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RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING AND EVALUATION OF HEPATOPROTECTIVE POTENTIAL OF *SWERTIA PETIOLATA* AGAINST THIOACETAMIDE INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

The hepatoprotective activity of two extracts of *Swertia petiolata* (hydro-alcoholic and aqueous extracts) was studied against thioacetamide induced liver damage in rats. The levels of serum aminotransferases (ALT, AST), alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (GGT) were increased owing to thioacetamide (TAA) insult. The extracts were able to decrease these increased levels. Hepatic malondialdehyde (MDA), an index of oxidative stress increased significantly after the induction of liver necrosis and decreased in extract treated rats. Significant reduction in the levels of reduced glutathione were observed in necrotic group and increased in extract pretreated experimental animals. Xanthin oxidase activity also increased significantly in TAA, and decreased in extract pretreated groups. Moreover, glutathione reductase, glutathione peroxidase, G-6-PD, SOD and catalase were decreased by thioacetamide, whereas level of these enzymes was found near normal in extract treated rats. The phytochemical investigation of the both extracts showed presence of flavonoids, steroids, triterpenoids, alkaloids, glycosides, carbohydrates, tannins, phenolic compounds etc. The histopathological examination of the liver tissue of all group of rats supported the hepatoprotective potential of extracts of *Swertia petiolata.*

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INTRODUCTION

Liver is the organ for metabolism and detoxification. Various components enter into the body, the toxins and drugs, viral infections (Hepatitis A, B, C, D, etc.) and microbial infections of *Entamoeba histolytica* can cause damages to the hepatocytes (Ping *et al*., 2010). The toxins absorbed from the intestinal tract gain access first to the liver resulting in a variety of liver ailments. Thus liver diseases remain one of the serious health problems. Conventional or synthetic drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects (Patil and Mall, 2012). Kashmir region of Himalaya is a rich source of diversified herbs and shrubs of medicinal importance. Among various species of plants of medicinal importance, the family *Gentianaceae* holds a distinct place, as various genera of this family are medicinally important and have been used over years in various parts of the world to treat different ailments. There are around 1000 species of this family. Among the plants often used in traditional medicine, *Swertia* species are quite important and have been used as crude drugs in Indian Pharmacopoeia. There are about 250 species of *Swertia,* distributed worldwide, out of which near about 32 species occur in India with 15 species in north west Himalaya. About 9 species of *Swertia* have been reported from Jammu and Kashmir. These grow in grasslands, slopes or alpine bugyal. A perusal of data reveals that the genus *Swertia* is heteromorphous as the species occupy habitat ranging from mesophytic, more or less xeric to temperate conditions from low to high (alpine) altitude (490—6250mts). These species are found in western Himalayas, in Sonamarg, Batote, Banks of Chenab, Gulmrg, Baderwah, Gilgit, Lidder valley, Sindh valley, Drass, Banihal, Aharbal, Zanskar and Banamarg. *Swertia* species are used as a tonic and febrifuge. These have been used as bitter tonic, febrifuge, anthelmintic, antimalarial and antidiarrheal, (Brahmachari *et al*., 2004). In Chinese traditional medicine, 20 species of this genus are being used for the treatment of hepatic, choleric and inflammatory diseases. The herb of *S. purpurascens* is used in Pakistan as a substitute of *S. chirata*, and in Japan *S. Japonica* is an important bitter stomachic. A fair number of *Swertia* plants have been used since the remote past for the treatment of various ailments, particularly in the Indian subcontinent. In Indian system of medicine, *Swertia chirata* is credited with anthelmintic and antidiarrhoeal properties and is prescribed in

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dyspepsia, in the debility of convalescence and generally in cases in which corroborant measures are indicated (Bhatia *et al*., 2004). Unlike other bitter drugs, it does not produce constipation, instead it tends to produce a regular action and causes a free discharge of bile. It is given as powder, infusion, tincture, or as a fluid extract. However, the predominant species *Swertia chirata,* though recorded in literature, has not been found yet from Kashmir. *Swertia petiolata* has been used traditionally and regionally for various ailments by local healers. The entire plant of *Swertia petiolata* is used in Tibetan medicine for its cooling potency (Joshi and Dhawan, 2005), antiinflammatory activity, and as febrifuge, and liver tonic. In Kashmir, the rhizome of the perennial herb is used traditionally in toothaches, in rheumatic diseases and to heal and protect wounds from infections by *Gujjar* and *Bakerwal* community and people living in mountainous regions, who locally call it 'momram'. The phytochemical investigation of the genus *Swertia* as carried out so far has afforded some 200 compounds with varying structural patterns. Among these xanthenoids, terpenoids, flavonids, alkaloids, irridoid glycosides and some other compounds with varying structural patterns and different pharmacological activities have been established.

Swertia Petiolata

S. petiolata is widely distributed in India, Pakistan, Tibet, East Afghanistan and China. In India, it is found in Jammu & Kashmir, Himachal pardesh, and U.P hills. It is widely distributed in the area of Jammu & Kashmir from Zanskar, Gilgit, Rinchagma, Kaspucha, Sangam, Rucham pass, Burzil pass, Pirpanchal, Kishanganga valley, Jehlum valley, Harmukh slope, Khuihama forest, Sarbal pass, Gulmarg, Deosai plains, Amaranth way, Apharwat, Sheshnag, Mahaguna pass, Khillanmarg, Banamarg etc. The flowers and fruits come in late July to September.

Swertia petiolata is perennial, erect, rhizomatous, simple or branched, glabrous herb. The rhizomes are covered with old petiole bases, and the stem terete and hollow. Radical and lower1-2 pairs of cauline leaves have long petiole, elliptic-oblong, spathulate, petiole dilated, connate near base, 5-nerved, upper cauline leaves usually opposite in 3's, narrowly lanceolate, sessile, amplexicaule, 3-nerved. Inflorescence lax, few to many flowered, panicle solitary axillary or axillary and terminal, cluster of 3-5 flowered peduncle terete, bracts foliaceous, sessile, narrowly elliptic lanceolate. Flowers pentamerous, variously colored, pedicels unequal, calyx lobes lanceolate or linear lanceolate, glabrous 1-3 nerved, margins denticulate, corolla lobes 5, oblong, glands 2, orbicular or ovoid, with ciliate fimbrial at base, anthers bluish, capsule oblong-ellipsoid, many seeded coloured reddish brown. *Swertia Petiolata* from Gulmarg and Banamarg areas of Kashmir valley at 2950-3500 mt was obtained and identified by consultant taxonomist Department f Pharmaceutical Sciences, University of Kashmir, Hazratbal Srinagar. The specimen was kept in the herbarium for reference. The extracts of these plants were analyzed and characterized as follows:

Physico-chemical characterization according to the WHO protocol

Physico-chemical parameters of the plant extract were evaluated as per standards laid down by World Health Organization (Quality control methods for medicinal plant materials, WHO Geneva, 1998) and determined as per the standards and methods laid down by Indian Pharmacopoeia (1996). These included:

Loss on drying

It is the loss of weight in % w/w resulting from water and volatile matter of any kind that can be driven off under specified conditions. Loss on drying was estimated as per the method described in Indian Pharmacopoeia (1996). Accurately weighed (2 g and 10 g) of the air dried drug was placed in the tarred crucibles (also weighed) and put in oven maintained at 105°C for five hours. The crucibles were then taken out, cooled and again weighed. The percentage loss was then calculated with reference to air-dried drug.

Extractive values

The extractive value (% yield obtained) was determined in the 50% alcoholic medium, which was used in this study. Alcohol dissolves most substances such as glycosides, resins, alkaloids, and the water dissolves water-soluble substances in the extract. Both cold and hot extractive values were determined. For cold extraction, the air-dried crude powder of the drug (20g) was macerated with 100 ml solvent (50% hydro-alcoholic) in a closed flask for 24 hours, shaking frequently and allowed to stand for 24 hours. It was filtered rapidly, taking precaution against the loss of solvent. The filtrate was evaporated to dryness in a tarred flat bottom dish, dried at 105°c to constant weight, and weighed. For hot extraction, the powdered herbs (20g) were packed in a Soxhlet apparatus separately in 50% hydro-alcoholic solution. Each extract was evaporated to dryness and the extractive value was calculated.

Ash values

This parameter was used for the determination of inorganic material such as carbonates, silicates, oxalates and phosphates. Heating causes the loss of organic material in the form of CO_2 leaving behind the inorganic components. Ash value is an important characteristic of a drug and with the help of this parameter, quality and purity of the drug can be tested. To determine the total ash, the ground drug (1 g) was incinerated in a silica crucible at temperature not exceeding 450^0C until free from carbon. The ash was then cooled and weighed to get the total ash content. Acid insoluble ash and water insoluble ash values were determined as follows: Acid insoluble ash – The ash was boiled with 25 ml dilute HCl (6N) for five minutes. The insoluble matter collected on an ash less filter paper was washed with hot water and ignited at a temperature not exceeding 450^0C to a constant weight. For determining the water insoluble ash, the ash of the selected herb was dissolved separately in distilled water and the insoluble part collected on an ashless filter paper and ignited at 450^oC to constant weight. By subtracting the weight of insoluble part from that of the ash, the weight of the soluble part of ash was obtained.

Determination of pH

pH of 1% and 10% solution was determined. Accurately weighed (1 g) amount of the extract was dissolved in appropriate volume of distilled water to make 1% or 10% solution. The pH of the filtrate was determined using glass electrode.

Tests for sterols

Salkowaski reaction

Few mgs of the residue of each extract were taken in 2 ml of chloroform and 2 ml of concentrated sulphuric acid was added from the side of the test-tube. The test-tube was shaken for few minutes. Development of red colour in the chloroform layer indicated the presence of sterols.

Liebermann's test

To a few mg of the residue in a test-tube, few ml of acetic anhydride was added and gently heated. The contents of the test-tube were cooled. Few drops of concentrated sulphuric acid were added from the side of the test-tube. A blue colour gave the evidence of the presence of sterols.

Test for alkaloids

Few mg of residue of the extract was mixed in 5 ml of 1.5% v/v hydrochloric acid and filtered, which was used for testing alkaloids as follows: addition of a saturated aqueous solution of picric acid (Hager's reagent) resulted in an orange yellow precipitate, indicating the presence of alkaloids.

Test for saponins (Foam Test)

A few mg of the test residue was taken in a test tube and shaken vigorously with a small amount of sodium bicarbonate and water. A stable, characteristic honeycomb like froth indicated the presence of saponins.

Test for tannins

The test residue of each extract was taken separately in water, warmed and filtered. Tests were carried out with the filtrate as follows: A 5% w/v solution of ferric chloride in 90% alcohol was prepared. Few drops of this solution were added to a little of the above filtrate. Dark green or deep blue colour indicated tannins.

Test for flavonoids

A small quantity to test residue was dissolved in 5 ml ethanol (95% v/v) and reacted with few drops of concentrated hydrochloric acid and 0.5 g of magnesium metal. The pink, crimson or magenta colour, developed within a minute or two, indicated flavonoids.

Test for proteins

Proteins were detected using the xanthoproteic test, in which a little amount of residue in water forms a yellow colour complex, which indicates the presence of protein. Aromatic amino acids (derivatives of benzene) can undergo reactions that are characteristic of benzene and benzene derivatives. Nitration of benzene ring with nitric acid is commonly referred to as the xanthoproteic test, when used to identify the presence of an activated benzene ring, because of the yellow color product; Greek, *xanthos:* yellow. Briefly, mixing a little amount (3-5 mg) of residue with 0.5 ml of concentrated nitric acid and 2.0 ml of distilled water gives yellow color, which indicates proteins.

Test for sugars

Presence of sugars was tested using Barfoed's test. The reagent was prepared by dissolving 13.3 gm of crystalline neutral copper acetate in 200 ml of 1% acetic acid solution. The test residue was dissolved in water and heated with a little of the reagent. Appearance of red precipitate of cuprous oxide formed within two minutes indicates the presence of monosaccharides.

Characterization of phytoconstituents by HPTLC method
Preparation of the extract

Test material was washed, dried and crushed in mixer grinder. Crushed material was subjected to extraction in a Soxhlet apparatus at 60-70^0 C for 6 hours continuously in 50 % ethanol. The extracted material was evaporated to dryness under reduced pressure at 40-50^0 C. The dried extract was diluted (2.0 mg/ml) with methanol and filtered through membrane filter (pore size: 0.45 micron) before loading onto the HPTLC plate. 10 µl of the filtrate was loaded on the HPTLC plate with the help of an automatic sampler for analysis.

Chromatographic Condition

A Camag HPTLC system equipped with an automatic TLC sampler Linovet 5, TLC scanner 3 and integrated software Win Cats version 3.0 was used for the analysis. HPTLC was performed on a pre-coated silica gel HPTLC plate of 0.20 mm layer thickness. Sample was applied to the plate as 8 mm wide bands in different volumes of standard solution with an automatic TLC sampler (Linovet 5) under a flow of N_2 gas, 10 mm from the bottom and 10 mm from the side. The space between two spots was 6 mm. The linear ascending development was carried out in a CAMAG twin trough chamber (10 cm x 10 cm), which was pre saturated with the mobile phase for 30 min at room temperature. The length of the chromatogram run was 8 cm. Toluene: ethyl acetate: formic acid (70:20:5) for *S. petiolata* was used as mobile phase. After the development, plate was dried in oven at 60^{0}C for 5 min. For subsequent development, after drying post chromatographic derivatization was carried out in sulphuric acid followed by heating at 130^{0}C for 10 min. For finger printing analysis, plate scanning was performed in absorption mode at various wavelengths under computerized CAMAG TLC scanner 3.1.

Preparation of extracts for Pharmacological evaluation

Shade dried rhizomes of *S. petiolata* were crushed in a mixer grinder. The crushed material was subjected to extraction in soxhlet apparatus at 60°-70°C for 6 hours continuously in hydro-methanol (20:80). The extracted material was evaporated to dryness under reduced pressure at 40°-50°C.

Experimental animals

Inbred pathogen free adult male wistar rats (180-200gm body weight), in an environmentally controlled room with a 12 h light-dark cycle at constant room temperature (24 ± 2^{0}C) and relative humidity (60 ± 15 %), were used throughout the study. Animals were acclimatized for one week before starting the experiment. A maximum of six rats were kept in polypropylene cages. Animals had free access to pellet diet

(Hindustan lever Ltd, Bombay, India) and water ad libitum. Guidelines issued by the CPCSEA for the care and use of laboratory animals were followed

Evaluation of Hepatoprotective activity

Rats were divided into six different groups, each consisting of six animals. Group-I animals received only saline and served as control group. Group-II animals received single dose of thioacetamide (300 mg/kg/b.w.), and Group-III & IV animals received aqueous (1000 mg/kg/b.w.) and hydro-alcoholic extracts (200mg/kg/b.w.) of *S. petiolata*. Group-V & VI animals received the aqueous (1000 mg/kg/b.w.) and hydro-alcoholic extracts (200mg/kg/b.w.) of *S. petiolata* and a single dose of thioacetamide (300 mg/kg body weight). The extracts were given orally for five consecutive days, and 18 hours post treatment with thioacetamide, the animals were sacrificed. Blood was drawn by eye vein (retro orbital) and allowed to clot. Serum was separated by centrifuging the clotted blood at 1,500 rpm for 10 min. Liver tissue was removed immediately after sacrificing the animals and washed in ice-cold saline, blotted and kept at -80°C for subsequent operations. Hepatic tissue was homogenized and subjected to sub-cellular fractionation. To evaluate the hepatoprotective effect of herb extracts serum and other biochemical parameters were investigated by standard procedure.

Serum preparation

Blood was collected from the animals through retro orbital vein, under light ether anaesthesia. The blood was allowed to clot in centrifuge tubes and then centrifuged at 4°c at 3000xg for 10 minutes to separate serum for enzymatic analysis (Mujeeb et. al 2011). The serum was refrigerated till enzyme analysis.

Processing of liver tissue

Livers were quickly excised, cut into pieces and washed thoroughly with ice-cold phosphate buffer (0.1M, pH 7.4). The tissues were blotted gently between the folds of a filter paper and a portion was cut and weighed. From this tissue, 10% homogenate was prepared in nine volumes of ice-cold phosphate buffer (0.1M, pH 7.4), containing 1.15% KCl, using a polytron homogenizer.

Sub cellular fractionation

The tissue lysate (10% homogenate) was centrifuged first at 800× g for 10 minutes in a cooling centrifuge to remove the nuclei and other cell debris. The aliquots so obtained were decanted into fresh centrifuge tubes and subjected to centrifugation at 10,500xg for 20 minutes to get post-mitochondrial supernatant (PMS). All the above mentioned and subsequent operations were carried out at 0^0C- 4^0C. All enzymatic estimations were essentially completed within 12 hours of sacrificing of the animals.

Biochemical estimations

Biochemical analyses from the serum and tissue preparations from the control as well as treated groups of animals were performed according to the methods described below. The group I receiving normal saline is referred to as normal control, whereas the group given the hepatotoxin alone is referred to as positive control. The extracts under evaluation were given orally for five consecutive days prior to inducing liver injury

Biochemical markers of hepatic injury

a) Aminotransferases

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity was estimated by using the kit supplied by Span diagnostics Ltd, New Delhi. The procedure of estimation was based on the method described by (Anita *et al*., 2011). The enzyme activity was expressed in U/ml. The assay mixture consisted of 0.1ml serum diluted to 1.0 ml with α-ketoglutarate-alanine buffer substrate (pH 7.4) in case of ALT and with α-ketoglutarate aspartate substrate (pH 7.4) in case of AST determination. Substrate for ALT was prepared by dissolving 29.2 mg α -ketoglutarate (2 mM) and 1.78 g dl-alanine (200mM) in small amount of 1N NaOH. Final volume was made to 100 ml with 0.1M phosphate buffer, pH 7.4. AST substrate was prepared by dissolving 29.2 mg α-ketoglutarate (2 mM) and 2.66g dl-aspartate (200 mM) in small amount of 1N NaOH. Final volume was made to 100 ml with 0.1M-phosphate buffer, pH7.4. After incubating the reaction mixture for 30 min at 37^0C, 0.5 ml of DNPH (19.5 mg dissolved in 100 ml of 1N HCl) was added to each test tube. To this 5 ml of 0.4N, NaOH was added and the samples were kept at room temperature for 30 min before recording the absorbance at 505 nm. The enzyme activity was calculated from the standard curve plotted using pyruvate (2 mM, stock).

b) Alkaline phosphatase

Serum alkaline phosphatase (ALP) activity was estimated by the method of (Venkidesh *et al*., 2010; Olaoluwa *et al*., 2015) The assay system consisting of 1.0 ml of alkaline-buffered substrate (pH 10.3 –10.4) in each test tube in a temperature controlled water bath at 37^0C for 5 min. The alkaline-buffered substrate was prepared by taking equal volumes of 0.1 M glycine buffer and 0.4 % p-nitro phenyl phosphate (PNPP). Glycine buffer was prepared by dissolving 0.75 gm of glycine and 0.95 mg of $MgCl_2$ in 75 ml of distilled water. To the above solution, 8.5 ml of 1 N NaOH was added and the final volume w as made to 100 ml with distilled water. To this incubated substrate, 0.1 ml of serum was added to each test tube at an interval of 30 sec. The tubes were kept at room temperature for 30 minutes. The absorbance was taken at 410 nm using water as blank. 50 µl of 1 M HCl was added to each test tube and the absorbance was again measured. The final absorbance was determined after subtracting the absorbance of sample before and after addition of HCl. Enzyme, activities were expressed in Eq. Unit/ml.

c) Gamma Glutamyl Transpeptidase (GGT)

Serum γ – glutamyl transpeptidase (GGT) activity was determined by the method of (Naoko *et al*., 2008) using γ – glutamyl p – nitro anilide as substrate. The assay system consisted of 0.8 ml buffer substrate and 0.2 ml serum. Substrate was prepared by dissolving 0.528 gm of 40mM glycyl glycine, 0.121 gm of 40mM γ-glutamyl p-nitroanilide, 0.223 gm of 11mM $MgCl_2$ in 100 ml of 185 mM Tris- buffer at pH 8.25. After 10 minutes of incubation at 37^0C, the reaction was terminated adding 1.0 ml of 25 % trichloro acetic acid. All the tubes were centrifuged at 1,500 xg for 10 minutes. The

clear supernatant was read at 405 nm. Results were expressed in n mole p – nitroanilide / mg protein.

Oxidative stress parameters

a) Lipid peroxidation

The consequences of the peroxidative breakdown of membrane lipids have been considered in relation to both the subcellular and tissue aspects of liver injury. The production of thiobarbituric acid reactive substance was measured by the method of Mihaela and Denisa (Mihaela and Denisa, 2012). This modified method uses trichloroacetic acid to eliminate interference caused by malondialdehyde precursors. To a reaction mixture in a total volume of 2.0 ml containing 1.8 ml phosphate buffer (0.1M, pH 7.4), 0.2 ml of the liver homogenate (10%w/v) was added. The reaction mixture was incubated at 37^0C in water bath shaker for 1 hour. The reaction was terminated by adding 1.0 ml of 10 % trichloroacetic acid followed by the addition of 1.0 ml of 0.67 % thiobarbituric acid. All the tubes were kept in boiling water bath for 20 minutes. The tubes were then cooled in ice and centrifuged at 2,500 xg for 10 minutes. The resulting supernatant containing thiobarbituric acid-reactive substances (TBARS) was measured by taking the absorbance at 432 nm against a reagent blank. The Results were expressed in n mole MDA formed / mg protein at 37^0C using a molar extinction coefficient of 1.56 x 105M-1cm-1.

b) Reduced glutathione

Low level of the reduced form of glutathione (GSH) has been associated with an increased production of reactive oxygen species and free radicals in liver injury (Hanna *et al*., 2006). Cytosolic reduced glutathione was determined by the method described by Anuradha *et al* (Anuradha and Sarwat, 2006) with slight modification. In this method, 1.0 ml of the liver homogenate was precipitated with 1.0 ml 4 % sulphosalicylic acid. The samples were then kept for 1 hour at 4^0C and centrifuged at 1,200xg for 15 minutes at 4^0C. The assay mixture consisted of 0.1 ml of above supernatant, 2.7 ml of phosphate buffer (0.01 M, pH 7.4) and 0.2 ml of freshly prepared 5,5'- dithiobis-2- nitrobenzene (DTNB) (40 mg in 10 ml of 0.1 M phosphate buffer, pH 7.4) in a total volume of 3.0 ml. The colour developed due to the formation of a yellow colored complex, 5-thio-2- nitrobenzoate, was measured immediately at 412 nm. Results were expressed in µ mole of GSH /gm of tissue.

Antioxidant enzymes

a) Catalase

Catalase activity was assayed by the method of Djordjevic (Djordjevic *et al*., 2010). The assay mixture consisted of 1.99 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml of H_2O_2 (0.019 M) and 10 µl PMS (10% w/v) in a total volume of 3.0 ml in a quartz cuvette. Decrease in absorbance due to the disappearance of H_2O_2 was recorded at an interval of 30 seconds up to 3 minutes at 230 nm spectrophotometrically. Catalase activity was calculated in terms of nmole H_2O_2 consumed/min/mg protein using the extinction coefficient of 0.081 x 10-1 M-1 cm-1

b) Glutathione peroxidase

The glutathione disulfide produced as a result of GPx activity is immediately reduced by GR thereby, maintaining a constant level of reduced glutathione in the reaction system. The essay takes advantage of the concomitant oxidation of NADPH by GR, which is measured at 340 nm. Specific activity of the enzyme was measured according to the procedure described by Rehmat *et al* (Rahmat *et al*., 2012). The reaction mixture in a 3 ml cuvette consisted of 1.53 ml of phosphate buffer (0.05M, pH 7.0) 0.1 ml of 1 mM EDTA, 0.1 ml of 1 mM NaN_3, 0.1 ml of 1 mM reduced glutathione, 0.1 ml of 0.2 mM NADPH, 0.01 ml of 0.25 mM H_2O_2 and 100 µl PMS in a final volume of 2.0 ml. The activity was measured in terms of decrease in absorbance at 340 nm. The enzyme activity was calculated as nmole NADPH oxidized/min/mg protein by using molar extinction coefficient of 6.22 x 103 M-1 cm -1

Glutathione metabolizing enzymes and Glucose-6-phosphate dehydrogenase

a) Glutathione reductase

Glutathione reductase (GR) catalyzes the reduction of GSSG using NADPH as a reductant. It is important to maintain high cellular reductive potential. Activity of glutathione reductase was assayed by the method described by Kavita (Kavita *et al*., 2013). The assay mixture taken in a 3.0 ml cuvette consisted of 1.68 ml of phosphate buffer (0.1 M, pH 7.4), 0.1 ml of 0.1 mM NADPH (freshly prepared by dissolving 0.833 mg in 10 ml of 0.1 M phosphate buffer pH 7.4), 0.1 ml of 0.5mM EDTA (1.86mg in 10 ml distilled water), 0.05 ml 1mM oxidized glutathione (freshly prepared by dissolving 6.126mg in 10 ml 0.1 M phosphate buffer, pH 7.4) and 70µl of PMS (10% w/v) in a final volume 2.0 ml. The activity was measured in terms of decrease in absorbance at 340nm at an interval of 30 sec for 3.0 minutes at room temperature. Enzymatic activity was calculated by measuring the disappearance of NADPH and results were expressed as n mole of NADPH oxidized / min / mg protein.

c) Glucose-6 phosphate dehydrogenase

Glucose-6 phosphate dehydrogenase (G6PD) activity was assayed by the method of Zaheer (Zaheer *et al*., 1967). The reaction mixture in a total volume of 3.0 ml in a cuvette consisted of 0.3 ml of tris-HCL buffer (0.05M, pH 7.6), 0.1 ml 0.1mM NADP (freshly prepared by dissolving 0.787 mg in 10 ml tris-HCL buffer, pH 7.6), 0.1 ml of 0.8 mM glucose-6-phosphate (freshly prepared by dissolving 2.43 mg in 10 ml of tris-HCL buffer), 0.1 ml 8.0 mM $MgCl_2$ (7.073 mg in 10 ml distilled water), 100 µl PMS (10 %w/v) and 2.30 ml distilled water. The changes in the absorbance were recorded at an interval of 30 seconds for 3.0 min at 340 nm and the enzyme activity was calculated as nmole of NADP reduced / min/ mg protein by using molar extinction coefficient of 6.22 x 103 M-1 cm-1.

Phase II drug metabolizing enzyme

The drug metabolism enzymes are broken down into two distinct families and characterized to the type of reactions they catalyse. These are Phase I and Phase II enzymes. Phase I enzymes consist of CYP 450 the well-characterized family of cytochromes. Enzymes involved in the process of detoxification such as the glutathione-S-transferase, and the N-acetyl transferases are known as Phase II enzymes. These are responsible for the inactivation of hazardous compounds such as drugs, toxins, and carcinogens prior to excretion.

***a)* Glutathione S-transferase**

Glutathione S-transferase (GST) is an important component of the cellular defense against oxidative stress. It is also involved in the biosynthesis of prostaglandins (Edalat, 2002). Cytosolic glutathione–S-transferase activity was determined by the method of Chikezie (Chikezie *et al*., 2009). The assay mixture taken in a 3.0 ml cuvette consisted of 1.65 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml 1 mM reduced glutathione (freshly prepared by dissolving 3.07 mg in 10 ml 0.1 M, phosphate buffer pH 6.5), 100µl of PMS (10% w/v), 50µl 1 mM freshly prepared 1-chloro 2, 4-dinitrobenzene (2.025mg in 5-7 ml of absolute alcohol; the solution was vortexed and the volume was made up to 10 ml with distilled water) in a final volume of 2.0 ml. The increase in absorbance corresponding to an increase in CDNB- conjugate formed was recorded at an interval of 30 sec for 3.0 minutes at 340 nm. Results were expressed as n mole CDNB conjugated formed / min / mg protein by using the molar extinction coefficient of 9.6 X 103 M-1cm-1.

Free radical scavengers

***a)* Superoxide dismutase**

SOD activity was measured as per protocol adopted by Mukhopadhyay (Mukhopadhyay *et al*., 2012). Pyrogallol auto-oxidation by super oxide radical (O_2) generated by univalent reduction of oxygen is inhibited by super oxide dismutase (SOD). SOD converts superoxide radical to H_2O_2, which does not interfere with the auto-oxidation process. For preparation of tris buffer 50 mM Tris and 1 mM EDTA were dissolved in distilled water, pH adjusted to 8.5 by HCI and for preparation of pyrogallol solution, 20 mM pyrogallol was dissolved in double distilled water. The solution was prepared freshly at the time of assay. For each sample 2 test tubes were taken and marked C as control and T as treated. In control test tube 2.9 ml tris buffer and 0.1 ml, pyrogallol was taken and in test sample 2.8 ml tris buffer, 0.1 ml pyrogallol and 0.1 ml PMS sample was taken. After induction period of 90 seconds, absorbance was recorded first in control and then in test every 30 seconds for 3 minutes at 420 nm. The induction period was allowed to achieve a steady state of authorization of pyrogallol. A rate of change of absorbance per minute in the control as well as test sample was noted to calculate the SOD activity. The increase in absorbance at 420 nm was observed after addition of pyrogallol.

Pyrogallol auto-oxidation per 3ml assay mixture is given by the formula:

SOD per ml of sample = (A-B /A * 50*100) * 10 (dilution factor)

Where, A = Difference of absorbance in 1 minutes in control, and B = Difference of absorbance in 1 minute in test sample. Results were expressed in units/mg protein.

***b)* Xanthine oxidase**

Xanthine oxidase was assayed by the method described by Stripe and Corta (Stripe and Corta, 1969) modified by Ali *et al* (Ali *et al*., 2000). The reaction mixture consisted of 0.2 ml of post mitochondria supernatant (PMS) diluted to 1 ml with tris-HCl buffer (0.5M, pH 8.1) incubated for 5 min at 37^0C. Reaction was initiated by adding 0.1 ml of 1 mM xanthine (1.52 mg in 10 ml distilled water). The reaction mixture was kept at 37^0C for 20 min. The reaction was terminated by the addition of 0.5 ml ice-cold perchloric acid (10% v/v in distilled water). After 10 min., 2.5 ml distilled water was added to the precipitated mixture, which was then centrifuged at 1,200 xg for 10 min. The clear supernatant was read at 290 nm. The results were expressed as µmoles of uric acid formed/mg protein. The activity of xanthine oxidase was calculated by using a 2 mM Stock solution of the Uric acid to prepare the standard curve.

Estimation of protein

Protein from each biological sample was estimated by the method of **(Lowry *et al*., 1951)**. Peptide bond forms a complex with alkaline copper sulphate reagent that gives blue color with Folin's reagent. Briefly 0.1 ml of the tissue sample (10 % w/v) was diluted to 1.0 ml with distilled water and 1ml of TCA (10 % w/v) kept over night at 4^0C for protein precipitation, then centrifuged at 800xg for 15 min. The supernatant was decanted and discarded and the pellet was dissolved in 5 ml lN NaOH by vortex. Finally, 0.1 ml of the diluted aliquot was taken and further diluted to 1 ml with distilled water before adding 2.5 ml alkaline CuSO4 reagent. The alkaline CuSO4 reagent was prepared by mixing Na_2CO_3 (4% w/v in 0.1N NaOH) and Na-K tartrate (2% w/v in distilled water) kept for 10 min to allow complex formation and then 0.25 ml of Folin's reagent (2N, diluted to 1N with distilled water) was added exactly after 30 min. The absorbance of the blue color was recorded at 680 nm. For standard BSA (bovine serum albumin, l mg/ml) was used.

Gross morphological examination

Gross morphology of the liver with naked eye can reveal various forms of hepatic injury. Besides liver, other organs as kidney, heart, spleen, lungs and stomach were also studied for any morphological changes.

Histopathological studies

After collecting the blood, animals in each group were sacrificed by cervical dislocation under light anesthesia in an ethically proper way. The animals were immediately dissected and the liver of each animal was removed and washed in ice-cold saline solution (0.9% NaCl). The extraneous material was removed from the tissue, and the tissue was blotted gently between the folds of a filter paper. A small portion of the liver was cut, and stored in a vial containing 10% buffered formalin for fixation. The buffered formalin was prepared by adding 25 ml of formaldehyde solution in 75 ml of phosphate buffer (0.1 M, pH-7.4). For histopathological studies, the small portions of the liver were cut and processed. This processing consisted of fixing the specimen and embedding the fixed tissue in paraffin. The embedded tissue was sliced into 5-6 µm thin slices, and processed. The sections were prepared and processed at pathology lab, Department of pathology, Government medical college Srinagar according to routine standard procedure (Carlos and Verónica, 2013). Briefly, the slides containing cut sections were dipped in 70 % alcohol, than washed in water and stained using haematoxylin-eosin. The slides processed routinely and finally mounted in D.P.X. and covered with glass cover slips and kept for drying at room temperature. Histopathological examinations of the slides were done by an expert pathologist (who was unknown with the treatment)

Statistical analysis

The level of significance between the two groups was based on Student's t test followed by the analysis of variance using software available on Internet (www.graphpad.com). The level of significance was chosen at $p<0.001$, $p<0.01$, and $p< 0.05$.

RESULTS

The yield of the hydro-alcoholic extract was found to be 20.3 % w/w. Similarly the herb was cold extracted with water for 24 hours with occasional stirring. The extract was filtered and dried and the yield calculated as 15.5% w/w.

Table 1. Physico-chemical analysis of *Swertia petiolata*

Physical Analysis

S.No	Particulars of Analysis	Value
1	Loss on drying at 105°C	8.75 %
2	Total ash	6.349 %
3	Acid insoluble ash	2.773 %
4	Alcohol soluble extractive	2.69 %
5	Water soluble extractive	2.60 %
6	pH of the extract at 21.4^{0}C	5.70

Chemical Analysis

S.No	Particulars	Result
	Alkaloids	Present
	Flavonoids	Present
	Proteins	Present
	Saponins	Present
	Sterols	Present
	Sugars	Absent
	Tannins	Present

HPTLC finger printing of extract

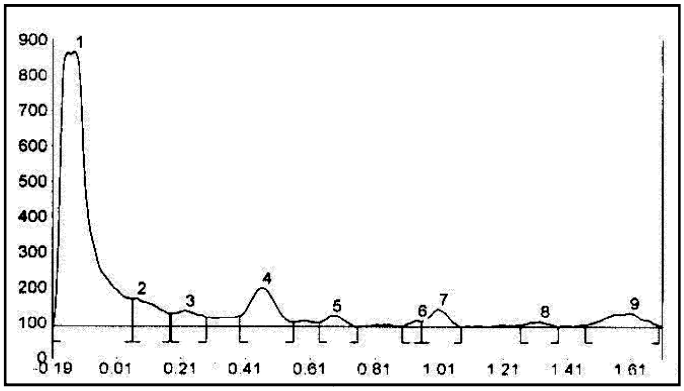

Figure 2. HPTLC chromatogram of *Swertia petiolata* showing prominent peaks representing the constituents present

Morphological findings

Morphological changes were studied in the texture of vital organs as liver, spleen, kidneys, heart, and stomach with the naked eye. No significant change in the average weight of livers was found in any of the group .The herb alone treated group also did not show any significant variation; neither did the group treated with the herbal extract and thioacetamide. The vital organs such as kidney, heart and spleen did not show any apparent changes.

Histopathological observation

Histology of the liver from control and treated rats was studied. Necrosis was found to be considerably low in the group of rats pretreated with the extracts of *Swertia petiolata.* Control group showed normal lobular architecture, while the necrotic liver tissue showed areas of necrosis and haemorrage. The cells were devoid of morphology.

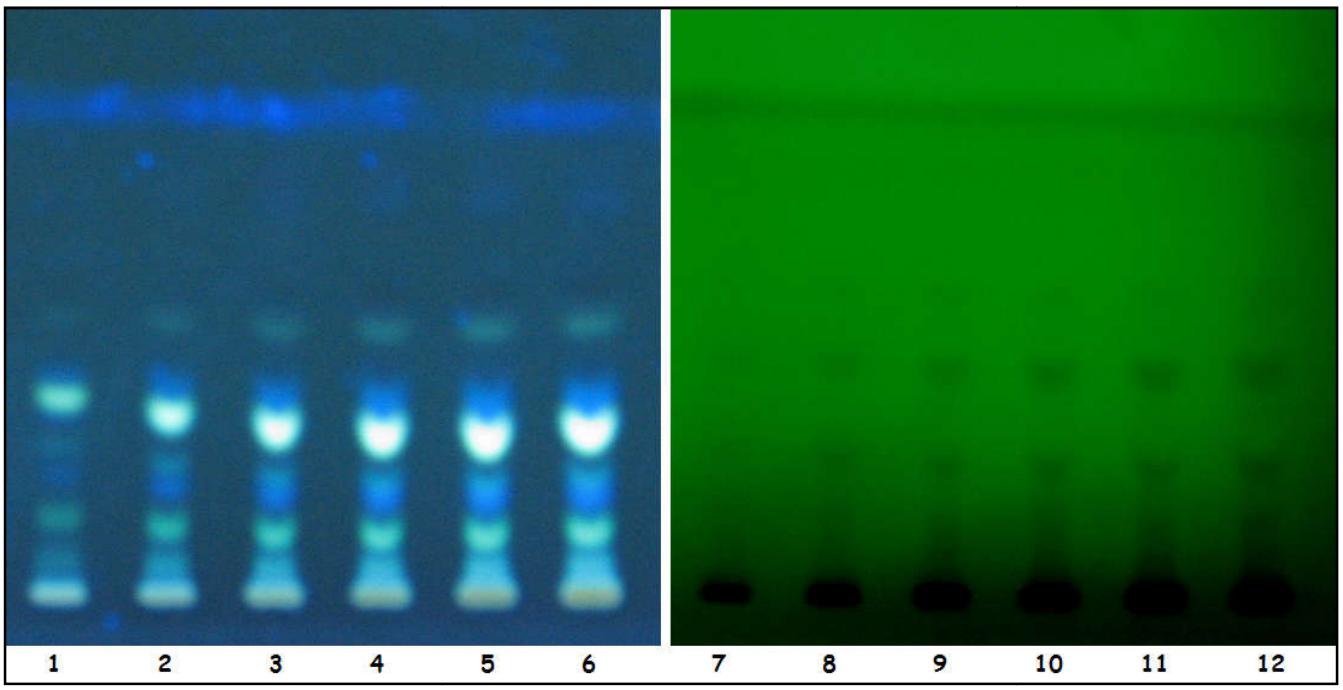

Figure 1. Photographic images of HPTLC analysis of *S. petiolata* Tracks (1-6) run at different volumes (1.0-6.0µl) (Bands 1-6 observed under fluorescent light at 366 nm, and 7-12 under UV at 254 nm)

Biochemical investigations

To study the effect of extracts on the liver, serum aminotransferases (ALT, AST), alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (GGT) activities were measured in different groups. Administration of thioacetamide caused significant increase in these enzyme levels, which were decreased by *Swertia* extracts. Hepatic lipid peroxidation was measured in terms of the hepatic malondialdehyde (MDA) levels in the tissue. MDA is an index of oxidative stress and it increased significantly after the induction of liver necrosis and decreased in extract treated rats. Marked reduction in the levels of reduced glutathione were observed in necrotic group and increased in extract treated experimental animals. The Mo–Fe–S flavin enzyme xanthine oxidase plays an important role in the metabolism of drugs and toxins.

Xanthine oxidase activity increased significantly in TAA, and decreased in extract pretreated groups. Glutathione reductase, glutathione peroxidase and catalase were decreased by thioacetamide, and normalized by the extracts. Results of the analysis of G6PD and SOD also showed decreased levels in TAA groups and were normalized by extract pretreatment.

Table 2. The effect of *Swertia petiolata* extracts on serum markers of liver injury

Groups	ALT	AST	ALP	GGT
NS	79.87±0.96	32.50±0.64	19.71±0.40	313.46±30.36
TAA	95.00±1.36	49.26±1.33	26.57±0.48	405.26±17.23
S.P(H/A)	78.78±1.17	32.17±0.39	19.05±0.16	311.21±8.56
S.P(Aq)	79.09±1.30	32.72±0.305	19.01±0.20	315.29±16.79
S.P(H/A)+TAA	84.17±0.78*	35.35±0.49*	20.81±0.33*	325.13±20.73*
S.P(Aq)+TAA	84.62±0.68*	35.58±0.41*	20.95±0.48*	327.43±46.06*

Data represents mean ± S.E.M (n=6). *p <0.05 which are statistically significant compared to the necrotic group (TAA). **NS:** group receiving normal saline and serving as normal control group; **TAA:** necrotic group received thioacetamide; **S.P (H/A) & (Aq):** *Swertia petiolata*, hydro-alcoholic and aqueous extracts; **S.P + TAA:** herb extracts plus thioacetamide. ALT, Units/ml; AST, Units/ml; ALP, Eq. units/ml; GGT, nmole p-nitroanilide/mg protein

Table 3. The effect of *Swertia petiolata* extracts on oxidative stress parameters

Groups	LPO	GSH	XO
NS	168.62 ± 8.00	112.94 ± 3.84	182.00 ± 7.13
TAA	622.52 ± 8.56	90.44 ± 2.84	212.00 ± 3.29
S.P (H/A)	169.66 ± 10.75	112.50 ± 6.36	180.00 ± 9.60
S.P (Aq)	169.94 ± 17.11	113.60 ± 6.07	186.68 ± 5.45
S.P (H/A) + TAA	203.33 ± 10.77**	103.68 ± 2.20*	190.83 ± 5.60***
S.P (Aq) + TAA	204.92 ± 5.54**	104.78 ± 4.89*	190.29 ± 7.12***

Data represents mean ± S.E.M (n=6). *p <0.02, **p <0.006, ***p <0.0001 which are statistically significant compared to the necrotic group (TAA). **NS:** group receiving normal saline and serving as normal control group; **TAA:** necrotic group received thioacetamide; **S.P (H/A) & (Aq):** Swertia petiolata, hydro-alcoholic and aqueous extracts; **S.P + TAA:** herb extracts plus thioacetamide. LPO, nmole of MDA/mg protein; GSH, µmole of GSH/g tissue; XO, µmole of uric acid/mg protein.

Table 4. The effect of *Swertia petiolata* extracts on antioxidant enzymes

Groups	GPx	GR	Catalase
NS	71.09 ± 5.42	38.40 ± 0.89	4449.59 ± 94.18
TAA	42.55 ± 1.78	22.13 ± 1.80	3081.28 ± 117.11
S.P (H/A)	71.46 ± 5.03	38.06 ± 1.33	4585.90 ± 75.38
S.P (Aq)	71.90 ± 4.11	39.91 ± 1.71	4656.63 ± 149.10
S.P (H/A) + TAA	65.60 ± 4.05**	30.82 ± 1.49**	3990.48 ± 148.14**
S.P (Aq) + TAA	65.56 ± 4.60**	30.58 ± 1.06**	3905.09 ± 228.26*

Data represents mean ± S.E.M (n=6). *p <0.02, **p <0.006, ***p <0.0001 which are statistically significant compared to the necrotic group (TAA). **NS:** group receiving normal saline and serving as normal control group; **TAA:** necrotic group received thioacetamide; **S.P (H/A) & (Aq):** Swertia petiolata, hydro-alcoholic and aqueous extracts; **S.P + TAA:** herb extracts plus thioacetamide. GPx, nmole of NADPH oxidized/min/mg protein; GR, nmole of NADPH oxidized/min/mg protein; Catalase: nmole of H_2O_2 consumed/min/mg protein

Table 5. The effect of *Swertia petiolata* extracts on glucose-6-phosphate dehydrogenase (G6PD) and superoxide dismutase (SOD)

Groups	G6PD	SOD
NS	11.29±0.415	2.07±0.075
TAA	6.98±0.56	1.41±0.05
S.P (H/A)	11.34±1.16	2.19±0.085
S.P (Aq)	11.78±0.315	2.29±0.075
S.P (H/A) + TAA	10.74±0.785**	1.95±0.055*
S.P (Aq) + TAA	10.99±0.52**	1.93±0.065*

Data represents mean ± S.E.M (n=6). *p <0.02, **p <0.006 which are statistically significant compared to the necrotic group (TAA). **NS:** group receiving normal saline and serving as normal control group; **TAA:** necrotic group received thioacetamide; **S.P (H/A) & (Aq):** Swertia petiolata, hydro-alcoholic and aqueous extracts; **S.P+ TAA:** herbal extracts plus thioacetamide. G6PD: n mole of NADP reduced/min/mg protein; SOD, units/mg protein.

DISCUSSION

Hepatic injury was induced by using intra-peritoneal injection of thioacetamide, which is known to cause hepatotoxicity in experimental rats and can produce hepatic necrosis (Shakir Ali *et al*., 2008). Thioacetamide induced hepatic injury is an experimental model widely used in hepatoprotective drug screening. This study shows, pre treatment of medicinal herb can prevent the acute hepatic damage induced by TAA. TAA is a thiono-sulfur-containing compound, which has liver-damaging and carcinogenic effects. Shortly after administration, it undergoes extensive metabolism by the mixed function oxidase system to acetamide, which has no liver necrotising properties, and thioacetamide-S-oxide. Thioacetamide-S-oxide is metabolized by cytochrome P-450 mono-oxygenases to further compounds, including the very reactive compound, thioacetamide-S-dioxide. The binding of this metabolite to tissue macromolecules may be responsible for hepatic necrosis, induction of apoptosis, perturbation of mitochondrial activity, and elevation of serum enzyme levels. Numerous studies in rats indicated the involvement of oxidative stress in the etiology of TAA-induced liver damage (Bruck *et al*., 2002). In the present study, a model of liver necrosis was produced by thioacetamide to evaluate the

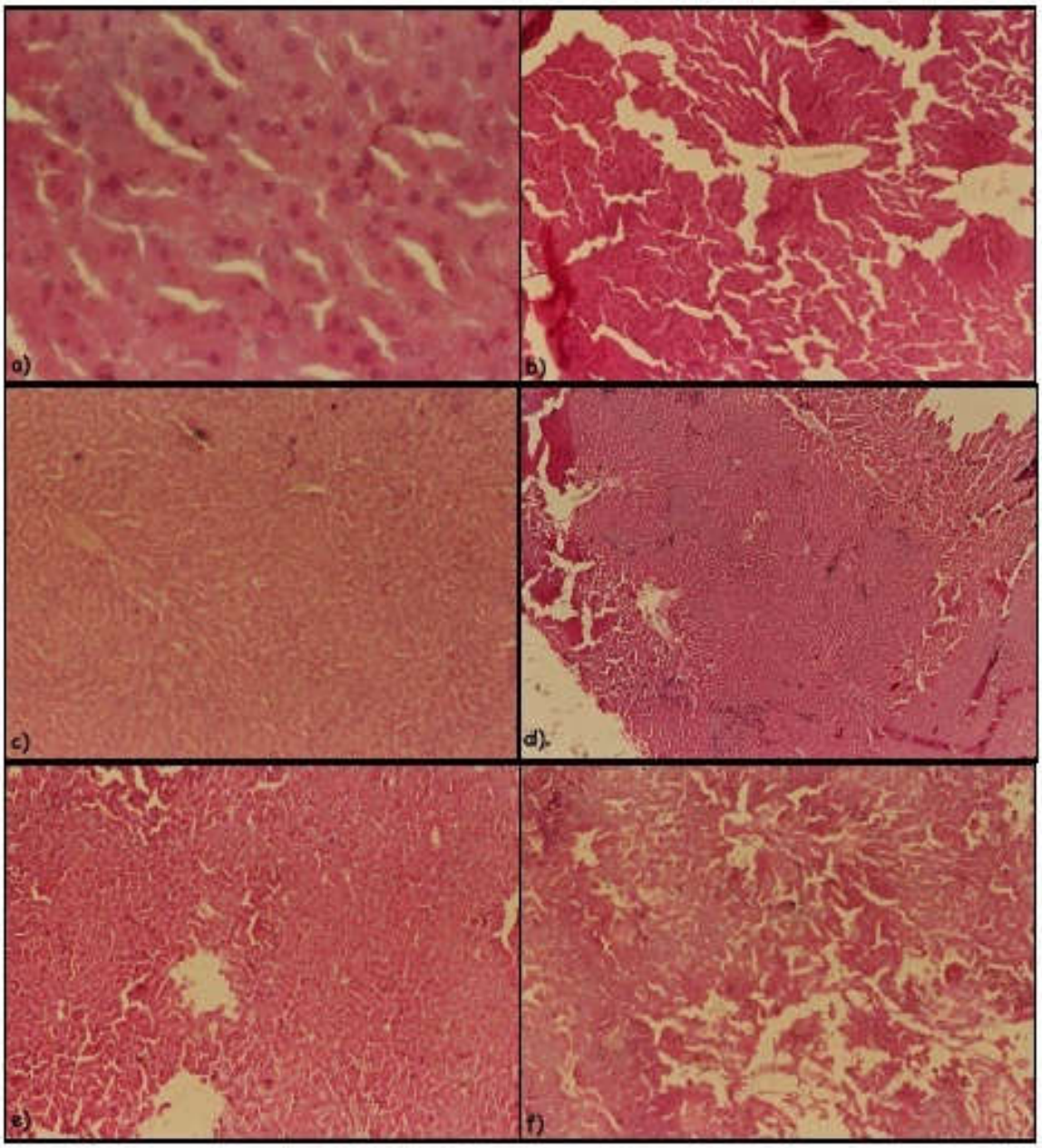

Figure 3. Summary of histopathological analysis of experimental rats. Normal control group received saline solution showing normal hepatocytes. a) necrotic group showing area of necrosis and sinusoidal dilatation. b). There are no changes in animals receiving aquous extract c) and hydro-alcoholic extract e), while necrotic animals that received aquous extract d) and hydro-alcoholic extract f) of *Swertia petiolata* showed sinusoidal dilatation

efficacy of our selected medicinal plant *Swertia petiolata* which shows hepatoprotective activity. In this regard, morphological and biochemical analysis was studied. In the morphological characteristics, there were no apparent pharmacological changes in the liver of rats treated with *Swertia* extracts; neither other organs viz (heart, kidneys, and spleen) showed any apparent change. Experimental animals treated with thioacetamide alone developed significant hepatocellular damage as was evident from a significant increase in the serum levels of AST, ALT, ALP, and GGT when compared with control (Iyanda and Adeniyi, 2011). The rise in serum levels of AST, ALT, ALP and GGT has been attributed to the damaged structural integrity of liver (Mihir *et al*., 2013), because these are cytoplasmic in location and are released into the circulation after cellular damage (Kew, 2000). The results indicate towards the protective role of extracts in liver necrosis. The extracts when given prophylactically could effectively prevent the liver injury induced by thioacetamide, as evidenced by decrease in the serum levels of ALT, AST, GGT, and ALP. Among the various phosphatases, ALP has attained much attention because of its location in the plasma membrane and possible role in active transport (Jeyachandran *et al*., 2007). Oxidative stress and its consequent lipid peroxidation have been considered involved in hepatic injury. Studies on thioacetamide-induced liver injury have demonstrated the generation of reactive oxygen species (ROS) and initiation of peroxidation reactions (Ali *et al*., 2001). The ROS either extract a hydrogen atom from unsaturated membrane lipids to initiate lipid peroxidation or react with the sulfhydryl compounds, triggering a chain of peroxidation reactions. These changes lead to cell injury. This study on *Swertia* extracts demonstrates that the extract could inhibit the injury induced by thioacetamide in rat. Measurement of lipid peroxidation and glutathione (reduced form, GSH, provided a

clear indication towards the antioxidant role of the extracts. Lipid peroxidation represents a degradative process in the tissue arising from the production and propagation of free radical reactions primarily involving membrane polyunsaturated fatty acids and the production of end products such as malondialdehyde and 4-hydroxynonenal (Poli, 2000). Data have been reported showing a progressive reduction in the activity level of lipid peroxidation and elevated level of reduced glutathione in necrotic animals (Bruck *et al*., 2001). In this study, we reported similar changes in the model, pretreatment of herbal extracts reversed this effect.

To evaluate the involvement of oxygen radicals in hepatic damage and potential defense of herbal extracts, we have measured activity of xanthine oxidase. In this study, TAA induced necrosis provokes xanthine oxidase activity which produces oxidative stress by generating ROS, indicating its role in this type of liver injury. Significant decrease of xanthine oxidase activity was observed in pretreated rats with herbal extracts followed by thioacetamide, which is in accordance with results of (Pawa and Ali, 2004). In addition to hepatic glutathione level, activity of enzymes involved in the glutathione redox cycle such as glutathione reductase was also determined. While in necrotic rats, it decreased markedly, in the test extract treated animals, the activity increased and reached almost up to normal value. The peroxide metabolizing enzyme, glutathione peroxidase, showed a similar pattern suggesting the generation of peroxides in hepatic injury. Catalase is known to catalyze the removal of hydrogen peroxide and therefore its upregulation may provide a compensatory or adaptive response against elevation in hydrogen peroxide (Pillai and Gupta, 2005). Over-production of ROS normally induces oxidative stress unless it was scavenged with endogenous antioxidants. Thus, overproduction of ROS could be attributed to the depletion of antioxidants. Glucose-6-phosphate dehydrogenase being a cytoplasmic enzyme, its main metabolic role is the production of NADPH in the monophasphate pathway and the defense against oxidizing agents. The results indicate a significant increase in glucose-6-phosphate dehydrogenase activity in necrotic groups, which were pretreated with the extracts. The antioxidant defense system is composed mainly of three enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The superoxide dismutases convert superoxide anion into H_2O_2 and O_2. Catalase catalyzes the dismutation of H_2O_2, forming neutral products as O_2 and H_2O. Gluthathione peroxidase catalyzes the reductive destruction of hydrogen and lipid hydroperoxides, using gluthatione as an electron donor (Esra *et al*., 2012). A significant decline in the level of liver superoxide dismutase in necrotic rats was observed in this study. The findings suggest that the extracts can protect the liver injury (necrosis), which is produced by the excessive production of ROS. However, the mechanism by which this effect is produced is not very clear. The effect appears to be due to the ability of extracts to somehow strengthen the antioxidant status of tissue, which is evident from results.

Conclusion

It can thus be concluded that *Swertia petiolata* shows good hepatoprotective activity against thioacetamide induced liver injury. Biochemical evidences as normalization of serum parameters involved in liver injury, oxidative stress parameters, free radical scavengers, phase II drug metabolizing enzymes, glutathione metabolizing enzymes, glucose-6-phosphate dehydrogenase and histopathological evidences provide support to these findings.

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