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

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

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 (CCl₄), 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 CCl₄ [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, CCl₄ is widely used a hepatotoxic agent [26]. The CCl₄-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 phoenicea) extract revealed its potent hepatoprotective effect against CCl₄-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 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 ailments [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 chrys), and high-quality chemicals such as CCl4, NaCl, KCl, K2S2O8, NaHPO4, H2O2, sodium nitrite, C2HClO2, 2,4-dinitrophenyl-hydrazine (DNPH), diazo reagent, sodium carbonate, aluminium trichloride, sylmarin, 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 CL3i; 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 ± 5°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 × 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 AAAF; orally administered with high dose (400 mg/kg) once daily, for 4 weeks. Group VIII: CCl₄ as in Group II + treatment with AAHF; 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 low dose (200 mg/kg), once daily, for 4 weeks. Group X: CCl₄ as in Group II + treatment with AAHF; orally administered with high dose (400 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 alpha-oxoglutarate (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 Suzuki 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 × 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 µL of 50 mM potassium phosphate buffer (pH = 5.0), 100 µL of 5.9 mM H₂O₂ and 35 µL 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 µL of supernatant was added to aliquot containing 600 µL of 0.052 mM sodium pyrophosphate buffer (pH 7.0) and 50 µL of 186 mM of phenazine methophsphate. To initiate the enzymatic reaction, 100 µL of 780 µM NADH was added. After 1 min, glacial acetic acid (500 µL) 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 buffer (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 µg/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 µm) of liver were cut using a microtome (SM2400; Leica, Germany). Preparations were lifted and transferred on glass material we 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 (UTUIX-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 µg/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:

Scavenging effect (% $\Delta A$) = $\left[ \frac{(A_c - A_s)}{A_c} \right] \times 100$

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 using methanol as a solvent. Standard solutions were tested at different concentrations of 500, 250, 125, 50, 25, 10, 5 and 2.5 µg/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 (µg/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 µg/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 µL of extract solution was added in 100 µL 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 µL of extract solution along with 100 µL 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 (µg/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-elusion 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 µm) 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 × 3 mm, 3.5 µm column and 1525 binary pump) to analyse the phenolic and flavonoid compounds in samples. The 717 plus autosampler (injection volume of 10 µL) 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 ± 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 ± standard deviation (SD) and linear regression was constructed by the graphic method using excel 2016 to calculate the IC$_{50}$ 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 (LD$_{50}$) 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
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 If 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 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% CCl₄) | 207.67 ± 4.51\(^a\) | 212.07 ± 3.76\(^a\) | 417.67 ± 5.60\(^a\) | 2.38 ± 0.19\(^a\) |
| III   | CCl₄ + S.D (silymarin 100 mg) | 62.05 ± 5.16\(^b\) | 77.03 ± 3.56\(^b\) | 191.17 ± 4.99\(^b\) | 0.77 ± 0.05\(^b\) |
| IV    | CCl₄ + 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     | CCl₄ + AAF (400 mg/kg) | 144.15 ± 5.58\(^b\) | 160.01 ± 5.32\(^b\) | 345.11 ± 5.22\(^b\) | 1.74 ± 0.13\(^b\) |
| VI    | CCl₄ + AACF (200 mg/kg) | 160.06 ± 5.85\(^b\) | 175.03 ± 5.73\(^b\) | 351.16 ± 6.28\(^b\) | 1.80 ± 0.03\(^b\) |
| VII   | CCl₄ + AAEF (400 mg/kg) | 148.17 ± 4.61\(^b\) | 167.33 ± 5.30\(^b\) | 340.18 ± 4.80\(^b\) | 1.68 ± 0.02\(^b\) |
| VIII  | CCl₄ + AABF (200 mg/kg) | 106.04 ± 4.28\(^cd\) | 120.03 ± 3.75\(^cd\) | 250.11 ± 4.27\(^cd\) | 1.14 ± 0.08\(^de\) |
| IX    | CCl₄ + AAAF (400 mg/kg) | 73.03 ± 5.19\(^def\) | 90.04 ± 5.05\(^ef\) | 194.03 ± 3.46\(^e\) | 0.78 ± 0.04\(^de\) |
| X     | CCl₄ + AAF (200 mg/kg) | 114.02 ± 5.20\(^c\) | 128.17 ± 2.90\(^c\) | 260.05 ± 4.47\(^c\) | 1.22 ± 0.06\(^cd\) |
| XI    | CCl₄ + AAF (400 mg/kg) | 80.67 ± 4.05\(^def\) | 103.06 ± 4.47\(^e\) | 226.33 ± 5.15\(^d\) | 0.92 ± 0.05\(^def\) |
| XII   | CCl₄ + AAF (200 mg/kg) | 92.93 ± 2.12\(^de\) | 107.05 ± 3.46\(^de\) | 248.10 ± 4.56\(^cd\) | 1.09 ± 0.03\(^de\) |
| XIII  | CCl₄ + AAF (400 mg/kg) | 53.05 ± 4.50\(^f\) | 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). \(^b\)P < 0.01 versus toxicant control (TC and Group II). The values with same letters do not have significant difference from each other.
## 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 ± 0.45b      | 33.54 ± 0.53a      | 30.565 ± 0.76b     |
| TC (25% CCl₄ only)                     | 33.39 ± 0.40f       | 12.78 ± 0.40f      | 11.527 ± 0.44f     |
| CCl₄ + silymarin (100 mg/kg)           | 65.5 ± 0.55a        | 35.293 ± 0.48a     | 26.763 ± 0.43c     |
| CCl₄ + AAHF (200 mg/kg)                | 39.492 ± 0.50f      | 20.028 ± 0.36f     | 16.218 ± 0.54f     |
| CCl₄ + AAHF (400 mg/kg)                | 42.53 ± 0.59a       | 24.252 ± 0.47a     | 21.358 ± 0.64a     |
| CCl₄ + AACF (200 mg/kg)                | 38.005 ± 0.33f      | 16.318 ± 0.46f     | 17.513 ± 0.48f     |
| CCl₄ + AACF (400 mg/kg)                | 44.175 ± 0.60f      | 27.055 ± 0.38f     | 22.028 ± 0.53f     |
| CCl₄ + AAEF (200 mg/kg)                | 63.73 ± 0.51ab      | 35.638 ± 0.48f     | 36.485 ± 0.54f     |
| CCl₄ + AAEF (400 mg/kg)                | 43.507 ± 0.57e      | 24.75 ± 0.55f      | 19.703 ± 0.59f     |
| CCl₄ + AABF (200 mg/kg)                | 57.338 ± 0.52e      | 30.988 ± 0.46f     | 30.462 ± 0.54f     |
| CCl₄ + AABF (400 mg/kg)                | 46.965 ± 0.30d      | 28.882 ± 0.65ef    | 26.252 ± 0.30f     |
| CCl₄ + AAAF (200 mg/kg)                | 61.815 ± 0.47f      | 35.885 ± 0.52a     | 34.695 ± 0.39f     |

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. $^gP < 0.01$ versus normal control (NC). $^a–fP < 0.01$ 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 X: CCl₄ + AABF 200 mg/kg (j) and Group XI: 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 IC_{50} 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 | 67.0 ± 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 |
| AAIF    | 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.

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), ACF (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, ACF, AAEF, AABF and AAAF) and standard AA, the IC_{50} values are 44.16, 73.75, 55.47, 30.20, 42.19, 25.51 and 31.90 µg/mL, respectively. The order of decreasing IC_{50} values of samples is AAHF > ACF > AAME > AABF > AA > AAEF > AAAF (with IC_{50} values of 73.75, 55.47, 44.14, 42.19, 31.90, 30.20 and 25.51 µg/mL, respectively). The lowest is the IC_{50} value of the sample, the highest is its antioxidant activity. AAAF showed the lowest IC_{50} value (25.51 µg/mL), with the highest antioxidant activity. Similarly, the promising high value of antioxidant activity is of AA (30.06 µg/mL) compared to the IC_{50} value of standard antioxidant AA with an IC_{50} 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 (AAE, AABF and AAAF) are presented in Table 4. TPCs of AAME and its more active fractions
(AAEF, AABF and AAAF) are 192.892 ± 0.814, 185.164 ± 0.836, 109.433 ± 1.771 and 165.304 ± 0.708 µg/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 µg/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-elusion 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).
nowadays for pharmaceutics. 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
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]. CCl₄ 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. CCl₄-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 against CCl₄-induced liver damage [66].

The results of our study confirmed the hepatoprotective effect of different solvent fractions of Aconogonon alpinum methanolic extract (AAME) against CCl₄-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 CCl₄ 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 CCl₄-induced hepatic injury [67]. The hepatoprotective effect of A. alpinum extracts on CCl₄-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 CCl₄-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. AAF, AAF, AABF and AAAF of AAME with two different doses (200 and 400 mg/kg) tested against CCl₄-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 CCl₄ 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 (AAAF, 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 CCl₄-induced toxicity. In current studies, when CCl₄ was induced, it caused injury (necrosis) with the destruction of liver structure and function, resulting in the increased level of lipid peroxidation caused by CCl₄ biotransformation into trichloromethyl (CCl₃) 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 H₂O₂ and O₂ while GSH reduces H₂O₂ to H₂O. 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 CCl₄ 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 CCl₄ intoxication. Our results are supported by the findings of previous studies, where Stachys pilifera ethanolic extract revealed considerable protection against CCl₄-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. AAF, AABF and AAAF with low and high doses (200 and 400 mg/kg) have detoxified the CCl₄-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 CCl₄ 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 CCl₄ 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 AAF, AABF and AAAF fractions with low and high doses (200 and 400 mg/kg) to respective groups of mice after CCl₄ 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 CCl₄ 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 CCl₄ 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 CCl₄ 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 CCl₄-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 CCl₄-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 CCl₄-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 CCl₄ toxicity with a strong hepatoprotective effect [10]. Studies have proved that the low IC₅₀ 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 CCl₄-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 hepato-protective 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 ethanolic extract of *P. orientale* caused significant reduction in serum levels of ALT, AST and ALP that were increased due to CCl$_4$-induced liver injury in mice, and this hepatoprotective effect of *P. orientale* was associated with antioxidant and anti-inflammatory 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 CCl$_4$-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 CCl$_4$ 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 CCl$_4$-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 CCl$_4$-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 CCl$_4$-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 CCl$_4$-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**

AAMF  Aconogonon alpinum aqueous fraction  
AABF  Aconogonon alpinum n-butanol fraction  
AACF  Aconogonon alpinum chloroform fraction  
AAEF  Aconogonon alpinum ethyl acetate fraction  
AAHF  Aconogonon alpinum n-hexane fraction  
AAEM  Aconogonon alpinum 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  
TC  Toxicant control  
TFCs  total flavonoid contents  
TPCs  total phenolic contents

**Acknowledgements:** This research work is a part of the PhD thesis of Mr. Muhammad Zakryya Khan. The authors are highly thankful to National Institute of Health (NIH), Islamabad, Pakistan, for providing facilities to conduct this research. Parts of research facilities provided by International Islamic University Islamabad (IIUI), Quaid-i-Azam University (QAU) Islamabad and Pir Mehr Ali Shah Arid Agriculture University Rawalpindi (PMAS-AAUR) during this research are also acknowledged.

**Ethical approval:** Institutional Bioethics and Biosafety Committee (IBBC) approved the study through assigned number no. IIUI (BIRBT)/FBAS-IBBC-2015-04 (approved on June 30, 2015).

**Conflict of interest:** The authors state no conflict of interest.

**Funding:** The research project was self-funded.

**Author contributions:** Study design was contributed by Muhammad Zakryya Khan, Muhammad Imran Shabbir, Muhammad Arshad Malik and Syed Aneel Gilani. Data analysis/interpretations were performed by Muhammad Zakryya Khan, Zafeer Saqib and Muhammad Arshad Malik. Write up of manuscript was contributed by Muhammad Zakryya Khan and Muhammad Arshad Malik. Critical revision/review was performed by Naqeebullah Jogeza, Mubin Mustafa Kiyani and Muhammad Arshad Malik. Supervision was done by Muhammad Arshad Malik. The final draft was approved by all the authors.

**References**

[1] Wang Z, Li Z, Ye Y, Xie L, Li X. Oxidative stress and liver cancer: etiology and therapeutic targets. review article. Hindawi Publishing Corporation. Oxid Med Cell Longev. 2016;1–10. doi: 10.1155/2016/7891574.

[2] Nazir T, Shakir L, Rahman Z, Najam K, Choudhary A, Saeed N, et al. Hepatoprotective activity of Foeniculum vulgare against paracetamol induced hepatotoxicity in rabbits. J Appl Pharm. 2020;12:270. doi: 10.35248/2376-0354.20.12.270.

[3] Tanweer S, Mehmood T, Zainab S, Ahmad Z, Shehzad A. Comparison and HPLC quantification of antioxidant profiling of ginger rhizome, leaves and flower extracts. Clin Phytosci. 2020;6:12. doi: 10.1186/s40816-020-00158-z.

[4] Hsouna AB, Dhibi S, Dhifi W, Mnif W, Nasr HB, Hfaiedh N. Chemical composition and hepatoprotective effect of essential oil from Myrtus communis L. flowers against CCL4-induced acute hepatotoxicity in rats. RSC Adv. 2019;9:3777–87.

[5] Ren X, Xin LT, Zhang M-Q, Zhao Q, Yue S-Y, Chen K-X, et al. Hepatoprotective effects of a traditional Chinese medicine...
formula against carbon tetrachloride-induced hepatotoxicity in vivo and in vitro. Biomed Pharmacother. 2019;117:1–8. doi: 10.1016/j.biopharm.2019.109190.

[6] Younis T, Khan MR and Sajid M. Protective effects of *Fraxinus xanthoxyloides* (Wall.) leaves against CCl4 induced hepatic toxicity in rat. BMC Complement Altern Med. 2016;16(1):1–13. doi: 10.1186/s12906-016-1398-0.

[7] Dutta S, Chakraborty AK, Dey P, Kar P, Guha P, Sen S, et al. Amelioration of CCl4 induced liver injury in Swiss albino mice by antioxidant rich leaf extract of *Croton bonplandianus* Baill. PLoS One. 2018;13(4):e0196411.

[8] Abdullah KMZ, Ahmad W, Ahmad M, Nisar M. Hepatoprotective effect of the solvent extracts of *Viola canecens* Wall. ex. Roxb. Against CCl4 induced toxicity through antioxidant and membrane stabilizing activity. BMC Complement Altern Med. 2017;17:10. doi: 10.1186/s12906-016-1537-7.

[9] Iweala EJ, Evbakhavbokun WO, Maduagwu EN. Antioxidant and hepatoprotective effect of *Cajanus cajan* in N-nitoso-diethylamine-induced liver damage. Sci Pharm. 2019;87(3):24. doi: 10.3390/scipharma87030024.

[10] Ullah R, Mansour S, Alsaid, Abdelaaty A, Shahat, Naser AA, et al. Antioxidant and hepatoprotective effects of methanolic extracts of *Zizia spinosa* and *Hammadada elegans* against carbon tetrachloride induced hepatotoxicity in rats. Open Chem. 2018;16:133–40.

[11] Gunathilaka TL, Samarakaon KW, Ranasinghe P, Peiris LDC. In vitro antioxidant, hypoglycemic activity, and identification of bioactive compounds in phenol fraction of *Garcinia eludis* (Gmelin) silica. Molecules. 2019;24(20):1–17. doi: 10.3390/molecules24203708.

[12] Bourhia M, Laasri FE, Aghmih K, Ullah R, Alqahtani AS, Mahmood HM, et al. Phytochemical composition, antioxidant activity, antiproliferative effect and acute toxicity study of *Bryonia dioica* roots used in North African alternative medicine. Intl J Agric Biol. 2020;23(3):597–602.

[13] Yildizhan K, Demirtaş OC, Uyar A, Huyüt Z, Çakır T, Keleş OF, et al. Protective effects of *Urtica dioica* L. seed extract on liver tissue injury and antioxidant capacity in irradiated rats. Braz J Pharm Sci. 2020;56(1):1–9. doi: 10.1590/s1753-979020201900013835A.

[14] Erzat MI, Okba MM, Ahmed SH, El-Banna HA, Prince A, Mohamed SO, et al. In-depth hepatoprotective mechanistic study of *Phyllanthus niruri*: in vitro and in vivo studies and its chemical characterization. PLoS One. 2020;15(1):0226185. doi: 10.1371/journal.pone.0226185.

[15] Kalsoom R, Haider MS, Chohan C. Phytochemical analysis and antifungal activity of some medicinal plants against *Alternaria* specie isolated from onion. J Anim Plant Sci. 2020;30(2):1–7.

[16] Mahmood N, Nazir R, Khan M, Iqbal R, Adnan M, Ullah M, et al. Phytochemical screening, antibacterial activity and heavy metal analysis of ethnomedicinal recipes and their sources used against infectious diseases. Plants (Basel). 2019;8(11):1–14. doi: 10.3390/plants8110454.

[17] Prinsi B, Morgutti S, Negrini N, Faoro F, Espen L. Insight into composition of bioactive phenolic compounds in leaves and flowers of green and purple basil. Plants. 2020;9(1):22. doi: 10.3390/plants9010022.

[18] Urrutia-Hernández TA, Santos-López JA, Benedi J, Sánchez-Muniz FJ, Velázquez-González VO, Arciniega MDL, et al. Antioxidant and hepatoprotective effects of *Croton hypoleucus* extract in an induced-necrosis model in rats. Molecules. 2019;24(4):2533. doi: 10.3390/molecules24122533.

[19] Adewusi EA, Moodley N, Steenkamp V. Medicinal plants with cholinesterase inhibitory activity. A review. Afr J Biotechnol. 2010;9(49):8257–76.

[20] Jayasuriya R, Ananth DA, Sivasudha T. Hepatoprotective and antioxidant activity of *Ipomoea staphylinia* Linn. Clin Phytosc. 2019;5:18. doi: 10.1371/journal.Plos16-019-0112-4.

[21] Wojdylo A, Nowicka P, Grimalt M, Legua P, Almansa MS, Amorós, A, et al. Polyphenol compounds and biological activity of caper (*Capparis spinosa* L.) flowers buds. Plants. 2019;8(12):539. doi: 10.3390/plants8120539.

[22] Ul-Haq I, Ullah N, Bibi G, Kanwal S, Ahmad M, Mirza B. Antioxidant and cytotoxic activities and phytochemical analysis of *Euphorbia walllichen* root extract and its fractions. Iran J Pharm Res. 2012;11(1):241–9.

[23] Mosquera OM, Correa YM, Buitrago DC, Niño J. Antioxidant activity of twenty-five plants from Colombian biodiversity. Mem do Inst Oswaldo Cruz. 2007;102(5):631–4.

[24] Preethi J, Jennila N, Velishty S. Hepatoprotective and antioxidant role of *Ziziphus jujuba* leaves on paracetamol induced hepatic damage in rats. J Dis Medicinal Plants. 2016;2(1–1):1–10. doi: 10.11648/j.jedmp.s.2016020101.

[25] Laszczyk M, Jager S, Scheffler A, Scheppeck CM. Physical, chemical and pharmacological characterization of a new oleogel-forming triterpene extract from the outer bark of birch (betulae cortex). Planta Med. 2006;72(15):1389–95.

[26] Ritesh KR, Suganya A, Dileepkumar HV, Rajasekhar Y, Shivananadappa T. A single acute hepatotoxic dose of CCl4 causes oxidative stress in the rat brain. Toxicol Rep. 2015;2:891–5. doi: 10.1016/j.toxrep.2015.05.012.eCollection 2015.

[27] Laouar A, Klibet F, Bourouga E, Benamara A, Boumendjel A, Chefrour A, et al. Potential antioxidant properties and hepatoprotective activities of *juniperus foinacea* berries against CCl4 induced hepatic damage in rats. Asian Pac J Tropical Med. 2017;10(3):263–9.

[28] Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurung R, Koirala N. Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. Plants. 2019;8(4):96. doi: 10.3390/plants8040096.

[29] Hashem MM, Salama MM, Mohammed FF, Tohamy AF, El-Deeb KS. Metabolic profile and hepatoprotective effect of *Aeschynomene elaphroxylon* leaves buds. Plants. 2019;14(1):e0210576.

[30] Shinwari ZK. Medicinal plants research in Pakistan. J Med Plant Res. 2010;4(3):161–76.

[31] Ahmed KS, Hamid A, Nawaz F, Hameed M, Ahmad F, Deng J, et al. Ethnomedicinal studies of indigenous plants in Kel village, Neelum Valley, Azad Kashmir, Pakistan. J Ethnobiol Ethnomed. 2017;13(1):68. doi: 10.1186/s13002-017-0196-1.

[32] Hakim FA, Haidy HA, Gadh, Radwan RA, Ayoub N, El-Shazly M. Biological and phytochemical review on the genus *Coccoidea* (Polygonaceae). Arch Pharm Sci ASU. 2019;3(2):180–19.

[33] Christopher PV, Joe LS, Tian M, MohantyBS, Parasuraman P, Al-Suede FSR, et al. Evaluation of methanol extract of
Polyergus minus Huds. Leaves for its hepatoprotective activity. Malay J Microbiol 2016;12(5):345–52.

[34] Chiu Y-J, Chou SC, Chiu CS, Kao CP, Wu KC, Chen CJ, et al. Hepatoprotective effect of the ethanol extract of Polygonum orientale on carbon tetrachloride-induced acute liver injury in mice. J Food Drug Anal. 2018;26(1):369–79.

[35] Yasin G, Khan MA, Shaheen N, Khan UJ. Palynological studies of Aconogonon (Polygonaceae) from Pakistan. Sarhad J Agriculture. 2015;31(1):16–21.

[36] Ch IM, Ahmad F, Maqbool M, Husain T. Ethno botanical inventory of flora of Maradori Valley District Forward Kahuta Azad Kashmir, Pakistan. Am J Res Communi. 2013;6(6):239–61.

[37] Qureshi AR, Ghufran AM, Gilani AS, Sultana K, Ashraf M. Ethno-botanical studies of selected medicinal plants of sudhan gali and Ganga Chotti hills Bagh, Azad Kashmir (Pakistan). Pak J Bot. 2007;39(7):2275–83.

[38] Mahmood A, Malik NR, Shinwari KZ, Mahmood A. Ethnobotanical survey of plants from Neelum, Azad Jammu & Kashmir, Pakistan. Pak J Bot. 2011;43:10–10.

[39] Vysochina GI, Khramova EP. Component composition of flavonols and their content in Aconogonon alpinum (All.) Schur Growing in the Altay. Chem Sustain Dev. 2010;18(18):477–83.

[40] Shah SMM, Sadiq A, Shah SMH, Khan S. Extraction of saponins and toxicological profile of Teucricum stocksianum boiss extracts collected from District Swat, Pakistan. Biol Res. 2014;47(65). doi: 10.1186/0717-6287-47-65.

[41] Chua LS, Lau CH, Chew CY, Dawood DAS. Solvent fractionation. Jeyadevi R, Sivasudha T, Rameshkumar A, Harnly JM, Lin LZ. Vysochina GI, Khramova EP. Component composition of flavonols and their content in Aconogonon alpinum (All.) Schur Growing in the Altay. Chem Sustain Dev. 2010;18(18):477–83.

[42] Chua LS, Lau CH, Chew CY, Dawood DAS. Solvent fractionation and acetone precipitation for crude saponins from Cardiospermum halicacabum fl. J Agriculture. 2015;3165. doi: 10.1186/0717-6287-47-65.

[43] Mahmood A, Malik NR, Shinwari KZ, Mahmood A. Ethnobotanical survey of plants from Neelum, Azad Jammu & Kashmir, Pakistan. Pak J Bot. 2011;43:10–10.

[44] Vysochina GI, Khramova EP. Component composition of flavonols and their content in Aconogonon alpinum (All.) Schur Growing in the Altay. Chem Sustain Dev. 2010;18(18):477–83.

[45] Shah SMM, Sadiq A, Shah SMH, Khan S. Extraction of saponins and toxicological profile of Teucricum stocksianum boiss extracts collected from District Swat, Pakistan. Biol Res. 2014;47(65). doi: 10.1186/0717-6287-47-65.

[46] Chua LS, Lau CH, Chew CY, Dawood DAS. Solvent fractionation and acetone precipitation for crude saponins from Cardiospermum halicacabum fl. J Agriculture. 2015;3165. doi: 10.1186/0717-6287-47-65.

[47] Mahmood A, Malik NR, Shinwari KZ, Mahmood A. Ethnobotanical survey of plants from Neelum, Azad Jammu & Kashmir, Pakistan. Pak J Bot. 2011;43:10–10.

[48] Vysochina GI, Khramova EP. Component composition of flavonols and their content in Aconogonon alpinum (All.) Schur Growing in the Altay. Chem Sustain Dev. 2010;18(18):477–83.

[49] Hussein L, Hamid AMS, Tahir M, Rehman K, Ahmed KZ. Hepatoprotective effects of methanolic extract of Alcea Rosea against acetaminophen-induced hepatotoxicity in mice. Bangladesh J Pharm. 2014;9:322–7. doi: 10.3329/ bjp.v9i3.19068.

[50] Maqsood F, Ibrahim T, Farooqi AA, Ahmed MS. Polygonum amplexicaule extract: an effective herbal cure to CCl4 induced liver damage in vivo. J Rare Disord Diagn Ther. 2017;3:4. doi: 10.21767/2380-7245.100057.

[51] Oriakh K, Patrick O, Ikechi U, Eze G. Hepatoprotective potentials of methanol extract of T. conophorum seeds of carbon tetrachloride induced liver damage in Wistar rats. Clin Phytosci. 2018;4:25. doi: 10.1186/s40816-018-0088-5.

[52] Parmar SR, Vashrambhai PH, Kalia K. Hepatoprotective activity of some plants extract against paracetamol induced hepatotoxicity in rats. J Herb Med Toxicol. 2010;4(2):101–6.

[53] Suzuki Y and Sakagishi Y. Determination of serum bilirubin by the Diazo method using the diazotized 3-nitroaniline reacting readily with the photoproducts of bilirubin. Jpn J Clin Chem. 1994;23:158–63.

[54] Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972;47:389–94.

[55] Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem. 1974;47(3):469–74.

[56] Moron MS, Depierre JN, Mannervik V. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochem Biophys Acta. 1979;582(1):67–78.

[57] Saleem M, Ahmed B, Karim M, Ahmed S, Ahmad M, Qadir MI, et al. Hepatoprotective effect of aqueous methanolic extract of Rumex dentatus in paracetamol-induced hepatotoxicity in mice. Bangladesh J Pharmacol. 2014;9:284–9.

[58] Kulisic T, Radonic A, Katalinic V, Milos M. Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 2004;85:633–40.

[59] Kalisz S, Oszmańska J, Kolniak-Ostek J, Grobelna A, Kieliszek M, Cendrowski A. Effect of a variety of polyphenols compounds and antioxidant properties of rhubarb (Rheum rhabarbarum). LWT-Food Sci Technol. 2020;118:108775.

[60] Gursoy N, Sarikurkcu C, Cengiz M, Solak MH. Antioxidant activities, metal contents, total phenolics and flavonoids of seven Morchella species. Food Chem Toxicol. 2009;47(9):2381–88.

[61] Ijaz S, Khan HMS, Anwar Z, Talbot B, Walsh JJ. HPLC profiling of Mimusops pudica polyphenols and their non-invasive biophysical investigations for anti-dermatoheiotic and skin reinstating potential. Biomed Pharmacother. 2019;109:865–75.

[62] Mekky RH, Fayed MR, El-Gindi MR, Abdel-Monem AR, Contreras MDM, Segura-Carretero A, et al. Hepatoprotective effect and chemical assessment of a selected Egyptian chickpea cultivar. Front Pharmacol. 2016;7:344. doi: 10.3389/ fphar.2016.00344.

[63] Jalil MA, Rahman SM, Rahman A, Ridwan B, Rashid, Rashid MA. Active fractions from Asparagus race-mosus Wild. With thrombolytic, membrane stabilizing and
free radical scavenging activities. Bangladesh Pharm J. 2015;18(2):183–6. doi: 10.3329/bpj.v18i2.4320.

[64] Zakaria ZA, Yahya F, Mamat SS, Mahmood D, Mohtarrudin N, Taher M, et al. Hepatoprotective action of various partitions of methanol extract of Bauhinia purpurea leaves against paracetamol-induced liver toxicity: involvement of the antioxidant mechanisms. BMC Complement Altern Med. 2016;16:175. doi: 10.1186/s12906-016-1110-4.

[65] Tsai JC, Chu IC, Chen YC, Lee MS, Hao FY, Hsieh MT, et al. Hepatoprotective effect of Coreopsis tinctoria flowers against carbon tetrachloride-induced liver damage in mice. BMC Complement Altern Med. 2017;17:139. doi:10.1186/s12906-017-1604-8.

[66] El-Toumy SAH, Salib JY, Shafik NH, Elkarim ASA, Salama A, Omara EAA, et al. Evaluation of hepatoprotective activity of *Polygonum aviculiforme* methanolic extract. J Appl Pharm Sci. 2019;9(11):54–9.

[67] Kokhdan EP, Ahmad K, Sagdehi H, Sagdehi HA, Dadgasy F, Daneshi N, et al. Hepatoprotective effect of *Stachys pilifera* ethanol extract in carbon tetrachloride-induce hepatotoxicity in rats. Pharma Biol. 2017;55(1):1389–93.

[68] Serairi-Jeiri R, Wannes WA, Hamdi A, Ksouri TR, Saidani-Tounsi M, Bourouai KMLN. Antioxidant and hepatoprotective effects of *Asparagus albus* leaves in carbon tetrachloride-induced liver injury rats. J Food Biochem. 2017;42(1):e12433. doi: 10.1111/jfbc.12433.

[69] Yu C, Wang CL, Jin X, Wu WK, Chan, Mckeehan ML. Increased carbon tetrachloride-induced liver injury and fibrosis in FGFR4-deficient mice. Am J Pathol. 2002;161(6):2003–10. doi: 10.1016/S0002-9440(10)64478-1.

[70] Park NY, Lee SJ, Mechesso AF, Boby N, Yixian Q, Yoon WK, et al. Hepatoprotective effects of gamma-aminobutyric acid-enriched fermented Hovenia dulcis extract on ethanol-induced liver injury in mice. BMC Complement Med Ther. 2020;20(1):75. doi:10.1186/s12906-020-2864-0.

[71] Abdelhafiez OH, Fawzy MA, Fahim JR, Desoukey SY, Krischke M, Mueller MI, et al. Hepatoprotective potential of *Malavaviscus arboreus* against carbon tetrachloride-induced liver injury in rats. PLoS One. 2018;13(8):1–18. doi:10.1371/journal. Pone.0202362.

[72] Yang J, Zhu D, Ju B, Jiang X, Hu J. Hepatoprotective effects of Gentianella turkestanae extracts on acute liver injury induced by carbon tetrachloride in mice. Am J Transl Res. 2017;9(2):569–79.

[73] Almey AA, Khan CAJ, Zahir S, Suleiman KM, Aisyah MR, Rahim KK. Total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of aromatic plants’ leaves. Int Food Res J. 2010;17:1077–84.

[74] Osadebe PO, Okoye FB, Uzor PF, Nnamani NR, Adiele IE, Obiano NC. Phytochemical analysis, hepatoprotective and antioxidant activity of *Alchornea cordifolia* methanol leaf extract on carbon tetrachloride-induced hepatic damage in rats. Asian Pac J Trop Med. 2012;5(4):209–93.

[75] Talluri MR, Gummadi VP, Battu GR. Chemical composition and hepatoprotective activity of *Saponaria officinalis* on paracetamol-induced liver toxicity in rats. Pharmacogn J. 2018;10(6):1196–201.

[76] Hassan RSM, Hossain MMD, Raushanara A, Mariam J, Mazumder EHM, Rahman S. DPPH free radical scavenging activity of some Bangladeshi medicinal plants. J Med Plants Res. 2009;3(11):875–9.

[77] Mansourian M, Mirzaei A, Azamneh V, Vakilpour H, Kokhdan EP, Doustimoitalghi AH. Hepatoprotective and antioxidant activity of hydroalcoholic extract of *Stachys pilifera*. *Benth* on acetaminophen-induced liver toxicity in male rats. Heliyon. 2020;6:03029.

[78] Devika M, Joshi H, Nalini MS. Phytochemicals, antioxidative and *in vivo* hepatoprotective potentials of *Litsea floribunda* (BL.) Gamble (Laureaceae) – an endemic tree species of the Southern Western Ghats India. Jordan J Biol Sci. 2016;9(3):163–71.

[79] Belguit-hadriche O, Ammar S, Contreras DM, Turki M, Carretero AS, Feki EA, et al. Antihyperlipidemic and antioxidant activities of Edible Tunisian *Ficus carica* L. fruits in high fat diet-induced hyperlipidemic rats. Plant Foods Hum Nutr. 2016;71(2):183–9.

[80] Atchou K, Lawson-Evi P, Metewo K, Bakoma B, Eklugadegbeka K, AklKokou K, et al. Antihyperglycaemic and antioxidant activities of *Crataeva adansonii* DC. ssp. *adansonii* leaves extract on ICR mice. J Drug Delivery Therapeutics. 2020;10(1–s):30–8. doi: 10.22270/jddt.v10i1.s.3855.

[81] Al-Rifai A, Aqel A, Al-Warhli T, Saitk M, Wabaidur, Zeid A, et al. Antibacterial, antioxidant activity of ethanolic plant extracts of some *Convolvulus* species and their DART-ToF-MS profiling. Evid-Based Compl Alt Med. 2017;1–9. doi: 10.1155/2017/5694305.

[82] Murugan R, Parimelazhagan T. Comparative evaluation of different extraction methods for antioxidant and anti-inflammatory properties from *Osbeckia parvifolia* Arn. – an in vitro approach. J King Saud Univ Sci. 2014;26:267–75.

[83] Mhalla D, Bouassida KZ, Chawech R, Bouaziz A, Makni S, Jialle L, et al. Antioxidant, hepatoprotective, and antidepressive effects of *Rumex tingitanus* extracts and identification of a novel bioactive compound. Bio Med Res Int. 2018:1–10. doi: 10.1155/2018/7295848.

[84] Rejaa ASS, Aleisa AM, Sayed-ahmed MM, Al-Shabanan OA, AbuOhashish HM, Ahmed MM, et al. Protective effect of rutin on the antioxidant genes expression in hypercholesterolemic male Westar rat. BMC Complement Altern Med. 2013;13:136. doi: 10.1186/1472-6882-13-136.

[85] Zhong K, Li XJ, Gou AN, Huang YN, Bu Q, Gao H. Antioxidant and cytoprotective activities of flavonoid glycosides-rich extract from the leaves of *Zanthoxylum bungeanum*. J Food Nutr Res. 2014;2(7):349–56. doi:10.12691/jfnr-2-7-4.

[86] Yang SY, Hong CO, Lee GP, Kim CT, Lee KW. The hepatoprotection of caffeic acid and rosmarinic acid, major compounds of *Perilla frutescens*, against ±BHP-induced oxidative liver damage. Food Chem Toxicol. 2013;55:92–9. doi:10.1016/j.fct.2012.05.042.