Evaluation of Hepatoprotective Potential of *Swertia tetragona* Edgew

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Abstract: *This study was designed to evaluate the hepatoprotective potential of aqueous and hydroalcoholic extracts of Swertia tetragona. Both the extracts of S. tetragona showed good hepatoprotective activity against thioacetamide induced liver injury. Biochemical evidences as normalization of Serum parameters(ALP,ALT,Alkaline Phosphatase and GGT), Oxidative stress parameters(lipid peroxidation and reduced glutathione), Free radical scavengers(catalase and glutathione peroxidase), Phase II drug metabolizing enzymes(glutathione-s-transferase), Glutathione metabolizing enzymes (glutathione reductase and G-6-PD), Free radical scavengers (SOD and xanthin oxidase) and Histopathological evidences provide support to these findings.*
Key words: *Swertia tetragona, Hepatoprotective activity, thioacetamide, serum aminotransferases*

I.   Introduction

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. 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 northwest 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, Gulmarg, Baderwah, Gilgit, Lidder valley,Sindh valley, Drass, Banihal, Aharbal, Zanskar and Banamarg.

These species have been used as bitter tonic, febrifuge, anthelmintic, antimalarial and antidiarrheal. [1] 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. *Swertia chirata* is considered the most important for its medicinal properties. The genus *Swertia* is one of the extensively phytochemically worked up herbs[2] and some 200 compounds with varying structural patterns have been reported. Amongst these xanthenoids terpenoids, flavonoids, alkaloids, irridoids and seco-irridoid glycosides and some other compounds with varying structural pattern are the important ones [3,4]. Xanthenoids are the major class of compounds among the chemical constituents of this genus, and since they often exhibit multidirectional biological activities, this spectacular segment of natural products has created a stir among pharmacologists and biologists. Particularly tetraoxygenated xanthones exhibit hypoglycaemic, antihepatotoxic, antimalarial, anti-inflammatory, antioxidant, antimicrobial, and anti-tumor properties.[5-7]

About 108 xanthones, 10 flavinoids, 5 alkaloids, 16 irridoids and seco-irridoid glycosides, some lignans, lactones and several other compounds have been identified in these species. Among these compounds, swertianolin, norswertianolin, swerchirin, amarogentin and swertiamarin have been described to possess antimicrobial activity. [8] These species have been used traditionally and regionally for various ailments by local healers. In Kashmir *Swertia tetragona* is mostly substituted for *S.chirata* and is locally used as anthelmintic, febrifuge and liver tonic.

Swertia tetragona [9] is an annual, erect, solitary or tufted branched herb, its stem is 12-48 cm tall, 4-angled (the angles narrowly winged), slightly lanceolate or lanceolate, acute, 2-7mm broad, bases alternate, scaberulus, nerves1-3, margins ± revolute, radical leaves withering at flowering, obovate, subacute, spathulate, 5-6 mm broad. Inflorescence panicle of solitary, axillary or solitary and terminal cluster of 3-5 flowers,

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peduncles slender, 4-lineolate, 0.6-4 cm long, 1-3 mm broad. Flowers are pentamerous, white, often with purple streaks, pedicellate, 5-8 mm long, pedicels thin, slender, unequal 0.5-2.6 cm long. Calyx nearly equal to corolla, lobes linear, linear-lanceolate, acute, 1-nerved, margins suberose, 3-8 mm long and 2 mm broad. Corolla lobes are ovate-lanceolate, acute, 4-7mm long and 2-3 mm broad, glands 2, submarginal at the base of lobes, margins subglabrous, stamens 5, filaments filiform, 3-5mm long, anthers greenish blue, minute, nearly hastate. Ovary is elliptic-oblong, 4- 5mm long and nearly 3 mm broad, sessile, stigma sessile, subreniform and rugose. Capsule is oblong-ovoid, sessile 0.6-1 cm long, sub-included at maturity. Seeds numerous, minute, subglobose, reddish brown and testa are alveolate. *Swertia tetragona* is bitter in taste. Bhat in 2009[7] reported the hypoglycemic activity of its hydroalcoholic extract.

II.   Liver Diseases

Liver is one of the largest organs in human body and is the chief site for intense metabolism and excretion. It has an important role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways of growth, fight against disease, nutrient supply, energy provision and reproduction. The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamins. Thus, maintenance of a healthy liver is a crucial factor for overall health and wellbeing. Unfortunately the liver is continuously and variedly exposed to environmental toxins and abused by poor drug habits & alcohol and prescribed & over-the-counter drugs, which can eventually lead to various liver ailments like hepatitis, cirrhosis and alcoholic liver disease. Thus, liver diseases are some of the fatal diseases in the world today. They pose a serious challenge to international public health. Modern medicines have little to offer for alleviation of hepatic diseases and also there aren't many drugs available for the treatment of liver disorders. Therefore, many folk remedies from plant origin are tested for their potential antioxidant and hepatoprotective activities. Many environmental toxins cause liver injury to humans, and despite new advances in hepatology, the treatment for liver diseases does not resolve the problems caused by these toxins. Acute hepatic failure frequently develops upon exposure of the tissue to viruses or numerous chemical agents and represents a complex process, characterized by simultaneous activation of multiple deregulated pathways that culminate in the loss of cell membrane integrity and thus, the leakage of cellular constituents.[10] Furthermore, despite the increasing need for agents to protect the liver from damage, modern medicine lacks a reliable liver protective drug. Therefore, there has been considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases. [11]

Medical science recognizes the liver for its metabolic, immune, and detoxification functions. Virtually all substances absorbed from the gastrointestinal tract pass through the liver before entering the central circulation, and at the same time, a site susceptible to damage. This is probably the reason why liver has a remarkable power of regeneration. An imbalance between liver cell death and regeneration may lead to hepatic injury and subsequently to its failure.

Functional liver comprises of four cell types: hepatocytes, sinusoidal epithelial cells, hepatic stellate cells and kupffer cells. All of them act as a potential target of liver injury and as potential targets of harmful mediators of hepatic cell damage, which may be because of necrosis, apoptosis, or both simultaneously.

Treatment modalities for liver diseases in allopathy

Not a single curative therapeutic agent has been found so far which could provide lasting remedy to patients suffering from hepatic disorders. In fact, remedies available in the modern system of medicine provide only symptomatic relief without any significant changes in the disease process. Moreover, their use is associated with severe side effects and chances of relapse, except some natural products claimed to be effective, no safe synthetic product is yet available for the management of hepatic disorders. Lack of effective, least toxic and curative hepatoprotectants has made the task difficult to discover new drugs. However, attempts are still being made to explore the potential of natural products, especially of plant origin for their antihepatotoxic potential.
The present study was undertaken to evaluate the herb pharmacologically (for its antihepatotoxic potential*).* The whole plant collected from Aharbal area of Kashmir (3100 mt) was used for the study.

III.   Experimental Design

Plant material
Swertia tetragona from Aharbal at 3100 mts altitude was collected and identified by consultant taxonomist Department of Pharmaceutical Sciences, University of Kashmir, Hazratbal Srinagar. A specimen was preserved in the herbarium for future reference.
Assessment of hepatoprotective activity
Experimental animals
Inbred pathogen free adult female wistar rats (220-240gm body weight), in an environmentally controlled room with a 12 h light-dark cycle at constant room temperature (24 ± 2ºC) 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.

Rats were divided into six groups, each consisting of six animals. Group-I animals received saline and served as control group. Group-II animals received single dose of thioacetamide (300 mg/kg body weight) and served as toxic control, Group-III & IV animals received aqueous and hydro-alcoholic extracts of *S. tetragona (to see whether the extracts themselves don't cause any disease)* and Group-V & VI animals received the extracts of *S. tetragona* plus a single dose of thioacetamide (300 mg/kg body weight). The plant extracts were given orally at the dose levels of 1 gm/kg body weight in case of the aqueous extract and 200 mg/kg body weight in case of the hydro-alcoholic extracts. The extracts were given for seven 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. 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.

Serum preparation

Blood collected from the animals through retro orbital vein was allowed to clot in centrifuge tubes and then centrifuged at 4°c at 3000xg for 10 minutes to separate serum for enzymatic analysis. [12] 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 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 fractionationk

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 10,500 xg for 20 minutes to get post-mitochondrial supernatant (PMS). All the above mentioned and subsequent operations were carried out at 0°C- 4°C. 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 was referred as normal control, whereas the group given the hepatotoxin alone as positive control. The extracts under evaluation were given orally for seven 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. [13] 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 (200 mM) 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°C, 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.,* 20 10 and Olaoluwa*et al.,* 2015. [14,1 5] 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 ºC 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 was 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. [16] 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 40mM γ-glutamyl p-nitroanilide, 0.223 gm 11mM $MgCl_2$ in 100 ml 185 mM Tris-buffer at pH 8.25. After 10 minutes of incubation at 37ºC, the reaction was terminated by adding 1.0 ml 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, 2012. [17] 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 liver homogenate (10%w/v) was added. The reaction mixture was incubated at 37ºC 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 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 37ºC 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.[18] Cytosolic reduced glutathione was determined by the method described by Anuradha *et al.,* 2006[19] with slight modification. In this method, 1.0 ml liver homogenate was precipitated with 1.0 ml 4 % sulphosalicylic acid. The samples were then kept for 1 hour at 4 ºC and centrifuged at 1,200xg for 15 minutes at 4ºC. 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 color 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 et *al.,* 2010.[20] The assay mixture consisted of 1.99 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml H_2O_2 (0.019 M) and 10µl 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 Khan *et al.,* 2012.[21] The reaction mixture in a 3 ml cuvette consisted of 1.53 ml phosphate buffer (0.05M, pH 7.0) 0.1 ml 1 mM EDTA, 0.1 ml 1 mM NaN_3, 0.1 ml 1 mM

reduced glutathione, 0.1 ml 0.2 mM NADPH, 0.01 ml 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 phosphatedehydrogenase
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 *et al.,* 2013.[22] 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 0.1 mM NADPH (freshly prepared by dissolving 0.833 mg in 10 ml 0.1 M phosphate buffer pH 7.4), 0.1 ml 0.5mM EDTA (1.86mg in 10 ml distilled water), 0.05 ml 1mM oxidized glutathione (freshly prepared by dissolving 6.126 mg 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 340 nm 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 *et al.,* 1965.[23] 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 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 pf 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 metabolizing enzymes are characterized by the type of reactions they catalyse. These are Phase I and Phase II enzymes. Phase I enzymes consist of CYP450 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.[24] Cytosolic glutathione−S-transferase activity was determined by the method of Chikezie *et al.,* 2009.[25] 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 *et al.,* 2012.[26] 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 minute 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[27] modified by Ali et al *et al.,* 2000.[28] The reaction mixture consisted of 0.2 ml post mitochondria supernatant (PMS) diluted to 1 ml with tris-HCl buffer (0.5M, pH 8.1)incubated for 5 min at 37ºC. 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 37ºC for 20 min. The reaction was terminated by the addition of 0.5 ml ice-cold perchloric acid (10% v/v in distilled water). After10 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. [29] 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 overnight at 4ºC 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 $CuSO_4$ reagent. The alkaline $CuSO_4$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 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. BSA (bovine serum albumin, l mg/ml) was used as a standard.

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

Animals were sacrificed by cervical dislocation under light anesthesia in an ethically proper way. The animals were immediately dissected and the livers 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. [30] 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 results have been presented as Mean + S.E.M. The level of significance between the two groups was based on Student's t test followed by the analysis of variance (ANOVA) using software available on Internet (www.graphpad.com). The level of significance was chosen at $p = <0.001$, $p = <0.01$, and $p = <0.05$.

IV. Results

Morphological findings

Morphological changes were studied in the texture of vital organs as liver, spleen, kidneys, heart, and stomach with the naked eye. These organs were then weighed to assess any change in their weights. As is evident from **Table 1,** thioacetamide caused significant reduction in the body weight of animals (average wt = 222.5 ± 2.5 p=0.007), whereas the extracts alone and in combination with thioacetamide did not cause any significant variation in the average weight of animals, which varied between 240-250 gms.

No significant change in the average weight of livers was found in any of the group **(Table 2)**. The vital organs such as heart and spleen did not show any apparent changes.The stomachs of herbs plus thioacetamide treated rats were bulged which was well distinguishable from other groups. There was significant increase in the average weight of stomachs in the herb treated groups.
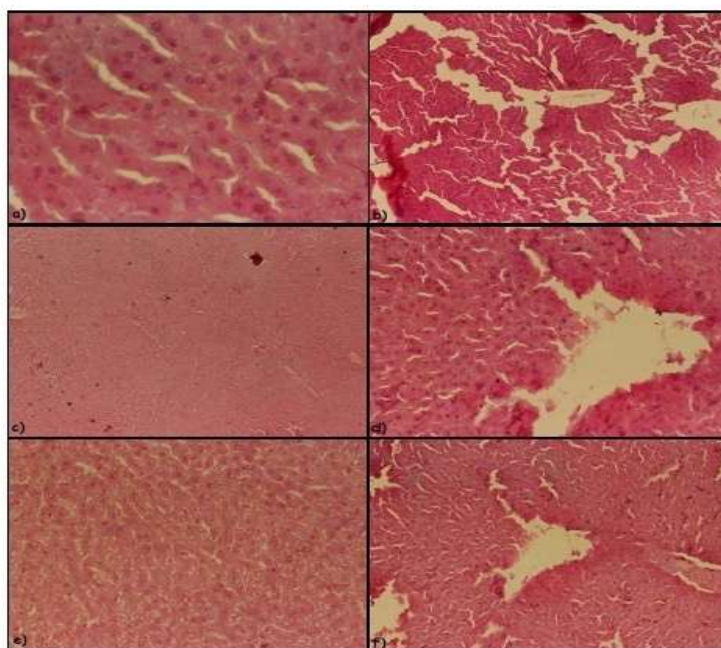
Histopathological observation

Gross morphology of the organs and histology of the liver from control and treated rats was studied. Necrosis was found to be considerably low in the group of rats pre-treated with the extracts of *Swertia tetragona* **(Figure 1).** Control group showed normal lobular architecture, while the necrotic liver tissue showed areas of necrosis and haemorrage. The cells were devoid of morphology.

Biochemical investigations

Serum amino transferases (ALT, AST), alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (GGT) activities were measured to assess the liver damage induced by thiaocetamide. As evident from **(Table 3)**, administration of thioacetamide caused significant increase in these enzyme levels, which were decreased by *Swertia tetragona* 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 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. XO activity increased significantly in TAA, and decreased in extract pre-treated groups **(Table 4)**.

Glutathione reductase , glutathione peroxidase, and catalase were decreased by thioacetamide, and normalized by the extracts**(Table 5)**. Results of the analysis of G6PD and SOD (also decreased in TAA groups and normalized by extracts) are shown in **(Table 6).**

Figure 1: 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) of *Swertia tetragona* showed congested vessels.

V.   Discussion

Hepatic injury was induced by intra-peritoneal injection of thioacetamide, which is known to cause hepatotoxicity in experimental rats and can produce hepatic necrosis. [31] Thioacetamide induced hepatic injury is an experimental model widely used in hepatoprotective drug screening. This study shows, pre-treatment of *Swertia tetragona* 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, (devoid of liver necrotizing 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.[32]

Morphologically there were no apparent pharmacological changes in the livers of rats treated with *Swertia tetragona*; neither any other organ (heart, kidneys, and spleen) showed any apparent change. However, the stomachs of the *Swertia* treated animals showed bulginess, which could be due to the laxative action of the extracts. 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.[33] The rise in serum levels of AST, ALT, ALP and GGT has been attributed to the damaged structural integrity of liver,[34] because these enzymes are cytoplasmic in location and are released into the circulation after cellular damage.[35] 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 i n 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.[36]

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.[37] 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 demonstrates that *S.tetragona* extracts 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 extract. 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.[38] Data is there to show a progressive reduction in the activity level of lipid peroxidation and elevated level of reduced glutathione in necrotic animals [39] In this study, we reported similar changes in the model, pre-treatment of herbal extracts reversed this effect.

To evaluate the involvement of oxygen radicals in hepatic damage and potential defense of herbal extracts, we measured activity of xanthine oxidase. In this study, TAA induced necrosis provoked 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.[40]

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 extracts 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.[41] Over-production of ROS normally induces oxidative stress unless it is 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 are 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.[42] A significant decline in the level of liver superoxide dismutasein 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. This is in accordance with our earlier work on *Swertia petiolata*, which showed almost similar results. [43]

VI.   Conclusion

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

In nut shell the present study describes the usefulness of *Swertia tetragona*, which has a great impact with regard to multidirectional pharmacological applications in indigenous systems of medicine. Pharmacological and biochemical results are very promising, which call for more-systematic research of these plant and its active principles.

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Table 1: The effect of *Swertia tetragona* extracts on the body weight of experimental rat.

Group	Body weight
NS	**242.50 ± 4.78**
TAA	222.50 ± 2.50
S.T (H/A)	250.00 ± 2.04
S.T (Aq)	241.25 ± 3.14
S.T (H/A) + TAA	240.00 ± 3.53*
S.T (Aq) + TAA	241.25 ± 2.39*

Data represents mean ± S.E.M (n=6). Groups that received TAA alone show a significant decrease in the body weights. *p <0.05 statistically significant NS: group receiving normal saline and serving as normal control group; TAA: necrotic group received thioacetamide; S.T (H/A) & (Aq): Swertia tetragona hydro-alcoholic and aqueous extracts; S.T + TAA: Swertia tetragona extract plus thioacetamide.

Groups	Liver (in gm)	Heart (in gm)	Kidney (in gm)	Spleen (in gm)	Stomach (in gm)
NS	8.03 ± 0.19	0.75 ± 0.01	0.71 ± 0.01	0.74 ± 0.01	4.00 ± 0.57
TAA	7.15 ± 0.20	0.77 ± 0.01	0.81 ± 0.01	0.77 ± 0.01	2.81 ± 0.02
S.T (H/A)	7.30 ± 0.11	0.73 ± 0.01	0.73 ± 0.01	0.74 ± 0.02	4.74 ± 0.09
S.T (Aq)	7.40 ± 0.15	0.74 ± 0.01	0.76 ± 0.02	0.72 ± 0.02	4.52 ± 0.22
S.T (H/A) + TAA	7.43 ± 0.16	0.73 ± 0.01	0.75 ± 0.02*	0.76 ± 0.02	5.74 ± 0.17*
S.T (Aq) + TAA	7.66 ± 0.15*	0.72 ± 0.01	0.75 ± 0.02*	0.75 ± 0.03	5.51 ±0.20*

Data represents mean ± S.E.M (n=6). Groups that received herb extracts + TAA Show a significant increase in the stomach weights, a significant decrease in kidney weights of herb+TAA groups as compared to necrotic group. There was no significant change in the average weights of other vital organs. *p <0.05= statistically significant NS: group receiving normal saline and serving as normal control group; TAA: necrotic group received thioacetamide; S.T (H/A) & (Aq): Swertia tetragona hydro-alcoholic and aqueous extracts; S.T + TAA: Swertia tetragona extract plus thioacetamide

Table 3: The effect of Swertia tetragona 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.T (H/A)	79.33 ± 1.02	32.67 ± 0.795	19.11 ± 0.51	311.63 ± 24.36
S.T (Aq)	79.26 ± 0.81	32.81 ± 0.26	19.05 ± 0.24	311.89 ± 23.79
S.T (H/A)+TAA	85.00 ± 0.42*	36.35 ± 0.82*	21.81 ± 0.43*	338.23 ± 23.16
S.T (Aq)+TAA	85.94 ± 1.47*	36.37 ± 0.26*	21.51 ± 0.16*	338.17 ± 7.64*

Data represents mean ± S.E.M (n=6). *p <0.05 = 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.T (H/A) & (Aq): Swertia tetragona hydro-alcoholic and aqueous extracts; S.T + TAA: Swertia tetragona extracts plus thioacetamide. ALT, Units/ml; AST, Units/ml; ALP, Eq. units/ml; GGT, nmole p-nitroanilide/mg protein.

Table 4: Showing the effect of *SwertiaTetragona* extracts on oxidative stress parameter.

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.T (H/A)	169.45 ± 14.59	113.60 ± 5.51	184.88 ± 5.95
S.T (Aq)	168.97 ± 0.81	168.97 ± 0.81	186.99 ± 4.73
S.T (H/A) + TAA	220.97 ± 15.52**	100.37 ± 2.11*	191.53 ± 5.13***
S.T (Aq) + TAA	221.38 ± 7.35**	101.47 ± 1.80*	192.55 ± 5.36***

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.T (H/A) & (Aq): Swertia tetragona hydro-alcoholic and aqueous extracts; S.T + TAA: Swertia tetragona extracts plus thioacetamide. LPO, nmole of MDA/mg protein; GSH, µmole of GSH/g tissue; XO, µmole of uric acid/mg protein

Table 5: showing the effect of *Swertiatetragona* extracts on antioxidant enzyme

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.T (H/A)	71.76 ± 4.65	39.65 ± 1.15	4542.18 ± 211.03
S.T (Aq)	71.05 ± 2.82	39.58 ± 1.55	4672.07 ± 310.78
S.T (H/A) + TAA	64.46 ± 5.15**	32.94 ± 3.12**	3876.03 ± 163.28**
S.T (Aq) + TAA	64.32 ± 2.87***	32.44 ± 1.13**	3849.79 ± 107.05**

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.T (H/A) & (Aq): Swertia tetragona hydro-alcoholic and aqueous extracts; S.T + TAA: Swertia tetragona 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 6: showing the effect of *Swertia tetragona* 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.T (H/A)	11.83±0.74	2.23±0.085
S.T (Aq)	11.52±0.615	2.29±0.05
S.T (H/A) + TAA	9.79±0.66*	1.89±0.025**
S.T (Aq) + TAA	9.84±0.78*	1.88±0.055*

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.T (H/A) & (Aq): Swertia tetragona hydro-alcoholic and aqueous extracts; S.T + TAA: Swertia tetragona extracts plus thioacetamide. G6PD: n mole of NADP reduced/min/mg protein; SOD, units/mg protein

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