400	55.71 ± 0.563
		500	68.99 ± 0.425
3	Diclofenac sodium	200	64.80 ± 0.557

Results are represented as Mean ± SEM; (N = 3).

Table 4
Percentage inhibition of haemolysis by *Ajuga bracteosa extracts*.

S. No	Extract/standard	Concentration μg/ml	Percentage inhibition
1	Methanol	100	28.55 ± 0.351
		200	49.10 ± 0.214
		300	63.10 ± 0.163
		400	69.08 ± 0.098
		500	72.40 ± 0.240
2	Aqueous	100	21.59 ±0 .506
		200	30.01 ±0 .088
		300	45.79 ± 0.429
		400	60.83 ± 0.323
		500	67.63 ± 0.492
3	Standard	200	65.03 ± 0.560

Values are expressed as Mean ± SEM; (N = 3).

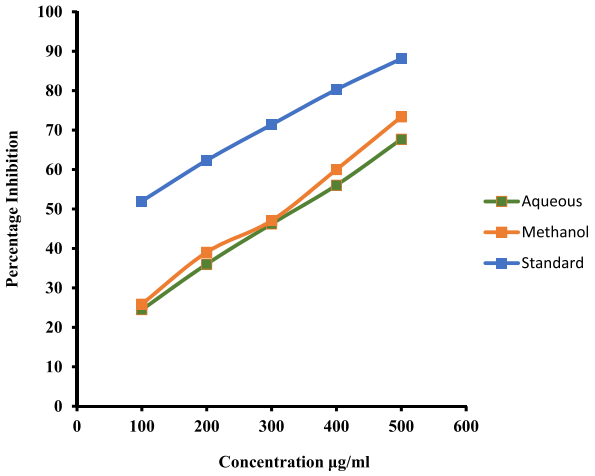

Fig. 1. DPPH radical scavenging activity of methanolic and aqueous extract with reference to standard ascorbic acid.

methanol extract has more Nitric oxide scavenging activity than aqueous extract. The IC_{50} value of methanolic extract was found to be 274 μg/ml, aqueous extract showed IC_{50} value of 338 μg/ml while standard ascorbic showed the IC_{50} value of 200 μg/ml.

3.5. Hepatoprotective activity

3.5.1. Serum analysis

The results of different extracts of *Ajuga bracteosa* on liver marker enzymes is represented in Table 6. It is evident from the table that control group treated with saline showed normal levels of ALP, ASP and ALT while paracetamol only group (negative control) showed elevated levels of these enzymes proving that larger doses of paracetamol induce liver damage. In the mice, the levels of ALP, ASP and

Table 5
Reducing power of aerial part extracts of *Ajuga bracteosa* at different dose levels.

S. No	Concentration (µg/ml)	Standard (Ascorbic acid)	Methanol extract	Aqueous extract
1	100	0.364 ±0 .003[a]	0.281 ± 0.004[b]	0.205 ± 0.005[c]
2	200	0.548 ± 0.003[a]	0.409 ± 0.006[b]**	0.387 ± 0.003[c]**
3	300	0.753 ±0 .001[a]	0.575 ± 0.006[b]	0.482 ± 0.005[c]
4	400	0.908 ±0 .002[a]	0.706 ± 0.002[b]	0.595 ± 0.003[c]
5	500	0.987 ± 0.001[a]	0.933 ± 0.013[b]	0.707 ± 0.008[c]

Absorbance is expressed as Mean ± SEM of triplicate experiments. Statistical significance was tested by ANOVA with Tukey's multiple comparison. Rows with different superscript letters (a,b,c,d) indicate highly statistical significance among groups. Superscripts with asterisk (**) demonstrate significant difference between the groups in the same row, $p < 0.01$.

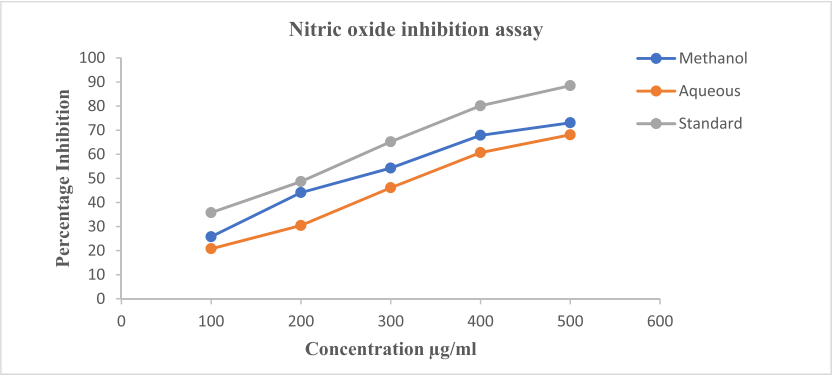

Fig. 2. Showing NO inhibition activity (% inhibition) of different extracts obtained from aerial parts of *Ajuga bracteosa* and ascorbic acid as standard.

Table 6
Effect of various extracts obtained from aerial parts of *Ajuga bracteosa* on serum enzymatic levels in mice liver injury brought on by paracetamol.

S. No	Treatment (n = 5)	Dose	ALT (IU/L)	ASP (IU/L)	ALP (IU/L)
I	Normal(control)	1 ml/kg	75.77 ± 0.96[a]	31.01 ± 0.64[a]	20.30 ± 0.62[a]
II	Toxic (control)	1000 mg/kg	99.21 ± 0.91[b]	53.44 ± 0.78[b]	32.57 ± 1.02[b]
III	Silymarin	100 mg/kg	81.78 ± 1.01[c#]	35.41 ± 0.91[c]	25.18 ± 0.87[c]
IV	Methanolic	200 mg/kg	85.78 ± 0.82[d]	36.93 ± 0.69[d]	27.89 ± 1.11[d]
V	Aqueous	1000 mg/kg	82.09 ± 1.33[e#]	35.77 ± 0.89[e]	27.35 ± 0.71[e]

Values are demonstrated as Mean±SEMof triplicate experiments. Statistical significance was tested by ANOVA with Tukey's multiple comparison. Columns with different superscript letters (a,b,c,d) indicate highly statistical significance among groups in the column. Superscripts with # demonstrate no significant difference between the groups in the same column, $p < 0.01$.

ALT were significantly decreased by treatment with methanolic and aqueous extract. Silymarin, the standard hepatoprotective agent also brought the levels of ALT, ASP and ALP down to 81.78 ± 1.01, 35.41 ± 0.91 and 25.18 ± 0.87 respectively.

3.5.2. Biochemical analysis

Both the extracts (aqueous and methanolic) were able to decrease lipid peroxidation (194.29 ± 6.87 and 199.45 ± 5.98

Table 7
Effect of various extracts of *Ajuga bracteosa* on lipid peroxidation, reduced glutathione and catalase.

S No.	Treatment	Dose	Lipid peroxidation (nmol/mg)	Reduced glutathione (nmol/litre)	Catalase (Unit/mg)
I	Normal (control)	1 ml/kg	175.54 ± 6.51[a]	115.71 ± 1.91[a]**	4539.03 ± 91.89[a]
II	Toxic (control)	1000 mg/kg	701.63 ± 7.41[b]	91.83 ± 2.54[b]	2978.42 ± 83.41[b]
III	Silymarin	100 mg/kg	189.31 ± 8.21[c]	113.51 ± 1.91[c]**	4394.33 ± 92.11[c]
IV	Methanolic	200 mg/kg	199.45 ± 5.98[d]	109.83 ± 1.85[d#]	4198.77 ± 98.12[d]
V	Aqueous	1000 mg/kg	194.29 ± 6.87[e]	108.91 ± 2.34[e#]	4174.82 ± 99.32[e]

Values are demonstrated as Mean±SEMof triplicate experiments. Statistical significance was tested by ANOVA with Tukey's multiple comparison. Columns with different superscript letters (a,b,c,d) indicate highly statistical significance among groups in the column. Superscripts with asterisk (**) demonstrate significant difference between the groups in the column. Superscripts with # demonstrate no significant difference between the groups in the same column, $p < 0.01$.

respectively) which was increased by high dose of paracetamol (701.63 ± 7.41). Silymarin under identical conditions reduced the lipid peroxidation to (189.31 ± 8.21). Levels of reduced glutathione were decreased by the toxic dose of paracetamol (91.83 ± 2.54),but were almost increased to normal levels (115.71 ± 91) by methanolic and aqueous extracts (109.83 ± 1.85 and 108.91 ± 2.34) respectively. Similarly, aqueous and methanolic extracts increased the catalase levels (4174.82 ± 99.32) and 4198.77 ± 98.12 respectively) which was decreased by the toxic dose of paracetamol (2978.42 ± 83.41). The results are presented in Table 7. Similarly, the levels of glutathione reductase, superoxide dismutase and glucose-6-phosphate dehydrogenase were increased by the treatment with extracts, which were decreased by the toxic dose of paracetamol. Xanthine oxidase levels were increased by the toxic dose of paracetamol and decreased by extracts. The results are summarised in Table 8.

3.6. Histopathological assessment

Histopathological findings (Fig. 3) showed that the hepatocytes of a control group had a normal architecture (Fig. 3A), whereas, hepatic areas with necrosis and sinusoidal dilations were observed in animals treated with the toxic dose of paracetamol (Fig. 3B).On the other hand, the animals treated with *Ajuga bracteosa* extracts showed only few congested vessels in otherwise normal hepatocytes (Fig. 3C and D). Moreover, in the Silymarin-administered mice hepatocytes were normal with very slight congestion in the portal regions (Fig. 3E)

4. Discussion

The rapid advancement of synthetic pharmaceuticals in the modern period has been followed with innumerable undesirable side effects [56]. In the field of drug development, the discovery and extraction of medicinal substances from plants is increasing day by day [57–61]. In our study the preliminary phytochemical screening of aerial parts of *Ajuga bracteosa* showed that all the extracts are rich source of phytochemicals. The methanolic and aqueous extracts revealed the presence of highest number of phytochemicals namely, triterpenoids, phytosterols, alkaloids, phenolics, flavonoids and, fixed oils. Petroleum ether extract showed negative result for alkaloids, proteins, anthraquinone glycosides and amino acids. Both methanolic and aqueous extracts stabilized the membrane of red blood cells.

Different concentrations of methanolic and aqueous extracts showed high inhibition of protein denaturation induced by heat and protected the RBC membrane against hypotonicity induced haemolysis. Denaturation disassembles the secondary and tertiary structure of proteins during inflammation, bringing loss of biological activity [62–64].As a result, the ability of methanolic and aqueous extract of *Ajuga bracteosa* to suppress protein denaturation was investigated as potential mode of action. Furthermore, lysosomal membrane breakage has been shown to produce pro-inflammatory indicators such as histamines, activated neutrophils and proteases near the local site of tissue injury during chronic inflammation. Therefore, stabilization of RBC membrane implies stabilization of lysosomal membrane as this membrane is alike to erythrocyte membrane [65]. Stabilizing membrane and inhibiting denaturation of proteins may be the potential mechanisms employed by plant-derived products for anti-inflammatory activities [66–71].

The stabilization of bio-membranes, protein denaturation inhibition and anti-proteinase effects produced by *Ajuga bracteosa* suggests that this plant can have an important role in traditional system of medicine in management of crippling diseases such as rheumatoid arthritis, ankylosing spondylitis etc. or can be an important ingredient in multidrug regimens for these diseases. Characterization of various compounds and their spectroscopic analysis can help in identification of molecule(s) responsible for the pharmacological action. This can lead to isolation of active compounds which will ultimately lead to evolution of potent molecules possessing specific activity and lesser side effects. DPPH is a fairly stable free radical, it is unaffected by some chain reactions. DPPH has a deep purple colour. Antioxidant compounds cause fading of this colour by scavenging free DPPH radical and transforming it into a

Table 8
Effect of *Ajuga bracteosa* extracts on glutathione reductase, G-6-PD, superoxide dismutase, and xanthine oxidase.

S. No.	Treatment	Dose	Glutathione reductase (μmoles/g liver tissue)	Glucose-6- phosphate dehydrogenase (Unit/mg protein)	Superoxide dismutase (Unit/mg) **Uni**	Xanthine oxidase (μmole/mg)
I	Normal (control)	1 ml/kg	40.01 ± 0.91^{a}	12.89 ± 0.51^{a}	$2.71 \pm 0.08^{a\#}$	190.51 ± 6.22^{a}
II	Toxic (control)	1000 mg/kg	19.57 ± 0.84^{b}	07.01 ± 0.39^{b}	1.51 ± 0.05^{b}	221.23 ± 9.30^{b}
III	Silymarin	100 mg/kg	$36.33 \pm 0.91^{c\#}$	$10.59 \pm 0.71^{c\#}$	2.91 ± 0.08^{c}	$196.84 \pm 7.42^{c**}$
IV	Methanolic	200 mg/kg	$35.42 \pm 0.59^{d\#**}$	$09.98 \pm 0.59^{d\#**}$	$2.53 \pm 0.05^{d\#}$	$195.63 \pm 8.23^{d**}$
V	Aqueous	1000 mg/kg	$33.99 \pm 0.94^{e**}$	$09.24 \pm 0.65^{e**}$	$2.51 \pm 0.05^{e\#}$	199.28 ± 6.32^{e}

Values are demonstrated as Mean ± SEM of triplicate experiments. Statistical significance was tested by ANOVA with Tukey's multiple comparison. Columns with different superscript letters (a,b,c,d) indicate highly statistical significance among groups in the column. Superscripts with asterisk (**) demonstrate significant difference between the groups in the column. Superscripts with # demonstrate no significant difference between the groups in the same column, $p < 0.01$.

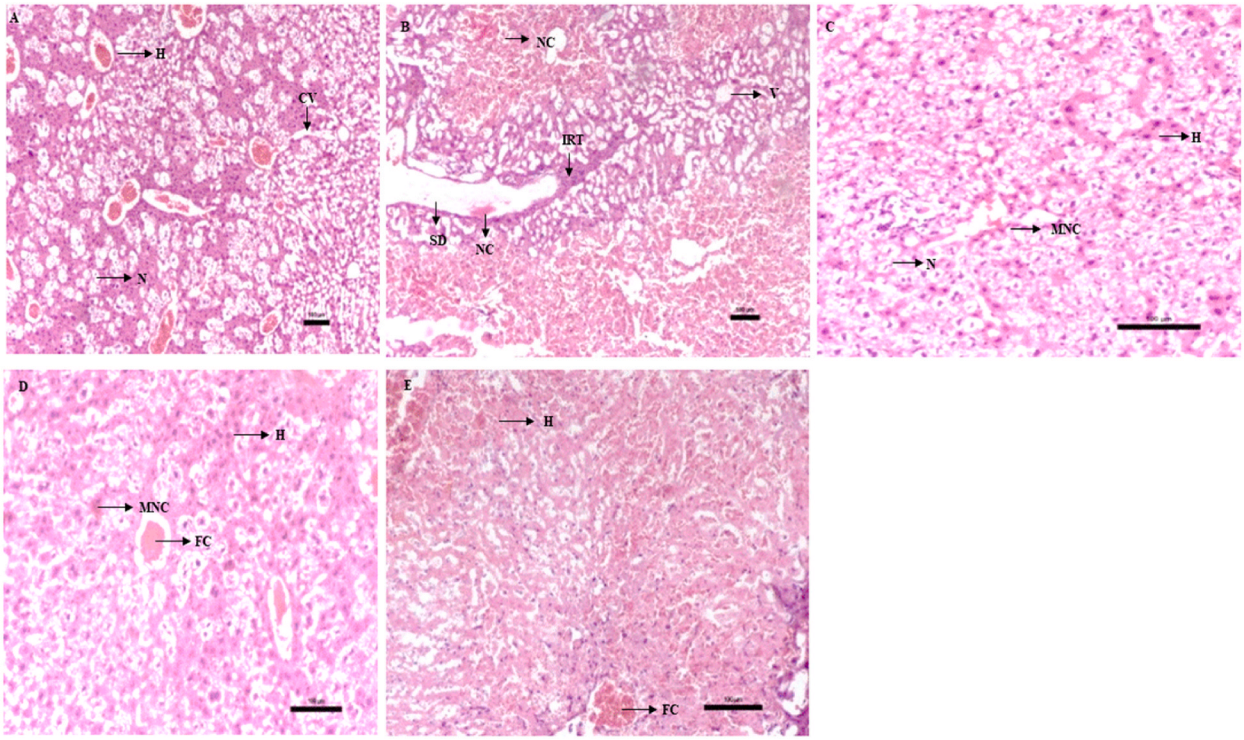

Fig. 3. Histopathological slides of mice liver from various groups **A:** Normal liver structure of mice normal nucleus (N), hepatocytes (H) and central vein (CV) **B:**Liver from mice treated with PCM (paracetamol) with severe necrotic (NC),vacuole (V),sinusoidal dilation (SD), and infiltration of portal tract (IPR) **C:** Liver from mice treated with aqueous extract with certain areas of mild necrosis (MNC) **D:**Liver from mice treated with methanolic extract with certain fatty changes (FC) but otherwise normal and undamaged hepatocytes (H) and hepatic cards. **E:** Hepatocytes in the silymarin-administered mice were normal, and there were only very fatty changes (FC).

2,2-diphenyl 1-hydrazine, a colourless compound which results in decrease in absorbance [72–74]. The ability of extracts of aerial parts of *Ajuga bracteosa* to scavenge DPPH free radical was found to be concentration dependent and highest for methanolic extract as compared to aqueous extract.

Endothelial cells and macrophages release nitric oxide. Nitric oxide is necessary for blood vessel dilatation to continue. The extent of scavenging is measured by amount of reduction in formation of purple azo dye [75]. Hepatocytes are the primary regulators of metabolic activity of liver. If this organ is altered, the body's metabolism will be disrupted [76]. Oxidative damage results from the higher level of nitric oxide generated by inducible nitric oxide synthase in macrophages. Peroxynitrite, a highly damaging substance formed when nitric oxide directly oxidises proteins of low density upon its combination with free radicals, causing irreparable damage to membrane of cells. The purple azo dye is formed by combination of nitrite ions with Griess reagent. The amount of nitrite decreases due to scavenging by methanolic and aqueous extracts. The capacity of *Ajuga bracteosa* extracts to scavenge nitric oxide radical was found to be concentration dependent, with methanolic extract having better activity than aqueous extract. Liver is the prime target of toxicity because of its role in detoxification and metabolization of drugs [77,78].

Hepatic cytochrome P-450 activates a portion of paracetamol to generate the highly reactive metabolite N acetyl-*p*-benzoquinone imine (NAPQI), which is responsible for the generation of toxic metabolites and has been linked to paracetamol hepatotoxicity [79]. N-acetyl-*p*-benzoquineimine, one of the toxic metabolites, can oxidise and alkylate intracellular GSH, leading to a depletion of GSH in the liver. This, in turn, increases lipid peroxidation by removing hydrogen from a polyunsaturated fatty acid, which ultimately causes liver damage from higher paracetamol dosages. Reactive metabolites have the ability to cause early cell stress in a variety of ways, such as via binding to lipids, enzymes and nucleic acids, and other cell components or by depleting glutathione (GSH) [80]. AST and Alt enzymes are released into the bloodstream during hepatic necrosis, which may be detected in the serum [81].The pool of ALT produced by hepatic parenchymal cells is considered a particular enzyme for detecting liver disorders. Regardless of that, AST measurement is taken as an important diagnostic marker being susceptible to mitochondrial distortion, particularly in centrilobular zone 3 [82,83]. The study's findings clearly show that PCM increased the serum levels of the liver enzymes AST, ALP, and ALT, suggesting hepatotoxicity in the animals. Methanolic extract at 200 mg/kg and aqueous extract 1000 mg/kg significantly lowered the levels of ALP, AST, and ALT in these PCM intoxicated groups. GSH levels were markedly increased in *extract* treated animals in comparison to the paracetamol treated animals in our study. Paracetamol toxicity causes oxidative stress by producing free radicals, reduction of the GSH, and death of hepatocytes [84,85]. Phenolic compounds and flavonoids are known for their antioxidant properties, which can help combat the formation of free radicals in the body. These free radicals can contribute to various clinical disorders, including liver

disorders, diabetes, cardiovascular disease, cancer etc. The extracts under investigation have a high proportion of these compounds that provides evidence for their potential preventive effects against liver disorders. This data holds significant promise for the development of preventive strategies or treatments aimed at enhancing liver health and addressing other conditions linked to oxidative stress.

Moreover, in our previous study this plant has shown good antioxidant potential due to presence of free radical scavenging phytochemicals [34]. The levels of hepatic malondialdehyde (MDA) in the tissue were used to determine hepatic lipid peroxidation. Malondialdehyde is an oxidative stress indicator.It decreased in extract-treated groups and increased dramatically after liver necrosis was induced in mice. Reduced glutathione levels were present in lower levels in the necrotic group and very high in the extract-treated group. In the metabolism of medicines and poisons Xanthine oxidase (XO), a Mo–Fe–S flavin enzyme plays an important role. In the PCM group, XO activity increased dramatically, whereas in the extract pretreated groups, it reduced significantly. The extracts normalized glutathione reductase and catalase, which were decreased by PCM. SOD and G6PD showed a declining tendency in the PCM group, but levels were normalized by extracts. When compared to control, PCM alone group acquired severe hepatocellular damage, as evidenced by a considerable rise in levels of AST, ALT and ALP.

These findings demonstrate that *Ajuga bracteosa* extracts possess a preventive role in liver injury. When taken prophylactically, the extracts could successfully prevent the liver damage caused by PCM, as evidenced by lower serum levels of ALP, AST and ALT. Hepatic damage is linked to oxidative stress and the resulting lipid peroxidation. To induce lipid peroxidation, ROS either take a hydrogen atom from unsaturated membrane lipids or combine with sulfhydryl molecules, triggering a series of peroxidation processes. Cell damage occurs as a result of these alterations. Our study on *Ajuga bracteosa* reveals that its extracts can prevent PCM-induced liver damage. However exact mechanism and active compounds involved in hepatoprotection need to be further investigated so as to devise/formulate cost effective drugs for liver disorders in a better way in this part of the world where around 80 % population have no access to costly allopathic drugs and these plant-based drugs being indigenous to them are easily available and cost effective. Evaluation of these results suggests that *Ajuga bracteosa* can be used alone or in combination with other agents as an antioxidant and an anti-inflammatory agent. However, identification and characterization of active constituents and further large-scale preclinical and clinical assessment of their *in vivo* pharmacological and therapeutic effects is important before its application in healthcare.

5. Conclusion

These findings indicate that *Ajuga bracteosa* contains important bioactive phytoconstituents particularly phenolics, flavonoids, alkaloids, tannins and steroids etc. In this study methanolic extract of *Ajuga bracteosa* showed significant hepatoprotective and antioxidant activities in paracetamol-induced mice hepatotoxicity. The phenolic and flavonoid components in extract which aid in scavenging free radicals and confer antioxidant potential, may be the reason for the plant's hepatoprotective effects. These phytoconstituents either individually or in combination are responsible for hepatoprotective, anti-inflammatory and antioxidant action. Hence, our observations give an idea that the bioactive compound/s present in *Ajuga bracteosa* can be used to develop a promising anti-inflammatory medicine that can be utilized to treat a variety of inflammatory illnesses. Additionally, the outcomes indicate that *Ajuga bracteosa* can be used as a natural antioxidant for disease prevention and promotion of longevity. The anti-inflammatory, hepatoprotective, and antioxidant capabilities of *Ajuga bracteosa* are the subject of ongoing research to better understand the plant's mode of action.

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Author's contribution

Conceptualization: GNB and TA, Methodology and Experiments: TA, SAM, Compilation of data: IJ, RB: Drafting of manuscript: TA, IJ, RB. SA performed statistics, review and revised the manuscript. All authors contributed to the writing of the final manuscript and approved the final version.

Ethics approval and consent to participate

Ethical approval of the study was given by Ethical committee, Department of Pharmaceutical sciences, University of Kashmir under approval number: F(IAEC-Approval) KU/2017/18.

Availability of data

The data is available with authors and will be shared upon a reasonable request.

CRediT authorship contribution statement

Tabasum Ali: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Ifat Jan:**

Writing – review & editing, Writing – original draft. **Rabiah Bashir:** Writing – review & editing, Writing – original draft. **Suhail Ahmad Mir:** Methodology. **Shafat Ali:** Writing – review & editing, Software, Formal analysis. **Ghulam Nabi Bader:** Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

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