

Research Article

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Investigation of polyphenol profile, antioxidant activity and hepatoprotective potential of *Aconogonon alpinum* (All.) Schur roots

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Abstract: Liver plays vital role in detoxification of exogenous and endogenous chemicals. These chemicals as well as oxidative stress may cause liver disorders. This study was aimed to evaluate the hepatoprotective effects of various fractions of *Aconogonon alpinum* methanolic extract against carbon tetrachloride (CCl₄)-induced liver toxicity in mice. First, hepatoprotective potential of various fractions of *A. alpinum* was assessed and then antioxidant activity and profiling of polyphenolic compounds were assessed. A total of 78 male albino mice (BALB/c) were randomly divided into 13 groups ($n = 6$); Group I (normal control), Group II (CCl₄

only), Group III (CCl₄ + silymarin 100 mg/kg) and Groups IV–XIII (CCl₄ + various fractions [200 and 400 mg/kg]). Hepatic biochemistry and liver injury were assessed by analysis of serum levels of hepatic enzymes and histopathological analysis, respectively. Results showed that polar fractions (ethyl acetate, *n*-butanol and aqueous fractions) exhibited highly significant ($P < 0.01$) reduction in increased level of liver biochemical parameters in a dose-dependent manner with consistent histopathological findings. Likewise, these fractions revealed strong antioxidant potential and polyphenolic compound contents. In conclusion, the present work has revealed promising antioxidant activity, polyphenolic profiling and potential hepatoprotective efficacy. Thus, the significant results unveil the study as a step forward towards evidence-based phytomedicine.

Keywords: *A. alpinum*, hepatocytes, liver biochemical parameters, phytochemicals

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1 Introduction

Liver is an important body organ, which regulates the chemical environment of the body by detoxification of exogenous and endogenous metabolites; it has been reported that liver damage is caused due to oxidative stress induced by free radicals [1]. Liver diseases have become global concern and deaths caused by hepatic diseases are increasing to alarming rate [2,3]. Liver is the major body organ for metabolism and is usually injured due to chemicals, drugs and pathogen infiltration (such as virus and bacteria) when the pathogens invade the body. Currently, the pharmacotherapy, liver transplant and surgery are options for the treatment of liver diseases but they have limited benefits associated with serious complications in the body [4]. According to World Health Organization, 10% of the world population

is suffering from liver diseases such as cirrhosis, hepatocellular carcinoma and liver fibrosis [5]. Toxicity is the most common risk factor of hepatic pathologies, which is contributed by alcohol, toxic chemicals, air pollutants, contaminated water and unhygienic food additives [6]. One of these chemicals is carbon tetrachloride (CCl_4), which is a potent environmental toxin emitted from chemical industries, and its presence in the environment is increasing at the alarming rate. In the human body, it causes liver damage through the free radical-mediated inflammatory process [7]. Exposure of liver to chemicals, drugs and toxic substances produces reactive oxygen species (ROS) that play a promising role in chronic disease development such as liver disorders [8,9]. In spite of advancement in modern medicines, currently there are hardly reliable drugs available to prevent liver diseases and they have adverse reactions. Currently, there is increasing interest to identify the potential plant products as antioxidants which would prevent liver toxicity [10].

Researchers have focused on natural products to discover the novel therapies to combat the oxidative stress of body with minimum side effects [11]. Plant-based food products and drugs are extensively used medicines to treat the different diseases to improve the community health. In the Ayurveda system, plants and herbal remedies have therapeutic importance since ancient times for prevention and treatment of diseases [3]. Various studies have shown that secondary metabolites of plants especially phenolic and flavonoid compounds have biological activities such as anti-bacterial, anti-fungal, anti-cancer, analgesic, anti-inflammatory and anti-tumoural effects [12]. The consumption of herbal medications against diseases is high and their usage will increase by patients suffering from liver diseases. The medicinal plants can act as supreme agents against any form of liver ailment [13]. The herbalism as an alternative treatment to diseases such as liver damage is considered the safe solution [14]. Due to the absence of reliable drugs in the modern system of medicines to treat the liver disorders, medicinal plants are recommended to evaluate the hepatoprotective agents which possess valuable bioactive compounds [4]. Secondary metabolites of plants play an important role against various diseases due to their medicinal value and they are the important source of powerful drugs all over the world [15,16]. Ethnomedicinal values of plants have been reported in various studies due to therapeutic values of phytochemicals (alkaloids, saponins, flavonoids and tannins) used for relieving the pain and effective treatment of different diseases [16]. Several studies have shown that phenolic and flavonoid compounds act as reducing agents and free radical scavengers [17]. Effects of lipid peroxidation and other oxidative damages to

liver are prevented by medicinal plants by their biological properties and hepatoprotective activities [18]. Hepatoprotective activities of plants are associated with phytochemicals such as alkaloids, coumarins, saponins, alkaloids, terpenes [19] and phenolic compounds acting as antioxidants [20]. Phytochemicals have potential antioxidant and radical scavenging effects, which prevent the body from various toxic metabolites and chemicals such as CCl_4 [8]. Phytochemicals and vitamins play an important protective role against ROS. Polyphenols, plant-based antioxidants, have important biological activities for pharmaceutical, food industry and health care benefits [21]. Systematic separation of the methanolic extract of medicinal plant (*Euphorbia wallichii*) with chloroform, ethyl acetate, *n*-hexane, *n*-butanol and aqueous fraction has proven biological activities [22]. Biological activities of the plant extracts are attributed to their antioxidant nature which is mostly determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [23]. Studies have shown that the antioxidant activity of phytochemicals plays an important role for the hepatoprotective effects against chemical toxicity induced in mice [24]. It is presumed that plant extract or herbal formulations prevent the liver injury and tend to normalize the toxic effect due to the presence of antioxidant phytochemicals (triterpenes, flavonoids and phenolic compounds) having radical scavenging effect [25]. To induce hepatotoxicity in laboratory animals for different pharmacological investigations and testing of both synthetic and natural products, CCl_4 is widely used a hepatotoxic agent [26]. The CCl_4 -induced toxicity leads to increase in the level of peroxides and free radicals that decrease the level of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) [20] and accelerates the liver damage which results in increase in the level of liver biochemical markers: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TB) in the blood serum [8]. Normal levels of antioxidant enzymes (CAT, SOD and GSH) lead to ROS degradation and decrease the hepatic damage by improving the antioxidant defence system [18]. Investigation of the medicinal plant (*Juniperus phoenice*) extract revealed its potent hepatoprotective effect against CCl_4 -induced toxicity and reduced the increased level of liver biochemical parameters (ALT, AST, ALP and TB) [27]. Phytochemicals such as phenolic and flavonoid compounds are strong antioxidants having correlation with concentration and antioxidant activity [28]. The liver diseases are life threatening, and conventional method of treatment is expensive as well as it has serious side effects. Alternative nutraceutical hepatoprotective agents are urgent medical treatment [29]. Finding safe, effective and protective drugs can provide an optimum solution to liver diseases.

Pakistan has diverse climatic and soil conditions for flora. About 6,000 species of plants are reported in Pakistan that possess medicinal values and 600 of them are reported in the Himalayan region. More than 75% of the population of this country uses the medicinal plants to treat various diseases recommended by herbal practitioners but their pharmacological investigation is limited [30]. The south region of Azad Jammu and Kashmir (AJ&K), Pakistan, is enriched with diverse flora, and medicinal plants have been less explored and investigated for pharmacological activities. Common people use these medicinal plants for the treatment of different diseases and ailment [31].

Polygonaceae is an important family of medicinal plants comprising 1,200 species and 48 genera [32]. Plants of this family (polygonaceae) are used as folk medicines in various countries worldwide due to their antioxidant activities. *Polygonum minus* Hud (family: Polygonaceae) has also been studied earlier for its hepatoprotective effect [33]. One important genera of family Polygonaceae is *Coccoloba* which possesses important biological activities (anti-oxidant, cytotoxic, anti-hyperglycemic, anti-inflammatory and anti-microbial activities) and its crude extract is rich in phytochemical compounds (flavonoids, phenolic acid, terpenoids, tannins, anthraquinones and volatile oils) that have been isolated and identified [32]. So far, the research data have shown that *Polygonum orientale* (family: polygonaceae) investigated for its hepatoprotective effect against CCl₄-induced liver injury in mice has shown the potent results [34].

Aconogonon alpinum (All.) Schur, an important medicinal plant, belonging to family polygonaceae is a wild herb distributed in various planting zones of forest glades, dry meadows, stony slopes, cliffs and alpine zone. It is widely spread in China, Mongolia, Japan and former USSR. In Asia and Western parts of North America, there are about 35 species of this genus. It grows at 2,000–4,000 m in open meadows, shady slopes and found to be distributed in Siberia, Turkestan and North Western Himalayas. There are five species of this genera recorded in Pakistan [35]. In AJ&K (Pakistan), it is found at high mountainous forest and alpine regions [36,37]. Local people use its seeds as emetic and purgative. From floral parts, sherbet is prepared for fever and menstruation [36]. It is reported that this plant is also used for the treatment of stomach problems and roots are carminative [38]. Folk people of the North region of AJ&K use it for the treatment of liver jaundice. About chemical contents, a report shows the flavonol complex, which is from Altay. Chemical contents reported are glycosides: astragalin, quercetin, avicularin, hyperoside,

quercetin-3,7-diglucoside, rutin, myricitrin, and aglycones: kaempferol, quercetin and myricetin from the above-ground parts of plant [39]. In spite of diverse ethnobotanical medicinal properties of *A. alpinum*, pharmacological investigations are largely obscure about the hepatoprotective effect in animal models earlier especially from AJ&K, Pakistan. Keeping in view the importance of phenolic and flavonoid compound content and folkloric importance of *A. alpinum* to treat the different diseases such as liver disorders, recent study has been intended to investigate the hepatoprotective effect of different solvent fractions of *A. alpinum* root methanolic extract (AAME) against CCl₄-induced toxicity in mice, their antioxidant potential as well as phenolic compound profiling of most active fractions.

2 Materials and methods

2.1 Chemicals/reagents and instrumentation

All the solvents (of analytical grade) and methanol of HPLC grade were purchased from Sigma Aldrich (Germany). Reference standards (epicatechin, ferulic acid, hyperoside, gallic acid [GA], caffeic acid, chlorogenic acid, luteolin, rutin, fisetin, apigenin-7-*o*-glucoside, naringenin, benzene-triol, apigenin and chrysin) and high-quality chemicals such as CCl₄, NaCl, KCl, K₂S₂O₈, Na₂HPO₄, H₂O₂, sodium nitrite, C₂HCl₃O₂, 2,4-dinitrophenylhydrazine (DNPH), diazo reagent, sodium carbonate, aluminium trichloride, silymarin, Folin–Ciocalteu reagent, quercetin and DPPH radical were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagent kits for the analysis of serum biochemical parameters (ALT, AST, ALP and TB) were purchased from Merck (Darmstadt, Germany). Formaldehyde, olive oil and normal saline were purchased locally from registered companies, and deionized water was available in the biotechnology laboratory. Round bottom flasks (5,000 mL), separating funnel, beakers, syringes, blood collection tubes, rotary evaporator (IKA RV10 control; Fisher Scientific, USA), centrifuge machine (IEC CL31; Thermo Fisher Scientific, UK), spectrophotometer (UV-VIS 4000; ORI, Germany), homogenizer (KT5 Basic; Korea Process Technology Co., Ltd, Korea), refrigerator (Dawlance, Pakistan), Vortex Mixer 24 (OMNI International, USA), Sledge microtome (SM2400; Leica, Germany), microplate reader (Bio Tek Instrument, Inc., VT, USA), light microscope (CX41; Olympus, Tokyo, Japan) and filter papers were available in the laboratory.

2.2 Plant material

Fresh whole plant specimens of *A. alpinum* were collected from localities of Kallamoola, District Haveli, AJ&K, Pakistan, in its flowering season (July–August). The plant was identified by plant taxonomist, Dr. Aneel Gilani (Associate Curator Botanical science Division), Pakistan museum of Natural history (PMNH), Islamabad, Pakistan. The voucher specimen of the plant (voucher specimen number 040991) was submitted to PMNH for future reference. Roots of plant specimens were separated from shoots, washed with tap water and allowed to dry under shade for 7 days.

2.3 Experimental animals

In this experiment, a total of 83 (78 males + 5 females) mice (BALB/c albino mice) of average weight of 30 ± 5 g were purchased from animal house of National Institute of Health (NIH), Islamabad, and were kept in stainless-steel cages under the same environment conditions ($25 \pm 5^\circ\text{C}$ for 12-h light/dark cycle). All mice were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Bioethics and Biosafety Committee (IBBC) of the International Islamic University Islamabad (IIUI), Pakistan, through assigned number no. IIUI (BI&BT)/FBAS-IBBC-2015-04 (approved on June 30, 2015).

2.4 Extraction and fractionation of plant material

Dried roots of *A. alpinum* were finely ground into powder (2 mm) using a mechanical grinder. Powder of *A. alpinum* roots was processed for the preparation of methanolic extract by the maceration process as described previously with little modification [40]. For this purpose, 2 kg (2,000 g) of plant material was treated for the cold maceration process. In this process, 2×1 kg ($2 \times 1,000$ g) of *A. alpinum* roots powder was soaked in methanol (3,000 mL) with 1:3 in two round bottom flasks (5 L each) separately and kept for 3 days with daily shaking. The material was filtered through filter papers (Whatman filter paper no. 1). This process of extraction was repeated in duplicate way. Methanol was evaporated using a rotary evaporator (IKA RV10 control; Fisher Scientific, USA) at 40°C under reduced pressure. All the extract was weighed and labelled as AAME. Fractionation of AAME was carried

out using liquid–liquid extraction. Different organic solvents mainly *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water were selected on increasing polarity basis to partition AAME to individual fractions [22]. AAME (200 g) was reconstituted in the water (200 mL). This aqueous suspension was subjected to solvent–solvent partition for five fractions. In this process, *n*-hexane (200 mL) was added in an aqueous suspension of AAME, vigorously shaken and separated by a separating funnel. This process was repeated in triplicate. Similarly, this procedure was adopted for all other solvents (chloroform, ethyl acetate and *n*-butanol) on increasing polarity basis and at the last aqueous reconstituted portion was an aqueous fraction. All fractions were dried separately by rotary evaporator (IKA RV10 control; Fisher Scientific, USA) at 40°C under reduced pressure [41]. These fractions were labelled as *A. alpinum* *n*-hexane fraction (AAHF), *A. alpinum* chloroform fraction (AACF), *A. alpinum* ethyl acetate fraction (AAEF), *A. alpinum* *n*-butanol fraction (AABF) and *A. alpinum* aqueous fraction (AAAF). The dried extracts were kept in refrigerator at 4°C to avoid contamination until further investigation.

2.5 Acute toxicity test

Acute oral toxicity of AAME was performed as per Organization of Economic Cooperation and Development (OECD-425) guidelines [42] with minor modifications. Test dose (2,000 mg/kg) was orally administered in five albino ($n = 5$ female) mice having uniform weight (30 ± 5 g). One mouse fasted overnight having access to water only and was treated with a test dose (2,000 mg/kg) of AAME. Mouse was observed for 24 h for behavioural changes and mortality. Animal survived and then four additional animals were tested sequentially with a test extract dose (2,000 mg/kg) of AAME; therefore, the total five animals were tested. All mice were closely monitored at least once after dosing during first 24 h. Tested animals were observed daily for 14 days [42].

2.6 Hepatoprotective effect of different solvent fractions of AAME

2.6.1 Experimental design

Experimental grouping was designed according to the procedure adopted earlier [43–45] with little modifications. Male mice ($n = 78$) under study were randomly divided into 13 groups ($n = 6$ in each group). The groups

were as follows: normal control (NC) group (Group I), CCl₄ toxicant control (TC) group (Group II), standard drug i.e. silymarin treatment group (Group III) and AAME fractions i.e. AAHF, AACF, AAEF, AABF and AAAF with low and high dose (200 and 400 mg/kg) treatment groups (Groups IV–XIII), respectively. Animals in all groups were allowed for access to laboratory diet and water ad libitum up to 28 days.

2.6.2 Treatment procedure

NC group (Group I) was only provided with laboratory diet and water ad libitum for 4 weeks. TC group (Group II): 25% CCl₄ (1 mL/kg) with dilution in olive oil was administered through intraperitoneal injections [46] once daily with 2-day interval for 13 days. Group III: CCl₄ as in Group II + oral administration of standard hepatoprotective drug (silymarin 100 mg/kg/d) up to 4 weeks [46]. Animals in Groups IV–XIII were treated as follows: Group IV: CCl₄ as in Group II + treatment with AAHF; orally administered with low dose (200 mg/kg), once daily, for 4 weeks. Group V: CCl₄ as in Group II + treatment with AAHF; orally administered with high dose (400 mg/kg) once daily, for 4 weeks (28 days). Group VI: CCl₄ as in Group II + treatment with AACF; orally administered with low dose (200 mg/kg) once daily for 4 weeks (28 days). Group VII: CCl₄ as in Group II + treatment with AACF; orally administered with high dose (400 mg/kg) once daily for 28 days. Group VIII: CCl₄ as in Group II + treatment with AAEF; orally administered with low dose (200 mg/kg), once daily, for 28 days. Group IX: CCl₄ as in Group II + treatment with AAEF; orally administered with high dose (400 mg/kg) once daily, for 4 weeks. Group X: CCl₄ as in Group II + treatment with AABF; orally administered with low dose (200 mg/kg) once daily, for 4 weeks. Group XI: CCl₄ as in Group II + treatment with AABF; orally administered with high dose (400 mg/kg) once daily, for 4 weeks. Group XII: CCl₄ as in Group II + treatment with AAAF; orally administered with low dose (200 mg/kg) once daily, for 4 weeks. Group XIII: CCl₄ as in Group II + treatment with AAAF; orally administered with high dose (400 mg/kg) once daily, for 4 weeks [45].

2.6.3 Blood and tissue sampling

Food and water were withdrawn after last dose administration of all fractions of AAME in all the groups for 12 h. On 29th day, animals (mice) were anesthetized with chloroform.

Blood was collected via syringes through cardiac puncture, placed in glass tubes (vacutainer), allowed to clot, for assessment of serum liver biochemical parameters and then mice were sacrificed. Liver was dissected out and rinsed with ice cold saline to remove the debris. One part was placed in 10% formalin for histopathological assessment and another part from each liver was frozen at –4°C for the preparation of liver tissue homogenate for the estimation of antioxidant enzyme activity [47].

2.7 Biochemical and histopathological analyses of mice

For the assessment of liver protective effect of various solvent fractions of *A. alpinum*, the biochemical analysis of liver marker enzymes in blood serum and antioxidant enzyme activity of liver homogenate was performed [6,47]. To further support the study about hepatoprotective effect of these extracts on liver against CCl₄-induced toxicity, liver histopathology was performed for each group of mice [48,47].

2.7.1 Measurement of serum biochemical parameters

Blood serum was separated by centrifugation (3,000 rpm for 15 min) with a centrifugation machine (IEC CL31; Thermo Fisher Scientific, UK) and preserved at –20°C for further analysis.

The activities of liver marker enzymes such as ALT, AST and ALP were estimated spectrophotometrically (UV-VIS 4000; ORI, Germany) using diagnostic kits [49–51]. Serum TB level was also estimated by the standard procedure [52,53].

2.7.1.1 Determination of ALT activity

The ALT activity was determined according to the method described by Masood *et al.* [50] and Oriakhi *et al.* [51] using ALT reagent kits purchased from Merck (Darmstadt, Germany).

The serum sample was added in buffered solution containing DL-alanine and α-ketoglutarate (pH = 7.4) and incubated for 30 min at 37°C. Then, 1.0 mM DNPH was added followed by addition of 0.4 M NaOH and then absorbance was read by a spectrophotometer (UV-VIS 4000; ORI, Germany) at 500 nm. The enzyme activity

was determined by a standard curve by using absorbance of test samples.

2.7.1.2 Determination of AST activity

The AST activity was determined according to the method described by Masood et al. [50] and Oriakhi et al. [51] using AST reagent kits purchased from Merck (Darmstadt, Germany).

The serum sample was added in buffered solution containing L-aspartate and α -oxaloglutarate (pH = 7.4) and incubated for 1 h at room temperature. Then, 0.1 mL of 3 mM nicotinamide adenine dinucleotide hydride (NADH) was added followed by addition of 0.1 mL of malate dehydrogenase (MDH). The absorbance was measured by a spectrophotometer (UV-VIS 4000; ORI, Germany) at 334 nm. The rate of absorbance is directly proportional to the AST activity that was determined by a standard curve.

2.7.1.3 Determination of ALP activity

The activity of ALP was determined by the method of Oriakhi et al. [51] using ALT reagent kits purchased from Merck (Darmstadt, Germany).

For this purpose, 0.5 mL of ALP substrate was put in test tubes and equilibrated for 3 min at 37°C. At time interval, 0.05 mL of each standard, control and sample was added to respective test tubes, mixed gently and then incubated for 10 min at 37°C; then ALP (2.5 mL) was added and absorbance was read at 590 nm by a spectrophotometer (UV-VIS 4000; ORI, Germany).

2.7.1.4 Determination of plasma TB

Serum TB was determined by the diazo-reaction method described by Sazuki et al. [53] using reagent kits purchased from Merck (Darmstadt, Germany).

In this method, 0.1 mL of serum was added in 3.9 mL of 50% methanol, then 0.1 mL of diazo reagent was added and solution was mixed well. The reaction mixture was allowed to stand for 30 min at 25°C and then absorbance was measured at 540 nm by a spectrophotometer (UV-VIS 4000; ORI, Germany) against reagent blank.

2.7.2 Antioxidant enzyme assay

For the assessment of the activity of antioxidant enzymes (CAT, SOD and GSH), phosphate-buffered

saline (PBS, 137 mM sodium chloride, 2.68 mM potassium chloride, 10.14 mM sodium phosphate dibasic and 1.76 mM potassium phosphate monobasic in 1,000 mL of distilled water at pH = 7.2) was used to prepare liver homogenates (10% w/v) according to the standard procedure [6,20]. An amount of 100 mg of tissue of each animal was homogenized and then centrifugation of homogenate was performed (IEC CL31; Thermo Fisher Scientific, UK) at $12,000 \times g$ at 4°C for 30 min to obtain the supernatant for the assessment of the following antioxidant enzymes.

2.7.2.1 CAT activity

The CAT activity was determined by the method of Sinha [54] and Younis et al. [6]. CAT reaction solution consisted of 625 μ L of 50 mM potassium phosphate buffer (pH = 5.0), 100 μ L of 5.9 mM H_2O_2 and 35 μ L of supernatant. After 1 min, the development of green colour was read at 590 nm against blank by a spectrophotometer (UV-VIS 4000; ORI, Germany). The CAT activity was expressed as units/mg protein (mmol of hydrogen peroxide consumed/min/mg protein).

2.7.2.2 SOD activity

The SOD activity was determined by the method of Marklund and Marklund [55] and Younis et al. [6]. For this purpose, 150 μ L of supernatant was added to aliquot containing 600 μ L of 0.052 mM sodium pyrophosphate buffer (pH 7.0) and 50 μ L of 186 mM of phenazine methophosphate. To initiate the enzymatic reaction, 100 μ L of 780 μ M NADH was added. After 1 min, glacial acetic acid (500 μ L) was added to stop the reaction. Absorbance was determined by a spectrophotometer (UV-VIS 4000; ORI, Germany) read at 470 nm against blank for 3 min after every minute. The enzyme activity was expressed as units/mg of protein.

2.7.2.3 Reduced GSH activity

To determine the reduced-GSH activity, the method of Moron et al. [56] was used, for which 0.5 mL of 10% trichloroacetic acid was added to tissue homogenate followed by centrifugation. To the resulting protein-free supernatant, 4 mL of 0.3 M sodium phosphate dibasic (pH 8.0) and 0.5 mL of 0.04% (w/v) 5,5-dithiobis-2-nitrobenzoic acid were added and absorbance of the

resulting yellow colour was read spectrophotometrically (UV-VIS 4000; ORI, Germany) at 412 nm and the results are expressed as $\mu\text{g}/\text{mg}$ protein.

2.7.3 Histopathological analysis

Histological examination of liver sections was performed by the well-developed procedure [46,48,57]. Liver slices from different groups were removed, washed with saline and then transferred in 10% buffered formalin in specimen bottles separately for 24 h for proper fixation. Then dehydration of liver specimens was performed by serial dilutions of alcohol (70%, 90% and absolute alcohol) and then cleared in xylene and embedded in paraffin wax in a hot air oven at 56°C for 24 h. Thin sections ($-6\ \mu\text{m}$) of liver were cut using a microtome (SM2400; Leica, Germany). Preparations were lifted and transferred on glass material and stained with basic stain (haematoxylin) for 40 s and counter stained with acidic stain (eosin) for 20 s and then observed under a microscope (CX41; Olympus, Tokyo, Japan) to see the changes in liver architecture and hepatocytes such as necrosis, inflammation and fatty infiltration [7,47,48]. Photographs of slides were taken using a camera (UTU1X-2; Olympus, Japan).

2.8 Antioxidant activity (DPPH assay)

The antioxidant activity of AAME as well as its fractions i.e. AAHF, AACF, AAEF, AABF and AAAF was determined by the procedure adopted by Kulisic *et al.* [58] and Kalisz *et al.* [59] with minor modifications, using DPPH as a free radical. IC_{50} was calculated with comparison of standard ascorbic acid (AA) to determine the antioxidant activity. For this purpose, DPPH (4 mg) was dissolved in 100 mL of methanol to prepare the solution. The DPPH solution (2,800 μL) was mixed in the methanolic extract and other fraction solutions (200 μL each) by addition in separate glass vials so as to lead to the final concentrations of 100, 70, 50, 25, 15 and 10 $\mu\text{g}/\text{mL}$, respectively. All the mixtures were shaken well and kept at room temperature (25–28°C) for 1 h. The absorbance of samples was measured at 517 nm spectrophotometrically (UV 4000; ORI, Germany). Methanol was used as blank, while mixture of DPPH (2,800 μL) solution and methanol (200 μL) was used as a negative control and AA was taken as a positive control. Percentage (%) inhibition was calculated using the following equation:

$$\text{Scavenging effect (\% A A)} = [(A_C - A_S) \div A_C] \times 100$$

where A_C means the absorbance of negative control and A_S means the absorbance of test sample. IC_{50} value was calculated by linear regression constructed by the graphic method ($y = 0.7283x + 26.7$, $R^2 = 0.9771$).

2.9 Quantitative analysis of phenolic and flavonoid contents

AAME and most active fractions i.e. AAEF, AABF and AAAF were subjected to quantitative analysis of total phenolic contents (TPCs) and total flavonoid contents (TFCs). Analysis was performed according to the previously standardized methods [11,20,60] with little modifications.

TPCs were estimated using the Folin–Ciocalteu reagent obtained from Sigma-Aldrich (St. Louis, MO, USA) using GA as standard. GA solution was prepared first and then serial dilutions were performed to make the final concentrations of 500, 250, 125, 50, 25, 10, 5 and 2.5 $\mu\text{g}/\text{mL}$, respectively. The sample solutions were also prepared by the similar procedure. All samples and GA solutions were tested in a similar manner by measuring the absorbance at the microscale by using a microplate reader (Bio Tek Instrument, Inc., VT, USA) at 544 nm. During this assay, in 4 μL of extract solution, 180 μL of distilled water was added and then 4 μL of Folin–Ciocalteu reagent afterwards (all this was done in three replicates using a 96-well microplate) for each sample extract. The plate was shaken vigorously with the addition of reagent. After 3 min, 12 μL of aqueous sodium carbonate (2%) solution was added and then the mixture was allowed to stand in the dark for 2 h with the application of intermittent shaking. A blank sample comprising 4 μL of extract solution, 180 μL of distilled water and 12 μL of aqueous sodium carbonate (2%) solution was used against the test samples to read the absorbance at 544 nm. Calculation of phenolic compound concentration was performed according to the regression equation ($y = 0.0043X + 0.0888$) constructed from a standard GA calibration curve having coefficient of correlation $R^2 = 0.9879$. Results were expressed as microgram per milligram of GA equivalent ($\mu\text{g}/\text{mg}$ GAE) of extract.

TFCs were determined by the aluminium chloride reagent method [11,60] using quercetin as a standard. Different concentrations of quercetin (500, 250, 125, 50, 25, 10, 5 and 2.5 $\mu\text{g}/\text{mL}$) solution and test samples were prepared using methanol as a solvent. Standard solutions were tested similar to test samples using aluminium trichloride (2% AlCl_3). The absorbance of standard solution was read at 405 nm against the blank quercetin solution. During this

assay, 100 μ L of extract solution was added in 100 μ L of 2% aluminium trichloride in methanol (all this was done in three replicates using a 96-well plate). After 10 min, the absorbance was read at 405 nm against blank samples (100 μ L of extract solution along with 100 μ L of methanol). The concentration of flavonoid compounds was calculated according to the equation obtained from the standard quercetin calibration curve. The regression equation was $y = 0.0051X + 0.0912$ and coefficient of correlation was $R^2 = 0.9964$. The results were expressed as microgram per milligram of quercetin equivalent (μ g/mg QE) of the extract.

2.10 High-performance liquid chromatography analysis of polyphenolic compounds

High-performance liquid chromatography (HPLC) analysis of methanolic extract (AAME) and its most active fractions (AAEF, AABF and AAAF) for hepatoprotective effect and antioxidant activity was performed by co-elution with reference standard compounds for the identification of phenolic and flavonoid compounds.

2.10.1 Preparation of samples

Stock solutions of phenolic compounds (reference standards) and the samples (AAME, AAEF, AABF and AAAF) were prepared in HPLC grade methanol (1 mg/mL) in amber Eppendorf tubes to avoid from effect of light. These mixtures were subjected to sonication (10 min) for proper mixing. All the samples were also filtered using Nylon membrane filters (0.45 μ m) in separate Eppendorf tubes.

2.10.2 HPLC methodology

HPLC analysis for phenolic compounds and samples was performed using Waters Breeze™ HPLC (USA) system (with a Zorbax SBC-18, 150 $\times$ 3 mm, 3.5 μ m column and 1525 binary pump) to analyse the phenolic and flavonoid compounds in samples. The 717 plus autosampler (injection volume of 10 μ L) was equipped with in-line Degasser and 2487 UV detector. Temperature of the column oven was set to 48 and 60°C and the flow rate to 1 mL/min. Run time for samples was 70 min each and all the detections of each sample were made at 330 nm. Two mobile phases gradient elution system was used. Mobile phase 1 (A) was phosphate buffer (pH 2.3, 85% orthophosphoric acid) whereas mobile phase 2 (B)

was methanol (HPLC grade). The elution of gradient was buffer/methanol 95:5 (0–52 min), 58:42 (52–57 min) then again with initial composition (57–60 min) having a flow rate of 1 mL/min and was let to run for another 10 min before injecting the next sample [61]. Identification of compounds was performed by comparison of retention time and absorption spectrum of these sample constituents with the reference compound used.

2.11 Statistical analysis

Data on animal model (mice) experiment were expressed as mean values $\pm$ SE with six animals in each group. Comparison between the groups was performed by one-way analysis of variance using Statistix 8.1, followed by the Tukey HSD test with $P < 0.01$ being statistically significant [62]. The values of scavenging effect (%) were expressed as mean $\pm$ standard deviation (SD) and linear regression was constructed by the graphic method using excel 2016 to calculate the IC₅₀ value for the antioxidant activity of samples.

3 Results

3.1 Acute toxicity test

It is established in our study that single dose (2,000 mg/kg) of crude methanolic extract of plant (AAME) administered orally in the mice observed for 24 h (acute toxicity) did not cause any toxic effect. There was not any gastrointestinal, morphological and behavioural change in mice, which made it clear that this plant was safe and non-toxic and oral lethal dose (LD₅₀) in mice was beyond 2,000 mg/kg of AAME.

3.2 Effect of different solvent fractions of AAME on liver biochemical parameters and histopathology

3.2.1 Liver biochemical parameters

Hepatoprotective effects of different fractions of *A. alpinum* (AAHF, AACF, AAEF, AABF and AAAF) with low and high doses (200 and 400 mg/kg) of each fraction on liver biochemical parameters (ALT, AST, ALP and TB) are represented in Table 1. The normal levels of liver

Table 1: Effect of different solvent fractions of AAME on serum biochemical parameters against CCl₄-induced toxicity in mice

Group	Treatment	Biochemical parameters			
		ALT (IU/L)	AST (IU/L)	ALP (IU/L)	TB (mg/dL)
I	Normal control (NC)	53.17 ± 5.38	63.08 ± 4.98	171.33 ± 5.38	0.66 ± 0.05
II	TC (25% CCl ₄)	207.67 ± 4.51 ^a	212.07 ± 3.76 ^a	417.67 ± 5.60 ^a	2.38 ± 0.19 ^a
III	CCl ₄ + S.D (silymarin 100 mg)	62.05 ± 5.16 ^{fg}	77.03 ± 3.56 ^{fg}	191.17 ± 4.99 ^e	0.77 ± 0.05 ^{def}
IV	CCl ₄ + AAHF (200 mg/kg)	156.99 ± 5.65 ^b	165.06 ± 4.51 ^b	358.05 ± 5.24 ^b	1.85 ± 0.15 ^b
V	CCl ₄ + AAHF (400 mg/kg)	144.15 ± 5.58 ^b	160.01 ± 5.32 ^b	345.11 ± 5.22 ^b	1.74 ± 0.13 ^b
VI	CCl ₄ + AACF (200 mg/kg)	160.06 ± 5.85 ^b	175.03 ± 3.75 ^b	351.16 ± 6.28 ^b	1.80 ± 0.03 ^b
VII	CCl ₄ + AACF (400 mg/kg)	148.17 ± 4.61 ^b	167.33 ± 3.50 ^b	340.18 ± 4.80 ^b	1.68 ± 0.02 ^{bc}
VIII	CCl ₄ + AAEF (200 mg/kg)	106.04 ± 4.28 ^{cd}	120.03 ± 3.75 ^{cd}	250.11 ± 4.27 ^{cd}	1.14 ± 0.08 ^{de}
IX	CCl ₄ + AAEF (400 mg/kg)	73.03 ± 5.19 ^{efg}	90.04 ± 5.05 ^{ef}	194.03 ± 3.46 ^e	0.78 ± 0.04 ^{def}
X	CCl ₄ + AABF (200 mg/kg)	114.02 ± 5.20 ^c	128.17 ± 2.90 ^c	260.05 ± 4.47 ^c	1.22 ± 0.06 ^{cd}
XI	CCl ₄ + AABF (400 mg/kg)	80.67 ± 4.05 ^{def}	103.06 ± 4.47 ^{de}	226.33 ± 5.15 ^d	0.92 ± 0.05 ^{def}
XII	CCl ₄ + AAAF (200 mg/kg)	92.93 ± 2.12 ^{cde}	107.05 ± 3.46 ^{cde}	248.10 ± 4.56 ^{cd}	1.09 ± 0.03 ^{de}
XIII	CCl ₄ + AAAF (400 mg/kg)	53.05 ± 4.50 ^g	74.10 ± 4.82 ^{fg}	184.04 ± 3.52 ^e	0.58 ± 0.04 ^f

Values are expressed as mean ± SEM of six mice in each group. The different letters a, b, c, d, etc. show the significant ($P < 0.01$) difference from each other. ^a $P < 0.01$ versus normal control (NC) group (Group I). ^{b–g} $P < 0.01$ versus toxicant control (TC and Group II). The values with same letters do not have significant difference from each other.

biochemical parameters (ALT, AST, ALP and TB) were 53.17 ± 5.38, 63.08 ± 4.98, 171.33 ± 5.38 IU/L and 0.66 ± 0.05 mg/dL in the normal group (Group I) which were increased to 207.67 ± 4.51, 212.07 ± 3.76, 417.67 ± 5.60 IU/L and 2.38 ± 0.19 mg/dL, respectively, on CCl₄ intoxication (Group II). Upon treatment with low dose (200 mg/kg) of fractions of *A. alpinum* (AAHF, AACF, AAEF, AABF and AAAF) orally administered in treatment groups (IV, VI, VIII, X and XII) after CCl₄ intoxication, the values of serum liver biochemical parameters (ALT, AST, ALP and TB) significantly ($P < 0.01$) decreased when compared with those of the CCl₄ TC group (Group II). The values of these biochemical parameters (ALT, AST, ALP and TB) were non-significantly ($P > 0.01$) decreased with treatment with high dose (400 mg/kg) of less polar fractions (AAHF and AACF) when compared among themselves in respective groups (IV–VII) as compared to other fractions. The dose-dependent effect was significantly improved with treatment of increasing polarity fractions (AAEF, AABF and AAAF) in respective groups (VIII–XIII) when compared among same fractions with low and high doses themselves (Table 1) compared to the CCl₄ TC group (Group II).

3.2.2 Antioxidant enzyme activity

The results of treatment effect of *A. alpinum* fractions on antioxidant enzyme activity are represented in Table 2. There is significant decrease in endogenous antioxidant enzymes such as CAT, SOD and GSH of liver by the

treatment of CCl₄ as compared to the NC group. Treatment with standard drug (silymarin 100 mg/kg) and *A. alpinum* fractions (AAHF, AACF, AAEF, AABF and AAAF) with low doses (200 mg/kg) have significantly ($P < 0.01$) increased the enzyme activity level as compared to the toxicant (CCl₄ treated) group (Table 2). The results showed that there was tremendous increase in the level of antioxidant enzymes (CAT, SOD and GSH) when high dose (400 mg/kg) of polar fractions (AAEF, AABF and AAAF) was administered in mice. The activity of AAEF, AABF and AAAF at a dose of 400 mg/kg was comparable to standard drug silymarin (positive control) and NC mice.

3.2.3 Histopathological analysis

The results of histopathological analysis under a light microscope of liver sections are shown in Figure 1a–m. The effect of AAHF with low and high dose (200 and 400 mg/kg) treatment after CCl₄ intoxication showed focal necrosis and inflammatory cell infiltration. There is mild constriction of central vein and hepatocytes are in process of mild recovering stage (Figure 1d–e). There is moderate degeneration of cells exhibited by moderate inflammatory cell infiltration and fatty degeneration; however, hepatocytes are moderately developed. Figure 1f and g shows the effect of AACF with low and high dose (200 and 400 mg/kg) treatment after CCl₄ intoxication, respectively, showing portal vein dilation, mild fatty degeneration and ballooning, but there is

Table 2: Effect of different solvent fractions of AAME on antioxidant enzyme activity against CCl₄-induced toxicity in mice

Treatment	Liver antioxidant enzymes activity		
	CAT (U/mg protein)	GSH (U/mg protein)	SOD (U/mg protein)
NC	62.837 ± 0.45 ^b	33.54 ± 0.53 ^a	30.565 ± 0.76 ^b
TC (25% CCl ₄ only)	33.39 ± 0.40 ^g	12.78 ± 0.40 ^g	11.527 ± 0.44 ^g
CCl ₄ + silymarin (100 mg/kg)	65.5 ± 0.55 ^a	35.293 ± 0.48 ^a	26.763 ± 0.43 ^c
CCl ₄ + AAHF (200 mg/kg)	39.492 ± 0.50 ^f	20.028 ± 0.36 ^f	16.218 ± 0.54 ^f
CCl ₄ + AAHF (400 mg/kg)	42.53 ± 0.59 ^e	24.252 ± 0.47 ^e	21.358 ± 0.64 ^d
CCl ₄ + AACF (200 mg/kg)	38.005 ± 0.33 ^f	16.318 ± 0.64 ^f	17.513 ± 0.48 ^{ef}
CCl ₄ + AACF (400 mg/kg)	44.175 ± 0.60 ^f	16.485 ± 0.46 ^f	22.028 ± 0.53 ^d
CCl ₄ + AAEF (200 mg/kg)	42.532 ± 0.69 ^e	27.055 ± 0.38 ^{cd}	25.003 ± 0.54 ^c
CCl ₄ + AAEF (400 mg/kg)	63.73 ± 0.51 ^{ab}	35.638 ± 0.48 ^a	36.485 ± 0.54 ^a
CCl ₄ + AABF (200 mg/kg)	43.507 ± 0.57 ^e	24.75 ± 0.55 ^d	19.703 ± 0.59 ^{de}
CCl ₄ + AABF (400 mg/kg)	57.338 ± 0.52 ^c	30.988 ± 0.46 ^b	30.462 ± 0.54 ^b
CCl ₄ + AAAF (200 mg/kg)	46.965 ± 0.30 ^d	28.882 ± 0.65 ^{bc}	26.252 ± 0.30 ^c
CCl ₄ + AAAF (400 mg/kg)	61.815 ± 0.47 ^b	35.885 ± 0.52 ^a	34.695 ± 0.39 ^a

Values are expressed as mean ± SEM of six mice in each group. The different letters a, b, c, d, etc. show the significant ($P < 0.01$) difference from each other. ^g $P < 0.01$ versus normal control (NC). ^{a–f} $P < 0.01$ versus toxicant control (TC). The values with same letters do not have significant difference from each other.

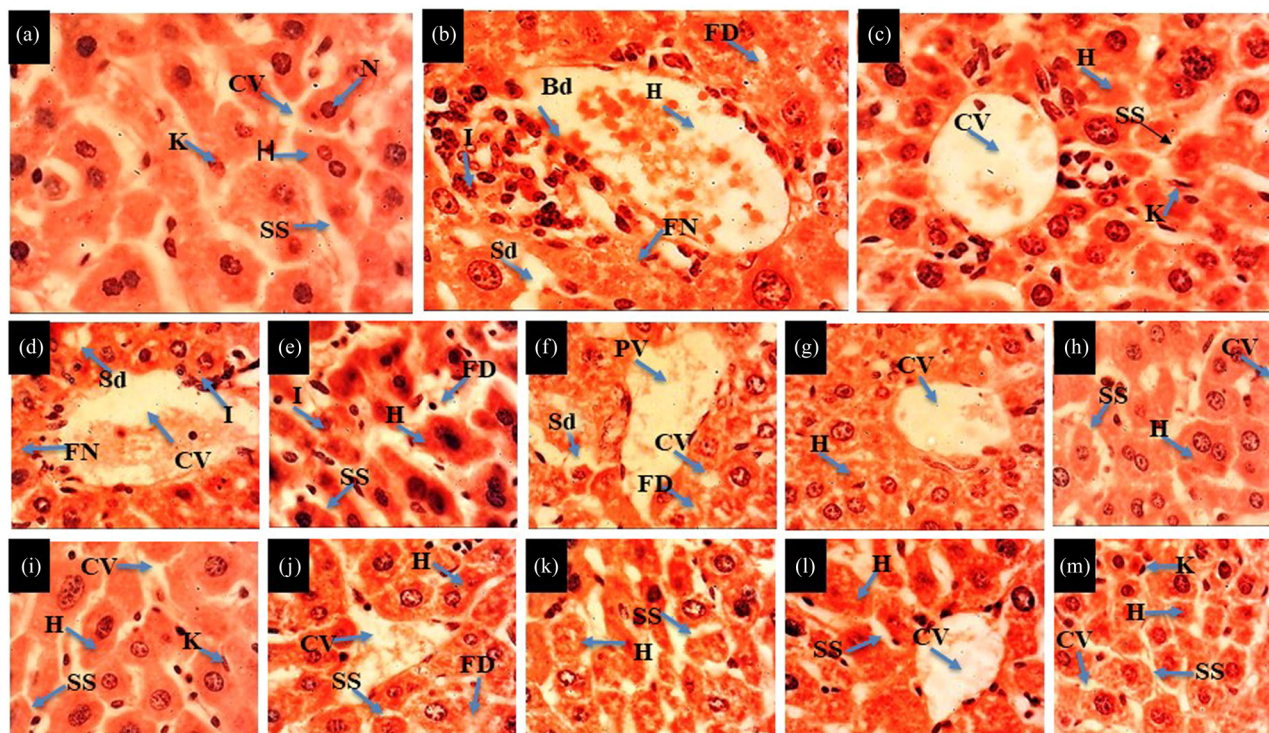


Figure 1: Photomicrograph of liver section of mice. Group I: normal control (NC) mice (a), Group II: 25% CCl₄ toxicant control (b) and Group III: standard drug (silymarin 100 mg/kg) control (c). Group IV: CCl₄ + AAHF 200 mg/kg (d) and Group V: CCl₄ + AAHF 400 mg/kg (e). Group VI: CCl₄ + AACF 200 mg/kg (f) and Group VII: CCl₄ + AACF 400 mg/kg (g). Group VIII: CCl₄ + AAEF 200 mg/kg (h) and Group IX: CCl₄ + AAEF 400 mg/kg (i). Group XC: CCl₄ + AABF 200 mg/kg (j) and Group XIC: CCl₄ + AABF 400 mg/kg (k). Group XII: CCl₄ + AAAF 200 mg/kg (l) and Group XIII: CCl₄ + AAAF 400 mg/kg (m). H&E magnification at 100×. Arrows representing, CV – central vein; FD – fatty degeneration; F – focal necrosis; H – hepatocytes; I – inflammatory cells infiltration; K – Kupffer cells; PV – portal vein; Sd – sinusoidal dilation and SS – sinusoids.

Table 3: Percent (%) DPPH radical- scavenging activity of *A. alpinum* extract and standard AA with IC₅₀ values

Extract	Radical scavenging effect (%) at different concentrations (µg/mL) ± SD						IC ₅₀ (µg/mL)
	10	15	25	50	70	100	
AAME	17.5 ± 1.8	24.5 ± 1.1	31.7 ± 2.3	66.3 ± 0.6	73 ± 1.8	91.2 ± 1.4	44.16
AAHF	8.4 ± 1.2	17.3 ± 0.8	22.2 ± 1.2	31.5 ± 1.3	48.0 ± 2.3	67.00 ± 2.4	73.75
AACF	7.5 ± 1.6	16.9 ± 1.2	33.2 ± 1.2	48.4 ± 2.9	65.6 ± 0.7	79.5 ± 0.9	55.47
AAEF	28.4 ± 1.2	36.6 ± 0.8	50.3 ± 1.1	69.5 ± 1.1	86.2 ± 0.7	96.4 ± 0.6	30.20
AABF	19.7 ± 0.4	27.00 ± 1.3	39.5 ± 2.4	58.4 ± 0.6	79.2 ± 2.1	89.5 ± 0.2	42.19
AAAF	33.2 ± 2.3	48.0 ± 0.0	57.2 ± 1.5	65.1 ± 1.9	66.2 ± 1.8	80.2 ± 2.3	25.51
A.A	29.0 ± 1.7	37.1 ± 2.1	48.2 ± 1.5	66.2 ± 0.8	81.4 ± 0.5	95.3 ± 1.2	31.9

Values are expressed as percent (%) absorbance of mean ± SD, $n = 3$ of each sample.

clear separation of hepatocytes with less loss of cellular boundaries (Figure 1f). Cellular structure exhibits that there is prominent central vein and hepatocytes in the regeneration process (Group I). Figure 1h and i shows the effect of AAEF with low and high dose (200 and 400 mg/kg) treatment after CCl₄ induction, respectively, which reveals that there is distinction of hepatocytes and central vein with clear sinusoidal spaces (Figure 1h). There is more cellular protection with clear central vein, and Kupffer cells and normal hepatocytes are distinctive by well-organized sinusoidal spaces (Figure 1i). This shows that AAEF has more dose-dependent recovery effect on damaged liver cells, which is also comparable to normal cells of liver (Group I). Figure 1j–k shows the effect of AABF with low and high dose (200 and 400 mg/kg) treatment after CCl₄ intoxication, respectively. There is moderate fatty change in the shape of central vein. Hepatocytes and sinusoids are distinctly seen (Figure 1j). There is also more recovery effect of damaged cells having clear sinusoidal space around the hepatocellular structure showing dose-dependent protective effect (Figure 1k). Figure 1l–m shows the result of effect of AAAF with low and high dose (200 and 400 mg/kg) treatment after CCl₄ intoxication, respectively. There is appearance of normal central vein, normal hepatocytes and more distinct separation by sinusoidal spaces (Figure 1l). Liver section in Figure 1m shows normal cellular architecture with sinusoidal spaces separation to normal hepatocytes. Highest cellular protection against CCl₄ damage is revealed by AAAF followed by AAEF and AABF in a dose-dependent manner.

3.4 DPPH radical scavenging activity of *A. alpinum*

Scavenging potential (%) of samples and standard AA against DPPH is presented in Table 3. In our results of the DPPH assay, methanolic extract (AAME) as well as its

different fractions i.e. AAHF, AACF, AAEF, AABF and AAAF of *A. alpinum* showed effective free radical scavenging activity. The highest scavenging activity is of AAEF (96.4 ± 0.6%) followed by those of AA (95.3 ± 1.2), AAME (91.2 ± 1.4), AABF (89.5 ± 0.2), AAAF (80.2 ± 2.3), AACF (79.5 ± 0.9) and AAHF (67.2 ± 2.4). The scavenging effect is increased on increasing the concentration of samples as indicated in Table 3. In the case of antioxidant activity of test samples (AAME, AAHF, AACF, AAEF, AABF and AAAF) and standard AA, the IC₅₀ values are 44.16, 73.75, 55.47, 30.20, 42.19, 25.51 and 31.90 µg/mL, respectively. The order of decreasing IC₅₀ values of samples is AAHF > AACF > AAME > AABF > AA > AAEF > AAAF (with IC₅₀ values of 73.75, 55.47, 44.14, 42.19, 31.90, 30.20 and 25.51 µg/mL, respectively). The lowest is the IC₅₀ value of the sample, the highest is its antioxidant activity. AAAF showed the lowest IC₅₀ value (25.51 µg/mL), with the highest antioxidant activity. Similarly, the promising high value of antioxidant activity is of AAEF (30.06 µg/mL) compared to the IC₅₀ value of standard antioxidant AA with an IC₅₀ of 31.90 µg/mL.

3.5 Quantitative analysis of TPCs and TFCs

The results of TPC and TFC analyses for AAME and more active fractions (AAEF, AABF and AAAF) are presented in Table 4. TPCs of AAME and its more active fractions

Table 4: Calculation of TPC and TFC of *A. alpinum* extracts

Extract	TPC (µg/mg GAE)	TFC (µg/mg QE)
AAME	192.892 ± 0.814	95.778 ± 0.404
AAEF	185.164 ± 0.836	132.279 ± 0.297
AABF	109.433 ± 1.771	128.267 ± 0.514
AAAF	165.304 ± 0.708	150.659 ± 0.489

Results have been expressed as mean ± SD, $n = 3$ for each value.

(AAEF, AABF and AAAF) are 192.892 ± 0.814 , 185.164 ± 0.836 , 109.433 ± 1.771 and 165.304 ± 0.708 $\mu\text{g}/\text{mg}$ GAE, respectively. The order of TPC in *A. alpinum* is AAME > AAEF > AAAF > AABF.

TFCs of the crude methanolic extract of *A. alpinum* (AAME) and its active fractions (AAEF, AABF and AAAF) are 95.778 ± 0.404 , 132.279 ± 0.297 , 128.267 ± 0.514 and 150.659 ± 0.489 $\mu\text{g}/\text{mg}$ QE, respectively. This showed that more polar solvents have high concentration of TFC. The order of TFC in *A. alpinum* is AAAF > AAEF > AABF > AAME (Table 4).

3.6 HPLC analysis of polyphenolic compounds

The HPLC method developed for the detection of phenolic and flavonoid compounds rendered a quick analysis of all the samples of AAME and its more active fractions i.e. AAEF, AABF and AAAF. The phenolic and flavonoid compounds were identified by co-elution with the reference standards used as indicated by the standard chromatogram in Figure 2. The presence of phenolic and flavonoid compounds in AAME and its active fractions (AAEF, AABF and AAAF) with chromatograms of peaks are indicated in Figures 3–6, respectively. Different reference standards of phenolic and flavonoid compounds, namely, GA, caffeic acid, chlorogenic acid, epicatechin, ferulic acid, hyperoside, luteolin, rutin, fisetin, apigenin-7-*o*-glucoside, naringenin, benzene-triol, apigenin and chrysin were eluted at 1.847, 10.070, 11.698, 15.256, 19.726, 29.110, 30.259, 30.893, 33.789, 35.810, 38.705, 42.768, 47.076 and 57.287 min, respectively,

as indicated in Figure 2. The HPLC chromatogram of AAME showed the seven peaks that coincided with the retention time of GA, chlorogenic acid, epicatechin, rutin, apigenin-7-*o*-glucoside, naringenin and chrysin (Figure 3). There were ten phenolic compounds detected in AAEF as indicated in Figure 4. The chromatogram of AAEF showed ten peaks that were closely coincided with GA, caffeic acid, chlorogenic acid, epicatechin, ferulic acid, luteolin, rutin, apigenin-7-*o*-glucoside, naringenin and chrysin, respectively. There were 14 phenolic compounds detected in AABF fraction whose peaks closely coincided with GA, caffeic acid, chlorogenic acid, epicatechin, ferulic acid, hyperoside, luteolin, rutin, fisetin, apigenin-7-*o*-glucoside, naringenin, benzene-triol, apigenin and chrysin, respectively, as indicated in Figure 5. From Figure 6, it is evident that the chromatogram of AAAF revealed the presence of 12 phenolic compounds having clear coincidence with caffeic acid, chlorogenic acid, epicatechin, ferulic acid, hyperoside, luteolin, rutin, apigenin-7-*o*-glucoside, naringenin, benzene-triol, apigenin and chrysin. In this plant, a higher number of phenolic and flavonoid compounds are identified in AABF (14) followed by AAAF (12), AAEF (10) and AAME (7), indicated in HPLC chromatograms (Figures 3–6).

4 Discussion

Medicinal plants have an important role in drug discovery. Due to increasing attention to side effects by the use of synthetic agents to cure the diseases, search for new, efficient and less toxic drugs is very important

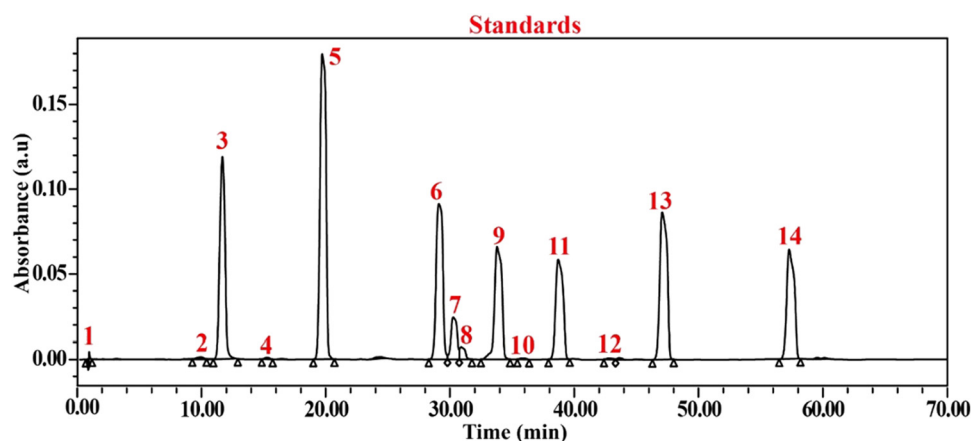


Figure 2: HPLC chromatogram of reference standards for flavonoids. 1: gallic acid (1.847 min), 2: caffeic acid (10.070 min), 3: chlorogenic acid (11.698 min), 4: epicatechin (15.256 min), 5: ferulic acid (19.726 min), 6: hyperoside (29.110 min), 7: luteolin (30.259 min), 8: rutin (30.893 min), 9: fisetin (33.789 min), 10: apigenin-7-*o*-glucoside (35.810 min), 11: naringenin (38.705 min), 12: benzene-triol (42.768 min), 13: apigenin (47.076 min) and 14: chrysin (57.287 min).

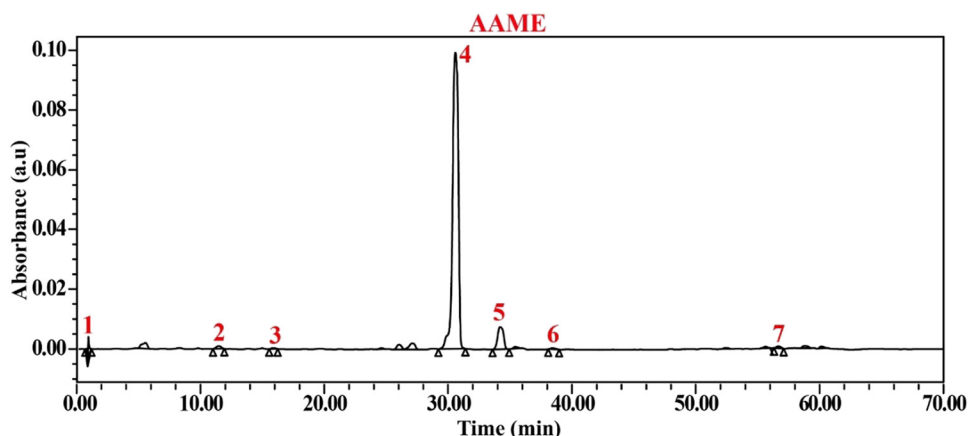


Figure 3: HPLC chromatogram of AAME. Peaks showing similar retention time to 1: gallic acid (0.960 min), 2: chlorogenic acid (11.479 min), 3: epicatechin (15.877 min), 4: rutin (30.591 min), 5: apigenin-7-*o*-glucoside (34.207 min), 6: naringenin (38.467 min) and 7: chrysin (56.663 min).

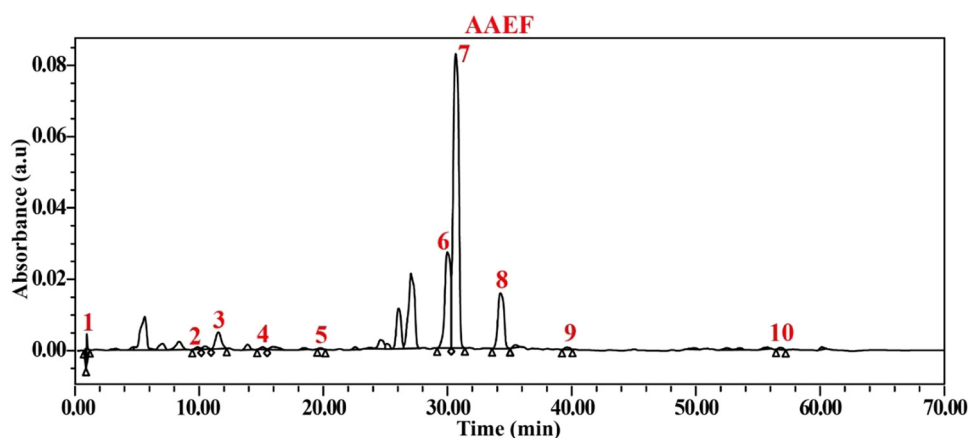


Figure 4: HPLC chromatogram of *A. alpinum* ethyl acetate fraction (AAEF). Peaks showing similar retention time to 1: gallic acid (0.970 min), 2: caffeic acid (9.888 min), 3: chlorogenic acid (11.550 min), 4: epicatechin (15.113 min), 5: ferulic acid (19.763 min), 6: luteolin (29.988 min), 7: rutin (30.660 min), 8: apigenin-7-*o*-glucoside (34.263 min), 9: naringenin (39.615 min) and 10: chrysin (56.784 min).

nowadays for pharmaceuticals. Natural compounds from medicinal plants play an important role to develop the drugs for the treatment of various kinds of diseases with remarkable contribution [12]. In most cases, the pharmacological activity of plants is limited to the crude extract only ignoring the separation of phytochemicals from the mixture by which consistent biological activities are not well characterized. Efficient herbal formulation can be better approached through solvent–solvent partition of mixture of compounds from their crude extract. When the secondary metabolites of plants are separated from the mixture, the specificity and efficacy are expected to be increased for standardization and characterization of plant-derived drugs. The partitioned

method is more important to increase the efficiency of herbal medicines [63]. Fractionation of AAME based on increasing polarity led to the separation of polar compounds through polar solvents and intermediate polar compounds by intermediate polar solvent in an efficient way [64].

Our body is protected by the antioxidant defence system. Multivariate causes involving propagation of chain of free radicals, lipid peroxidation and cellular membrane destruction, generation of ROS and occurrence of oxidative stress are pathogenesis of liver damage [14]. The biochemical parameters such as ALT, AST and ALP are key biomarkers whose level in the serum reflects the physiological activities of liver. ALT is

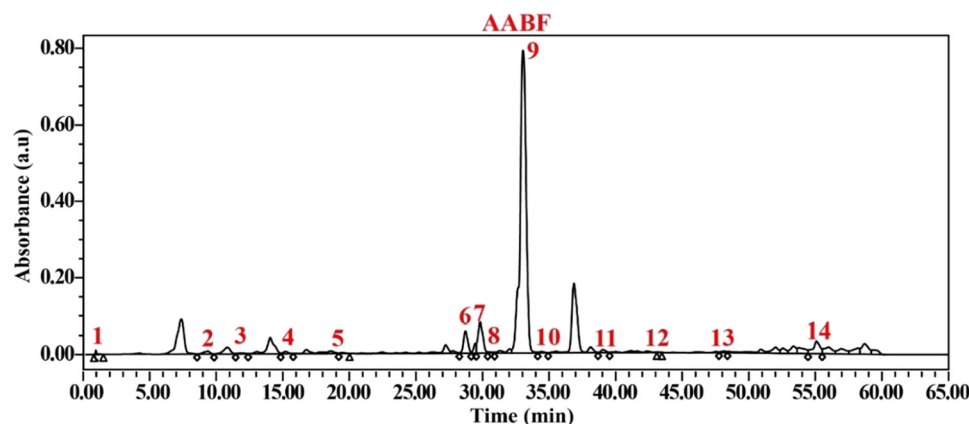


Figure 5: HPLC chromatogram of AABF. Peaks showing similar retention time to 1: gallic acid (1.263 min), 2: caffeic acid (9.366 min), 3: chlorogenic acid (11.870 min), 4: epicatechin (15.221 min), 5: ferulic acid (19.438 min), 6: hyperoside (28.723 min), 7: luteolin (29.822 min), 8: rutin (30.508 min), 9: fisetin (33.043 min), 10: apigenin-7-*o*-glucoside (34.444 min), 11: naringenin (39.054 min), 12: benzene-triol (43.104 min), 13: apigenin (47.538 min) and 14: chrysin (56.962 min).

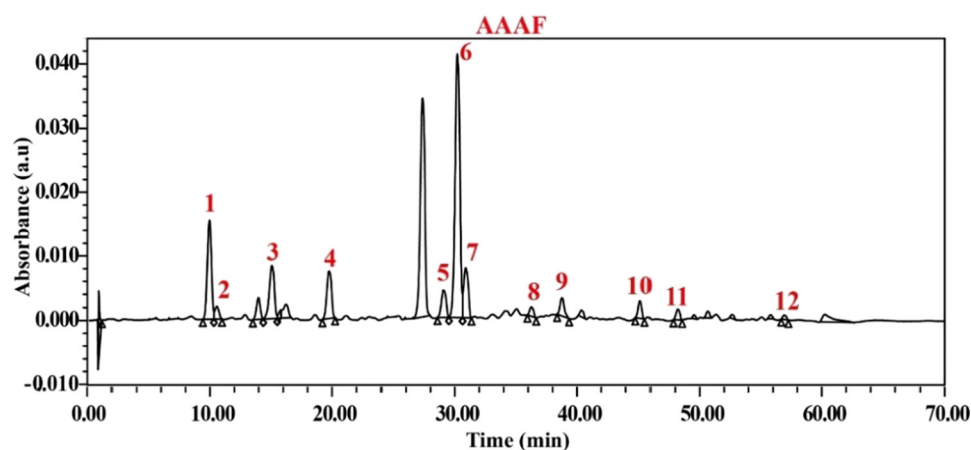


Figure 6: HPLC chromatogram of AAAF. Peaks showing similar retention time to 1: caffeic acid (9.969 min), 2: chlorogenic acid (10.569 min), 3: epicatechin (15.075 min), 4: ferulic acid (19.765 min), 5: hyperoside (29.076 min), 6: luteolin (30.208 min), 7: rutin (30.891 min), 8: apigenin-7-*o*-glucoside (36.265 min), 9: naringenin (38.781 min), 10: benzene-triol (45.156 min), 11: apigenin (48.213 min) and 12: chrysin (56.920 min).

the main metabolic enzyme that is present in intracellular parts, while AST is mostly bound in mitochondria. An oxidative stress induction leads to the circulation of these enzymes in the blood serum as a result of proliferation of free radicals and ROS generation [65]. CCl_4 has been widely used as an hepatotoxic agent in animal models to study the hepatoprotective effect of medicinal plants. Experimental damage causes histopathological changes in the liver; as a result of toxicity, the metabolic enzymes are released into blood. The leakage of these enzymes indicates necrosis, ballooning degeneration and cellular infiltration of liver tissues. CCl_4 -induced toxicity generates the ROS and triggers inflammation that leads to the development of hepatic damage and results in the decrease of antioxidant enzymes [65].

The use of natural antioxidants and plants has become the focus interestingly, and herbal medicines are generally considered a well-known complementary therapeutic strategy to combat the liver disorders [13]. Research has demonstrated that plant extracts are composed of phytochemical compounds that prevent the liver damage by increasing the antioxidant defence system by suppression of lipid peroxidation against CCl_4 -induced liver damage [66].

The results of our study confirmed the hepatoprotective effect of different solvent fractions of *Aconogonon alpinum* methanolic extract (AAME) against CCl_4 -induced toxicity. One way for the estimation of extent of hepatic damage is through the estimation of serum level of cytoplasmic enzymes such as ALT, AST, ALP and also

the level of serum TB. The results of this study about hepatoprotective effect of different solvent fractions of AAME showed that CCl_4 significantly caused the hepatic injury that increased the levels of serum biochemical parameters (ALT, AST, ALP and TB) due to leakage and decreased the levels of antioxidant enzymes (CAT, SOD and GSH; Tables 1 and 2). Treatment effect of solvent fractions of AAME has significantly reduced the increased level of serum biochemical parameters and increased the antioxidant enzymes. Reports show that lipid peroxidation, reduced activity of antioxidant enzymes and generation of free radicals are main reasons of CCl_4 -induced hepatic injury [67]. The hepatoprotective effect of *A. alpinum* extracts on CCl_4 -induced toxicity might be concerned with alleviating the increased level of serum biochemical parameters by restoring the injuries of liver, increasing the antioxidant enzyme level in the serum by detoxification of oxidative stress and suppressing the generation of ROS [5]. Extraction method concerning the solvent choice affects the activity of the obtained extract. Previous study has proved that the water extract of plant has shown better hepatoprotective effect than the ethanolic extract against CCl_4 -induced liver damages, suggesting the difference of active ingredients in different solvent extracts where the marked reduction in the serum level of ALT and AST was revealed with the enhancement of antioxidant enzyme (SOD) and amelioration of hepatic histopathological changes [5].

The results of this study about the hepatoprotective effect of different solvent fractions i.e. AAHF, AACF, AAEF, AABF and AAAF of AAME with two different doses (200 and 400 mg/kg) tested against CCl_4 -induced liver damage in mice (Table 1) showed that the levels of liver serum biochemical parameters (ALT, AST, ALP and TB) significantly ($P < 0.01$) decreased as compared to those of the TC group (Group II), which were increased due to CCl_4 intoxication by intraperitoneal injections as compared to the NC group (Group I). The levels of these serum biochemical parameters were shown to further reduced with the treatment effect of high dose (400 mg/kg) of increasing polarity fractions (AAEF, AABF and AAAF) as compared to high dose (400 mg/kg) treatment of less/non-polar fractions (AAHF and AACF), showing that more polar fractions exhibited high protective effect against CCl_4 -induced toxicity. In current studies, when CCl_4 was induced, it caused injury (necrosis) with the destruction of liver structure and function, resulting in the increased level of lipid peroxidation caused by CCl_4 biotransformation into trichloromethyl (CCl_3) free radical in liver by the cytochrome P450 (CYP) system [68] causing

the membrane lipid disintegration, membrane damage and reduction in the levels of antioxidant enzymes (CAT, SOD and GSH) in the mice (Table 2) and elevated the levels of liver biochemical enzymes (ALT, AST, ALP and TB) in serum by leakage, and high levels of these enzymes indicate that hepatotoxicity has occurred [20,69,70]. CAT eliminates ROS in the liver, whereas SOD converts superoxide anions into H_2O_2 and O_2 while GSH reduces H_2O_2 to H_2O . The reduction in the concentration of antioxidant enzymes (CAT, SOD and GSH) indicates that oxidative stress was induced in mice associated with ROS production due to CCl_4 intoxication [7]. It has been observed in previous investigation that irradiated mice have liver tissue damages and when treatment was performed with *Urtica dioica* L. seed extract, the injured liver tissues were restored with significant increase in antioxidant enzymes (CAT, SOD and GSH) with reduction of oxidative stress and normalization of liver degeneration [13]. The extracts of *A. alpinum* (with 200 mg and 400 mg/kg) in our investigation have reversed the elevated level of serum biochemical parameters by recovering the effect of injuries of liver caused by CCl_4 intoxication. Our results are supported by the findings of previous studies, where *Stachys pilifera* ethanolic extract revealed considerable protection against CCl_4 -induced hepatoprotection in rats [67] and ethanol-induced hepatotoxicity in mice; reported to treated with fermented *Hovenia dulcis* extract that prevented hepatic damage by enhancing the antioxidant defence system [70].

Treatment effect of fractions i.e. AAEF, AABF and AAAF with low and high doses (200 and 400 mg/kg) have detoxified the CCl_4 -induced toxicity and restored the elevated levels of liver biochemical markers in the serum and increased the antioxidant enzyme (CAT, SOD and GSH) activity as compared to the CCl_4 toxicant group (Group II) in a dose-dependent manner showing the marked hepatoprotective effect comparable to the treatment effect of standard drug (silymarin 100 mg/kg) group (Group III) and NC group (Group I), and this hepatoprotective effect of solvent fractions is further supported by histological examination of livers (Figure 1a–m). Loss of cellular architecture, ballooning and degeneration of hepatocytes, inflammatory cell infiltration, dilated sinusoidal and focal necrosis showed that the damage of livers was caused by CCl_4 intoxication (Figure 1b) as compared to NC mice (Figure 1a). Improvement of regeneration was seen by the well-recovered hepatocytes separated by clear sinusoids in groups treated with AAEF, AABF and AAAF fractions with low and high doses (200 and 400 mg/kg) to respective groups of mice after CCl_4 intoxication [10,71], as indicated in Figure 1(h–m).

The highest hepatoprotective activities of *A. alpinum* fractions (AAEF, AABF and AAAF) in a dose-dependent manner after CCl_4 intoxication are due to the nature of phytochemicals partitioned in these solvent fractions with high contents of phenolic and flavonoid compounds that have detoxified the toxic metabolites generated due to CCl_4 intoxication and declined the ROS and lipid peroxidation [71,72] by improvement in levels of CAT, SOD and GSH [20,70], which is attributed to intermediate and more polar compound mixture (flavonoids, tannins, saponins and polyphenols) in these fractions [73]. The liver protective effect of extracts was triggered by increase in the antioxidant defence system and suppression of leakage of serum biochemical liver markers. The antioxidant system associated with SOD, GSH and CAT was improved by administration of extracts, which is the evidence of repair of hepatic tissues which were damaged due to CCl_4 intoxication [66]. In the current investigation, histopathological analysis of livers strongly supports the biochemical assessment for hepatoprotective effect [2].

The study is also supported by earlier investigation performed on *Alchornea cordifolia* against CCl_4 -induced toxicity in mice [74] and different solvent fractions (ethyl acetate, petroleum ether and water) of the methanolic extract of *Bauhinia purpurea* leaves against paracetamol-induced liver toxicity in mice [64], especially our investigation about the hepatoprotective effect of most active fractions (AAAF, AAEF and AABF) of *A. alpinum* root against CCl_4 -induced liver damage in mice which is time and dose dependent [75].

According to results, the underlying mechanism for the protective effect of *A. alpinum* extract in the CCl_4 -induced liver injury model is not clearly understandable but these extracts of *A. alpinum* have rich phenolic compounds that exhibit varieties of biological and pharmacological activities including anti-oxidant, anti-inflammatory and hepatoprotective effects [14,67]. Hepatoprotective effect of any herbal product is associated with antioxidant activity and free radical scavenging effect due to phytochemical constituents such as phenolic and flavonoid compounds [14,76]. Previous study has shown that the ethanolic extract of medicinal plant such as *Stachys pilifera* has strong hepatoprotective activity due to radical scavenging and antioxidant activity that has the capability to diminish the oxidative stress by inhibiting protein oxidation and boosting the antioxidant enzyme activity [77].

In this study, the highest antioxidant activity was exhibited by AAAF followed by AAEF and AABF, as compared to standard antioxidant AA (Table 3). This is due to the nature of phytoconstituents extracted in

aqueous and ethyl acetate solvents and in a concentration-dependent manner [10]. The highest scavenging effect (%) by our fractions i.e. AAEF, AABF and AAAF to DPPH revealed their highest antioxidant activities against ROS and peroxides generated during CCl_4 toxicity with a strong hepatoprotective effect [10]. Studies have proved that the low IC_{50} value or close to standard AA value of the extract has good antioxidant activity with ethyl acetate and aqueous extract compared to other solvent extracts [78]. Hepatoprotective and antioxidant effects of plant extract are due to phytochemical contents, especially the phenolic and flavonoid compounds associated with anti-inflammatory, hepatoprotective and antioxidant activity [12,79].

In this study, the highest hepatoprotective is exhibited by the aqueous fraction of *A. alpinum* due to the antioxidant property (Table 3) of phenolic and flavonoid constituents (Table 4). Similar study reported earlier about the *Phyllanthus niruri* aqueous extract, which showed that the significant hepatoprotective effect against CCl_4 -induced liver damage in mice was associated with antioxidant properties of phenolic compounds [14]. Polyphenols and flavonoids are important secondary metabolites of plants that have therapeutic benefits and potential sources of antioxidants [11]. Phytochemical compounds would act alone or in synergy due to their antioxidant potential [80].

In this study, the total contents of phenolic compounds were determined using the Folin-Ciocalteu reagent and this is the widely used method due to its simplicity [12]. It is evident from the results obtained for the analysis of TPC; there is highest TPC in AAME followed by AAEF, AAAF and AABF (Table 4). The TPC in ethyl acetate fraction is higher than butanol and aqueous fractions [81]. High TPC in AAME is due to the mixture of all phytochemical (polyphenolic) compounds. Our investigation showed the agreeable findings of TPC in the crude methanolic extract and most active fractions (AAEF, AABF and AAAF) of *A. alpinum* with those of the previous study on another plant extract [82]. There is high level of TPC in *A. alpinum* than reported in earlier study performed by Laouar et al. [27] for a different plant (*Juniperus phoenicea* berries) with aqueous and methanolic extracts. The structure of phytochemical compounds and oxidative linkage formation are attributable to the diversity of these compounds due to which polyphenols play role in some important biological activities [64]. In this study, it is evident that AAAF has the highest TFC followed by AAEF, AABF and AAME (Table 4). The concentration of TFC in these fractions is due to separation of flavonoids on the basis of increasing polarity.

As we advance towards the purity of compounds by the fractionation process, the concentration of particular group of compounds is increased. The important phenolic compounds mainly consist of flavonoids and they are more concentrated in polar fractions (AAEF, AABF and AAAF), which is more effective for extracting the phenolic compound from plant materials [81]. Phenolic and flavonoid contents of plants in different parts may vary due to environmental factors such as sun exposure, rainfall and soil type [11]. These active phytochemical ingredients such as phenolic and flavonoid compounds are responsible for the antioxidant activity associated with the different biological activities such as anti-carcinogenic, anti-inflammatory and hepatoprotective properties [27,78]. The fractionation caused the separation of high TFC in polar solvent fractions (AABF and AAAF) and impaired potent hepatoprotective effect, which is attributed to the high antioxidant activity [24,83].

In previous study on *P. orientale* L. (Polygonaceae), it was revealed that treatment with the ethanoic extract of *P. orientale* caused significant reduction in serum levels of ALT, AST and ALP that were increased due to CCl₄-induced liver injury in mice, and this hepatoprotective effect of *P. orientale* was associated with antioxidant and anti-proinflammatory activities [34]. Studies have demonstrated that the methanolic extract of *Polygonum equisetiforme* (family: Polygonaceae) showed substantial reduction in ALT and AST levels in the serum and increased the levels of antioxidant enzymes (CAT, SOD and GSH) in Sprague–Dawley rats and this liver protective effect was due to synergistic action of flavonoids [66]. Similarly, another study has shown that members of genus *Polygonum* have strong antioxidant activity and they are used for the treatment of many ailments, particularly, for the liver damages. *Polygonum amplexicaule* (genus: *Polygonum*, family: Polygonaceae) rhizome methanolic extract showed strong hepatoprotective effect against CCl₄-induced liver damage in albino mice [50]. The earlier study has shown that the methanolic extract of *Polygonum minus* Hud (family: polygonaceae) leaves has a prominent hepatoprotective effect against CCl₄- and paracetamol-induced toxicity in rats [33].

The main mechanism by which polar fractions of *A. alpinum* root extracts in our investigation had significant hepatoprotective effect against CCl₄-induced toxicity is associated with antioxidant properties of phytochemicals such as phenolic compounds including the flavonoids (Table 4) that have elevated the antioxidant defence system and reduced the levels of liver biochemical parameters (ALT, AST, ALP and TB) by recovering the damaged liver in mice [18].

Quality of polyphenolic compounds is further studied by chromatographic techniques. In our result, HPLC chromatograms (Figures 3–6) showed the presence of phenolic and flavonoid compounds: highly present in *n*-butanol fraction (14) followed by aqueous fraction (12), ethyl acetate fraction (10) and methanolic extract (7). There were no data recorded about compounds identified from the roots of *A. alpinum*. In another study, some of the phytochemical constituents (chlorogenic acid, quercetin, myricetin, vitamin C and kaempferol) identified by HPLC from the aqueous methanolic extract of *Rumex dentatus* (polygonaceae family) have known hepatoprotective effects [57]. GA, catechin, rutin vanillic acid, quercetin and 3,4-dimethoxy benzoic acid were also identified by the HPLC method in another investigation on *Asparagus alba* leaves separated by the hot aqueous extract with strong antioxidant, free radical scavenging and hepatoprotective effects against CCl₄-induced liver injury [68]. Phytoconstituents (protocatechuic, caffeic acid and rutin) have potent hepatoprotective effects due to antioxidant and free radical scavenging potential [71,84,85]. This study for identification of compounds from fractions correlated with the above findings. The hepatoprotective activities of chlorogenic acid, quercetin, kaempferol [57], caffeic acid [71,86], protocatechuic acid [71] and rutin [71,79] are already known, which strongly support the arguments about our findings of hepatoprotective effect of most active fractions (AAEF, AABF and AAAF) of the *A. alpinum* root methanolic extract against CCl₄-induced liver damage in mice due to strong antioxidant potential. The pharmacological and biological activities of the *A. alpinum* root extract discussed in current research are mainly associated with polyphenol compounds that have been detected by HPLC. The data obtained about studies of pharmacological activities of different plants of family Polygonaceae also provide strong evidence and are supportive for our investigation on *A. alpinum* root extracts that have revealed the hepatoprotective effect and promising antioxidant potential associated with phenolic compounds.

5 Conclusion

Based on our results of investigation, it is concluded that polar solvent fractions AAEF, AABF and especially AAAF of AAME have exhibited significant hepatoprotective effect against CCl₄-induced toxicity in a dose-dependent manner due to strong antioxidant

properties. The hepatoprotective effect of polar fractions of *A. alpinum* is attributed to the combination of different phytoconstituents of bioactive phenolic and flavonoid compounds with antioxidant potential and these compounds are synergistic in action for bioactivity. The findings of this study suggest that *A. alpinum* roots could be recommended as a potential source of hepatoprotective agent against liver damages induced due to chemical toxicity. Moreover, the experimental analysis justifies the traditional claims and use of *A. alpinum* as folklore medicine for the treatment of liver disorders.

Abbreviations

AAAF	<i>Aconogonon alpinum</i> aqueous fraction
AABF	<i>Aconogonon alpinum</i> <i>n</i> -butanol fraction
AACF	<i>Aconogonon alpinum</i> chloroform fraction
AAEF	<i>Aconogonon alpinum</i> ethyl acetate fraction
AAHF	<i>Aconogonon alpinum</i> <i>n</i> -hexane fraction
AAME	<i>Aconogonon alpinum</i> root methanolic extract
AJ&K	Azad Jammu and Kashmir
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
Bd	Bleeding
CAT	Catalase
CV	Central vein
DNHP	2,4-Dinitrophenylhydrazine
DPPH	2, 2-Diphenyl-1-picryl-hydrazyl
FD	Fatty degeneration
FN	Focal necrosis
GA	Gallic acid
GSH	Glutathione
H	Hepatocytes
HPLC	High-performance liquid chromatography
I	Inflammatory cell infiltration
K	Kupffer cells
N	Nucleus
NC	Normal control group
ROS	Reactive oxygen species
SD	Sinusoidal dilation
SOD	Superoxide dismutase
SS	Sinusoids
TB	Total bilirubin
TB	Total bilirubin
TC	toxicant control
TFCs	total flavonoid contents
TPCs	total phenolic contents

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