HEPATOPROTECTIVE AND ANTIOXIDANT EFFECTS OF TOTAL EXTRACTS AND STEREOIDAL SAPONINS OF *SOLANUM XANTHOCARPUM* AND *SOLANUM NIGRUM* IN PARACETAMOL INDUCED HEPATOTOXICITY IN RATS

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Summary

The hepatoprotective and antioxidant effects of total extracts and steroidal saponins of *Solanum xanthocarpum* (Sx) and *Solanum nigrum* (Sn) on paracetamol induced hepatotoxicity and the possible mechanism involved therein were investigated in rats. The total extracts and steroidal saponins of Sx and Sn at two dose levels (100 and 200 mg/kg, p.o.) and a standard hepatoprotective herbal drug silymarin (25mg/kg b.w/day p.o. for 7 days) were administered to the paracetamol (500mg/kg p.o x 7days) intoxicated rats. The degree of protection was measured by using biochemical parameters, such as, serum transaminase (SGOT and SGPT), alkaline phosphatase (ALP), total bilirubin and total protein estimation. Further, the effects of total extracts and steroidal saponins of Sx and Sn on lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) status were estimated. The total extracts and steroidal saponins of Sx and Sn at the above doses produced significant hepatoprotective effects by attenuating the activity of serum enzymes, bilirubin and lipid peroxidation, while they significantly increased the levels of GSH, SOD and CAT in a dose dependent manner. The effects of the total extracts and steroidal saponins of Sx and Sn were comparable to those of the standard drug, silymarin. The antioxidant effects of the compounds may be responsible, at least partly, for the beneficial effects. These compounds have provided remedial measures against the deleterious effects of toxic metabolites of paracetamol. The hepatoprotective and antioxidant effects of steroidal saponins of Sx and Sn were found to be greater than those of the total extracts.

Keywords: *Solanum xanthocarpum, S. nigrum*, steroidal saponins, Hepatoprotective effect, Antioxidants, Paracetamol, Silymarin

Introduction

Liver is the most important organ in maintaining the homeostasis of the body. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction. It plays a pivotal role in regulating metabolism of endogenous as well as xenobiotic substances [1], secretion and storage. Liver has the great capacity to detoxicate toxic substances and synthesize useful chemical principles. Therefore, damage to the liver infected by hepatotoxic agents is of great concern [2, 3]. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders [4, 5]. Most of the hepatotoxic chemicals damage liver cells primarily by producing reactive species which form covalent bond with the lipids of the tissue. However inbuilt protective mechanisms combat the hazardous reactions associated with the free radicals. Due to excessive exposure to hazardous chemicals, sometimes the free radicals generated are so high that they overpower the natural defensive system leading to hepatic damage and cause jaundice, cirrhosis and fatty liver [6]. Production of the reactive species manifests in tissue thiol depletion, lipid peroxidation, plasma membrane damage etc., culminating into severe hepatic injury [7].

Paracetamol is a well known antipyretic and analgesic agent. A number of reports indicate that overdose of paracetamol can produce centrizonal hemorrhagic hepatic necrosis in humans and experimental animals [8, 9]. Paracetamol toxicity is caused by the reaction metabolite N-acetyl-*p*-benzoquinoneimine (NAPQI), which is partly metabolized by cytochrome P-450 [10]. This species causes severe oxidative damage and glutathione depletion leading to liver necrosis. Introduction of cytochrome or depletion of hepatic glutathione is a prerequisite for paracetamol-induced hepatotoxicity [11, 12].

In spite of major advances in modern medicine, there are no potent drugs with sustainable effects that stimulate liver functions, protect liver damage or help to regenerate hepatic cells [13]. In absence of consistent liver-protective drugs in modern medicine, there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders over the years [14].

An ideal approach for the treatment of hepatotoxicity should not preclude the antioxidant potential of the drug, as noxious free radicals generated from hepatotoxic agents cause irreparable damage to hepatic cells. Both *Solanum xanthocarpum* and *S. nigrum* are known for their antioxidant activities [15]. *S. nigrum* fruit extracts are reported to have hepatoprotective activity against CCL_4 induced hepatic damage [16]. The hepatoprotective and antioxidant effects of total extracts as well as of one of the most important group of compounds of both the plants, viz. steroidal saponins, against paracetamol induced hepatotoxicity constitute the subject of this study.

Materials and Methods

Chemistry:

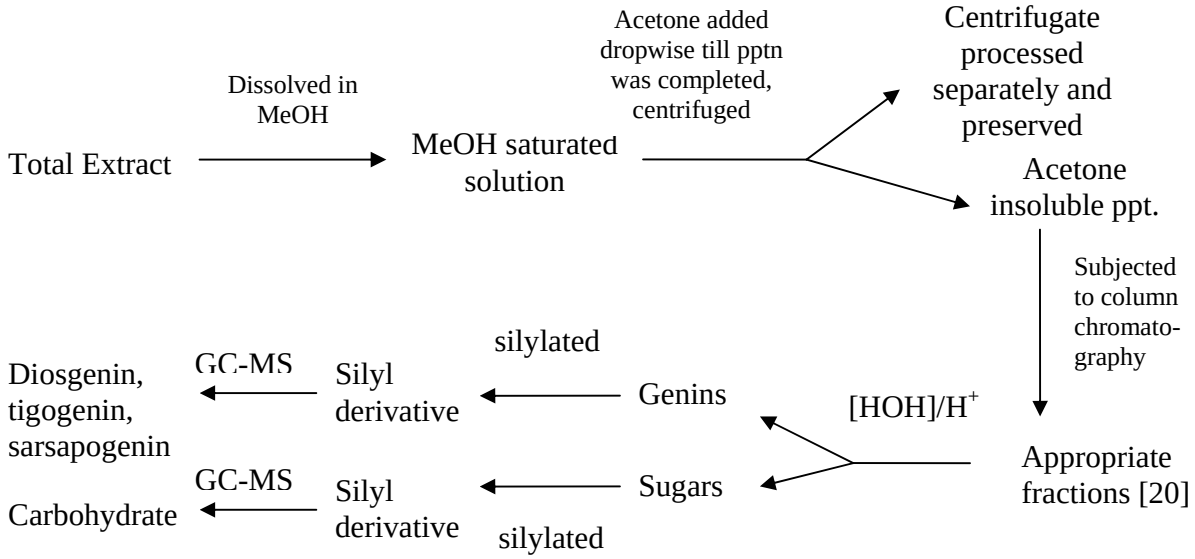
Test samples- Authenticated plant materials of *S. xanthocarpum* and *S. nigrum*, cultivated in the Western Himalayas, were obtained from Indian Herbs Ltd, Saharanpur (U.P.). Each specimen, of the two samples, has been preserved in our file as reference.

Extraction *S. xanthocarpum* (Sx) and *S. nigrum* (Sn):

Dried and powdered whole plant material of each species was separately extracted with aqueos-methanol (40-60) at a temperature of 70°C for 4 hrs. The solvent was evaporated under reduced pressure and the dried and powdered extractives, thus obtained, were used in the animal study.

Extraction and isolation of spirosta-steroidal saponins of *S. xanthocarpum* and S. nigrum:

Dried and powdered whole plant of Sx and Sn were continuously (Soxhlet) extracted (6h) with aqueous-methanol (40:60). The solvent was evaporated under reduced pressure. The total extractives were subjected to solvent-gradient separation followed by comprehensive column chromatographic and spectroscopic analyses, using markers, where possible. In a typical experiment the major bioactive fraction(s), viz. the spirosta-steroidal saponins were processed as follows (Scheme-I).



Scheme-1: A general method of isolation of spirosta-saponins and component moieties thereof.

Drugs and chemicals- 1-Chloro-2, 4-dinitrobenzene [CDNB], Bo-vine serum albumin (Sigma chemical St. Louis, MO, USA), Thiobarbituric acid, Nitroblue tetrazolium chloride (NBT) (Loba Chemie, Bombay, India), 5,5'-dithio *bis*-2-nitrobenzoic acid (DTNB). Silymarin was purchased from Ranbaxy Laboratories, Indore, used as standard drug; paracetamol was purchased from La-Chemico Pvt. Ltd, Kolkata. The solvents and/or reagents obtained were of analytical grade and procured from Merck-India.

Pharmacology:

Animals- Albino rats (Sprague Dawley strain) of either sex, 3-4 months old and weighing around 180 to 240 gm, procured from Central Research Institute (Ayurveda), Govt. of India, Salt Lake City, Kolkata, were used. The animals were housed in an animal room with alternating light-dark cycle of 12 hr each. The animals were acclimatized for at least 7 days to the laboratory conditions before conducting experiments. Experiments were carried out between 0900 h and 1700 h. The study was conducted in accordance with Good Laboratory Practice (GLP) Regulations of WHO (WHO Document, 1998). The "Principles of laboratory animal care" (NIH Publication # 85-23, 1985) were also followed in the study. The 'Institutional Animal Ethics Committee' (IAEC) approved the experimental protocol.

Experimental protocols:

Animals were divided into 11 groups and each group comprised of 6 rats.

Gr-1: NC- Normal Control: Treated with vehicle only (5ml/kg b. wt normal saline).
Gr-2: PC-Paracetamol Control: The animals received paracetamol (500 mg/kg p.o) once daily for 7 days and then treated with vehicle only
Gr-3: Paracetamol + Sx-100: The animals received paracetamol (500 mg/kg p.o) once daily + treated with Sx (100mg/kg, p.o.) conjointly for 7 days.
Gr-4: Paracetamol + Sx-200: The animals received paracetamol (500 mg/kg p.o) once daily + treated with Sx (200mg/kg, p.o.) conjointly for 7 days.
Gr-5: Paracetamol + Sn-100: The animals received paracetamol (500 mg/kg p.o) once daily + treated with Sn (100mg/kg, p.o.) conjointly for 7 days.
Gr-6: Paracetamol + Sn-200: The animals were received paracetamol (500 mg/kg p.o) once daily + treated with Sn (200mg/kg, p.o.) conjointly for 7 days.
Gr-7: Paracetamol + Sx/saponin-100: The animals received paracetamol (500 mg/kg p.o) once + treated with Sx/saponin (100mg/kg, p.o.) conjointly for 7 days.
Gr-8: Paracetamol + Sx/saponin-200: The animals received paracetamol (500 mg/kg p.o) once daily + treated with Sx/saponin (200mg/kg, p.o.) conjointly for 7 days.
Gr-9: Paracetamol + Sn/saponin-100: The animals received paracetamol (500 mg/kg p.o) once daily + treated with Sn/saponin (100mg/kg, p.o.) conjointly for 7 days.
Gr-10: Paracetamol + Sn-200/saponin: The animals received paracetamol (500 mg/kg p.o) once daily + treated with Sn/saponin (200mg/kg, p.o.) conjointly for 7 days.
Gr-11: Paracetamol +Silymarin: The animals received paracetamol (500 mg/kg p.o) once daily+ Silymarin (25 mg/kg p.o.) conjointly for 7 days.

The bio-chemical parameters were determined after 18 hr fasting after administration of the last dose of treatment.

Biochemical studies

Blood was obtained from all animals by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and utilized for the estimation of various bio-chemical parameters namely SGPT, SGOT [17], SALP [18], serum bilirubin [19] and protein content [20] was measured.

After collection of blood samples the rats were sacrificed and their livers excised, rinsed in ice cold normal saline followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10 % w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation [21]. A part of homogenate after precipitating proteins with trichloroacetic acid (TCA) was used for estimation of glutathione [22]. The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of SOD [23] and CAT [24] activities.

Lipid peroxidation

The tissues were homogenized in 0.1 M buffer (pH 7.4) with a Teflon-glass homogenizer. Lipid peroxidation in this homogenate was determined by measuring the amounts of malondialdehyde (MDA) produced primarily, according to a published procedure [21]. 0.2 ml of tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml of 8% TBA were added. The volume of the mixture was made up to 4 ml with distilled water and then heated at 95°C on a water bath for 60 min using glass balls as condenser. After incubation the tubes were cooled to room temperature and final volume was made to 5 ml in each tube. 5.0 ml of butanol:pyridine (15:1) mixture was added and the contents were vortexed thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min, the upper organic layer was taken and its OD read at 532 nm against an appropriate blank without the sample. The levels of lipid peroxides were expressed as nM MDA/mg Protein using an extinction coefficient of $1.56 \times 105\ M^{-1}cm^{-1}$.

Estimation of Reduced Glutathione (GSH)

To measure the reduced glutathione (GSH) level, the tissue homogenate (in 0.1 M phosphate buffer pH 7.4) was taken and processed according to a published procedure [22]. The homogenate was added with equal volume of 20% trichloroacetic acid (TBA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. The supernatant (200 µl) was then transferred to a new set of test tubes and added 1.8 ml of the Ellman's reagent (5, 5'-dithio bis-2-nitrobenzoic acid) (0.1mM) was prepared in 0.3M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes make upto the volume of 2ml. After completion of the total reaction, solutions were measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from standard curve from known GSH.

Estimation of Superoxide Dismutase (SOD)

SOD activity of the liver tissue was analyzed by a published procedure [23]. Assay mixture contained 0.1 ml of sample, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml phenazine methosulphate (186 µM), 0.3 ml of 300 µM nitroblue tetrazolium, 0.2 ml NADH (750 µM). Reaction was started by addition of NADH. After incubation at 30°C for 90 s, the reaction was stopped by the addition of 0.1 ml glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 ml of n-butanol. Mixture was allowed to stand for 10 min, centrifuged and butanol layer was separated.

Color intensity of the chromogen in the butanol layer was measured at 560 nm spectrophotometrically and concentration of SOD was expressed as units/mg protein.

Estimation of Catalase (CAT)

Catalase activity was measured by a published procedure [24]. 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 ml of freshly prepared 30 mM H_2O_2. The rate of decomposition of H2O2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein.

Statistical analysis- Statistical analysis was carried out using Prism software ver.4.0 statistical software (Graph pad software Inc). All the results were expressed as Mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA followed by Dunnett's test. In the entire test, the criterion for statistical significance was $p<0.05$.

Results

Effect of Sx, Sn whole plant extracts and steroidal saponins thereof on hepatic parameters in paracetamol intoxicated rats

The hepatoprotective effects of the total extracts and steroidal saponins of Sx and Sn were determined. Total extracts of both Sx and Sn (100 and 200 mg/kg, p.o. for 7 days), showed significant dose dependent attenuation of elevated levels of SGOT, SGPT, alkaline phosphatase, and total bilirubin contents with a concomitant increase in total protein content. The adverse effects were due to paracetamol induced hepatotoxicity. The steroidal saponins (100 and 200 mg/kg, p.o., for 7 days) of both the plants also showed significant dose dependent beneficial activity. The results were compared with those of a standard hepatoprotective drug, silymarin (25 mg/kg b.w.). The results are incorporated in Table 1.

Antioxidant effect of Sx, Sn whole plant extracts and steroidal saponins thereof on the liver of paracetamol intoxicated rats

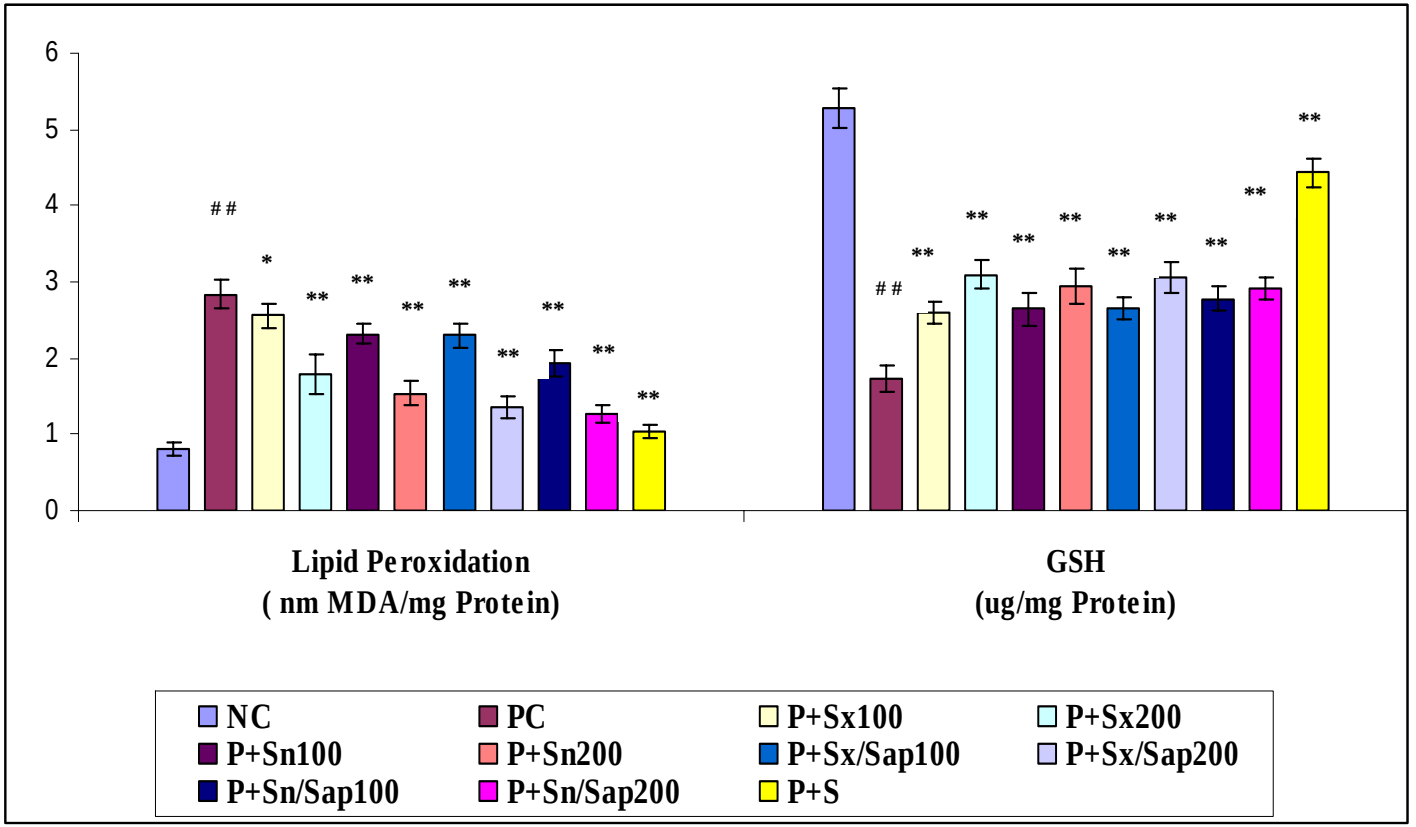
The findings of antioxidant effects of total extracts and of steroidal saponins of Sx and Sn on rat liver tissue, lipid peroxidation, glutathione, and antioxidant enzyme levels, such as SOD and CAT, were incorporated in Figures 1 and 2. Lipid peroxidation level (expressed in term of malonedialdehyde (MDA) formation) was significantly increased in the paracetamol- control intoxicated rats when compared with the normal rats. Treatment with total extracts and steroidal saponins of Sx and Sn at the doses of 100mg and 200mg/kg significantly inhibited the increase in MDA levels. Glutathione, SOD and CAT levels were significantly and dose dependently increased in the test compound treated groups. The effects of total extracts and of steroidal saponins of Sx and Sn were compared with those of a standard drug, silymarin. The results are incorporated in Figures 1 and 2.

Table 1: Effect of Sx, Sn whole plant extracts and steroidal saponins thereof on hepatic parameters in paracetamol intoxicated rats

Groups	Hepatic Parameters					
	Liver Weight (gm/ 100 g b.w)	**SGOT (U/L)**	**SGPT (U/L)**	**ALP (U/L)**	**Total Bilirubin**	**Total Protein (mg/dl)**
Gr-1	3.31±0.24	53.92±3.60	44.43±3.04	27.75±4.26	0.53±0.04	7.09±0.30
Gr-2	6.27±0.36$^{\#\#}$	117.9±6.65$^{\#\#}$	96.60±7.10$^{\#\#}$	61.75±3.65$^{\#\#}$	2.15±0.09$^{\#\#}$	5.51±0.29$^{\#\#}$
Gr-3	4.99±0.73**	109.6±6.68*	89.09±6.89	55.81±4.41*	2.02±0.09	6.02±0.18**
Gr-4	4.09±0.15**	91.75±2.73**	64.17±5.56**	43.55±2.82**	1.39±0.12**	6.53±0.20**
Gr-5	5.09±0.60*	108.9±5.16*	78.03±4.27**	48.95±2.62**	1.54±0.15**	6.23±0.18**
Gr-6	4.16±0.37**	81.91±1.63**	60.65±2.96**	41.46±2.78**	1.16±0.09**	6.86±0.12**
Gr-7	4.89±0.48**	108.8±4.67*	88.08±8.63*	52.91±2.67**	1.91±0.15**	6.28±0.40**
Gr-8	4.02±0.26**	76.39±3.96**	64.14±3.56**	43.20±3.77**	1.37±0.08**	6.79±0.15**
Gr-9	4.87±0.56**	95.62±5.25**	73.55±4.22**	46.89±3.87**	1.42±0.10**	6.51±0.19**
Gr10	3.87±0.21**	67.41±4.68**	55.26±3.71**	38.36±2.27**	0.94±0.08**	6.92±0.16**
Gr-11	3.49±0.20**	59.09±3.39**	49.18±1.65**	31.16±3.72**	0.69±0.06**	7.16±0.13**

Data represented as Mean ± SD; for 6 rats.
$^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; in comparison to Group 1 (normal control) rats treated with vehicle.
$*p<0.05$; $**p<0.01$; $***p<0.001$; in comparison to Group 2 (negative control) rats paracetamol induced hepatotoxic and treated with vehicle.

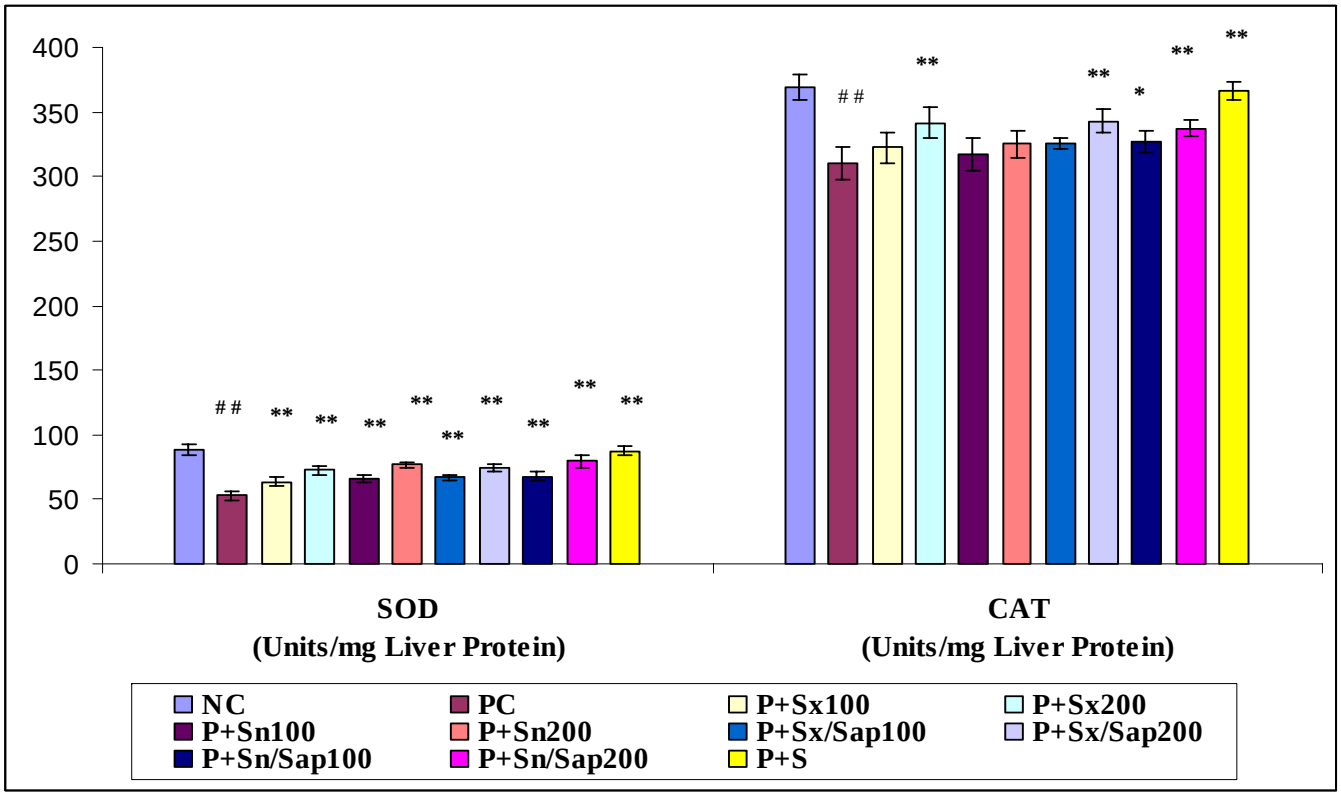
Figure-1. Effect of Sx, Sn whole plant extracts and steroidal saponins thereof on lipid peroxidation and GSH content in paracetamol intoxicated rats



$^{\#}p<0.05$; $^{\#\#}$ $p<0.01$; $^{\#\#\#}$ $p<0.001$; in comparison to Group 1 (normal control) with Group 2 (paracetamol control) treated with vehicle.

$^{*}p<0.05$; ** $p<0.01$; *** $p<0.001$; in comparison to Group 2 (paracetamol control)

Figure-2. Effect of Sx, Sn whole plant extracts and steroidal saponins thereof on SOD and CAT in paracetamol intoxicated rats



$^{\#}p$<0.05; $^{\#\#}$ p <0.01; $^{\#\#\#}$ p *<0.001;* in comparison to Group 1 (normal control) with Group 2 (paracetamol control) treated with vehicle.

*p<0.05; ** p <0.01; *** p *<0.001;* in comparison to Group 2 (paracetamol control)

Discussion

The hepatotoxicity of paracetamol has been attributed to the formation of toxic metabolites when a part of paracetamol is activated by hepatic cytochrome P-450 [10] to highly reactive noxious metabolite, N-acetyl –*p*-benzoquinoneimine (NAPQI) [25]. NAPQI is initially detoxified by reduced glutathione (GSH) to form mercapturic acid. However, when the rate of NAPQI formation exceeds the rate of detoxification by GSH, it oxidizes tissue macromolecules such as lipid or –SH group of protein and alters the calcium homeostasis after depleting GSH.

Paracetamol intoxication elevates the levels of SGOT, SGPT, ALP and total bilirubin, which indicates hepatotoxicity. High levels of SGOT indicate liver damage, such as that due to viral hepatitis as well as cardiac infarction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury [26]. Serum ALP and bilirubin are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure [27]. In the present study, it was found that total extracts as well as steroidal saponins of Sx and Sn significantly and dose dependently improved the abovementioned adverse hepatic biochemical conditions in paracetamol induced hepatotoxic rats. These findings suggested the hepatoprotective effects of total extracts and steroidal saponins of Sx and Sn. This was further substantiated by the improvement of total protein content of serum and of liver weights by the test compounds despite of paracetamol treatment in rats. The hepatoprotective effects of steroidal saponins were even better than those of the total extracts of both Sx and Sn.

Lipid peroxidation has been postulated to be the destructive process of liver injury due to paracetamol administration [28]. In the present study, elevations in the levels of end products of lipid peroxidation in liver of rats, treated with paracetamol, were observed. The increase in MDA level in liver suggested enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with total extracts as well as steroidal saponins of Sx and Sn significantly and dose dependently reversed these changes. Hence, it may be postulated that, the hepatoprotective action of the test samples was due to their antioxidant effect. Here also, the hepatoprotective effects of steroidal saponins were found to be better than those of the total extracts of both Sx and Sn.

SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide [30, 31]. In the present study, it was observed that, total extracts as well as steroidal saponins of Sx and Sn caused a significant and dose dependent increase in the hepatic SOD activity of the paracetamol intoxicated rats.

Catalase is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red cells and in liver. Catalase decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals [32]. In the present study, it was observed that, total extracts as well as steroidal saponins of Sx and Sn caused a significant and dose dependent increase in the hepatic catalase activity of the paracetamol intoxicated rats.

Glutathione is one of the most abundant tripeptide, a non-enzymatic biological antioxidant present in the liver. Its functions are concerned with the removal of free radical species and maintenance of thiol proteins and as a substrate for glutathione Peroxidase and GST [29]. In the present study, the decreased level of GSH was associated with enhanced lipid peroxidation in paracetamol treated rats. Administration of total extracts as well as steroidal saponins of Sx and Sn significantly and dose dependently increased the level of glutathione.

In conclusion, the total extracts as well as the steroidal saponins of both Sx and Sn, exhibited potent hepatoprotective and antioxidant effects in paracetamol induced hepatotoxic rats. Since the steroidal saponins were found to be more active agents than the total extracts, these compounds are projected as the major bioactives of Sx and Sn.

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