

HEPATOPROTECTIVE AND ANTIOXIDANT EFFECTS OF NATURALLY OCCURRING WITHASTEROID METAL ION CONJUGATES OF *WITHANIA SOMNIFERA* IN PARACETAMOL INDUCED HEPATOTOXICITY IN RATS

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Summary

The role of metal ions when in conjugation with the withasteroids of *Withania somnifera* [WS], in intervening paracetamol induced hepatotoxicity in rats was investigated. The resultant hepatoprotective and antioxidant effects of a standardized *Withania somnifera* extract (WSE), comprising of a number of conjugated metal ions, and the possible mechanism involved therein was investigated. WSE (100mg/kg, p.o. x 7 days) and a standard hepatoprotective herbal drug silymarin (25mg/kg, p.o. x 7 days) were administered conjointly with the paracetamol (500mg/kg, p.o. x 7days) treatment to rats. The degree of reversed of toxicity by the test compound was measured by determining the weight of the liver and hepatic parameters, such as, serum transaminase (SGOT and SGPT), alkaline phosphatase (ALP), total bilirubin and total protein levels. Further, the effects of WSE on lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase activity (CAT) were determined. WSE (100mg/kg, p.o. x 7 days) produced significant hepatoprotective effect by preventing the increase in weight of the liver, serum enzymes, bilirubin and lipid peroxidation while it significantly arrested the decrease in levels of GSH, SOD and CAT. WSE/excluded-I, (100mg/kg, p.o. x 7 days), comprising of metal ions strongly conjugated with withasteroids and oligosaccharides, exhibited trends of further improvement, compared to those of WSE in these parameters. In contrast, metal ions-depleted bioactives of WSE, viz., WSE/excluded-II, (100mg/kg, p.o. x 7 days) showed only marginal effects. These bioactivities were more pronounced at higher dose levels (200 mg/kg, p.o. x 7 days) of the two extracts, WSE and WSE/excluded-I, indicating dose-dependant relationships which were comparable to those of the standard drug, silymarin. The antioxidant effects of the metal ion-conjugated withasteroids may be responsible, at least partly, for the beneficial effects that provided remedial measures against the deleterious effects of toxic metabolites of paracetamol.

Keywords: *Withania somnifera*, Withasteroid-metal ion conjugates, Hepatoprotection, Antioxidants, Paracetamol, Silymarin.

Introduction

Liver is a very important organ in maintaining homeostasis of the body. It is involved in almost all the biochemical pathways related to growth, to 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 centrilobular 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. Glycowithanolides of *Withania somnifera* have been reported to induce significant antioxidant effect in the rat brain frontal cortex and striatum, by increasing the activity of the oxidative free radical scavenging enzymes. [15]. Metal ions in conjugation with withasteroids have a profound bearing on the antioxidant activity of *Withania somnifera* [16]. The glycowithanolides of *Withania somnifera* are reported to have hepatoprotective activity on iron-induced hepatotoxicity in rats [17]. The hepatoprotective and antioxidant effect of *Withania somnifera* extract (WSE) (containing mixtures of native withasteroid-metal ion conjugates) and metal ion-depleted corresponding withasteroids were evaluated against paracetamol induced hepatotoxicity in rats.

Materials and Methods

Chemistry:

Test samples- Authenticated plant material of WS, cultivated in the Western Himalayas, was obtained from Indian Herbs Ltd, Saharanpur (U.P.). A specimen of the sample has been preserved in our file for further reference.

Extraction of WS- Dried and powdered plant material (root and leaf) of WS was hot extracted with water for 2 hr. The extract was concentrated under reduced pressure and then spray dried. The yield of total extractives was ca. 8% (w/w). The different categories of bioactive constituents present in WSE were determined by HPLC and HPTLC, and the undesirable constituents were removed as before [18].

Separation of differently conjugated metal ions from WSE- In a typical experiment, WSE was dissolved in a minimum volume of water and passed, successively, through a weak (Amberlite IRC-86 H⁺) and a strong cation exchange resin (Dowex-50 H⁺) column. The excluded fractions (WSE/excluded-I&II respectively) were evaporated under reduced pressure and the residue was analysed for metal ions by spectrophotometric, titrimetric and/or AAS methods.

The retarded fractions (WSE/retarded-I&II obtained from weak and strong cation exchange resin columns, respectively), comprising the constituents remained in the resin columns, were eluted with 2N-HCl and each acidic solution was evaporated under reduced pressure. The residues were analysed for metal ions.

Drugs and chemicals- 1-Chloro-2, 4-dinitrobenzene [CDNB], bovine 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.

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 9 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.

Gr-3: Paracetamol + WSE-100: The animals received paracetamol (500 mg/kg p.o.) once daily + treated with WSE (100mg/kg, p.o.) conjointly for 7 days.

Gr-4: Paracetamol + WSE-200: The animals received paracetamol (500 mg/kg p.o.) once daily + treated with WSE (200mg/kg, p.o.) conjointly for 7 days.

Gr-5: Paracetamol + WSE/excluded/I-100: The animals received paracetamol (500 mg/kg p.o.) once daily + treated with WSE/excluded/I (100mg/kg, p.o.) conjointly for 7 days.

Gr-6: Paracetamol + WSE/excluded/I -200: The animals received paracetamol (500 mg/kg p.o.) once daily + treated with WSE/excluded/I (200mg/kg, p.o.) conjointly for 7 days.

Gr-7: Paracetamol + WSE/excluded/II-100: The animals received paracetamol (500 mg/kg p.o.) once + treated with WSE/excluded/II (100mg/kg, p.o.) conjointly for 7 days.

Gr-8: Paracetamol + WSE/excluded/II-200: The animals received paracetamol (500 mg/kg p.o.) once + treated with WSE/excluded/II (100mg/kg, p.o.) conjointly for 7 days.

Gr-9: 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 h-fasting after administration of the last dose of treatment.

Biochemical studies

Blood was obtained from all animals from heart after mild anesthesia with diethyl ether. 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 [19], SALP [20], serum bilirubin [21] and protein content [22] 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 [23]. A part of homogenate after precipitating proteins with trichloroacetic acid (TCA) was used for estimation of glutathione [24]. 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 [25] and CAT [26] 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 [23]. 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.

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 [24]. 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 μ l) 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 the standard curve from marker GSH.

Estimation of Superoxide Dismutase (SOD)

SOD activity of the liver tissue was analyzed [25]. 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 μ M), 0.3 ml of 300 μ M nitroblue tetrazolium, 0.2 ml NADH (750 μ M). 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 [26]. 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 H₂O₂. 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 (Graphpad software Inc). All the results were expressed as Mean \pm 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

The differently conjugated metal ions present in WSE were separated and estimated [27]. The abundance of the metal ions was found to be in the decreasing order of: Ca²⁺, Mg²⁺, Fe²⁺/Fe³⁺, Zn²⁺ and Cu²⁺.

Effects of WSE fractions on hepatic parameters in paracetamol intoxicated rats- The hepatoprotective effect of differently metal ions-conjugated compounds present in WSE was investigated. Administration of WSE and WSE/excluded-I (100 mg/kg, p.o.) to rats inhibited the augmentation in SGOT, SGPT, alkaline phosphatase, total bilirubin and caused concomitant decrease in total protein content due to paracetamol induced hepatotoxicity. However, in case of WSE/excluded-II fraction (depleted metal ions conjugation of withasteroids) the effect was marginal and statistically insignificant. The effects of WSE and WSE/excluded-I were compared with those of silymarin (25 mg/kg). The results are incorporated in Table 1.

Table 1: Effects of WSE fractions on hepatic parameters in paracetamol intoxicated rats

Groups	Liver weight and Hepatic Parameters					
	Liver Weight (gm/ 100 g b.w)	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Total Bilirubin	Total Protein (mg/dl)
Normal control	3.31±0.09	54.74±0.85	44.88±1.49	27.75±1.74	0.53±0.02	7.09±0.12
Paracetamol control	6.26±0.15 ^{##}	118.56±1.76 ^{##}	98.90±1.87 ^{##}	61.75±1.49 ^{##}	2.15±0.04 ^{##}	5.51±0.12 ^{##}
Paracetamol+WSE-100	5.71±0.13*	111.29±1.63*	81.47±1.47**	55.25±1.81*	1.82±0.05**	6.02±0.07**
Paracetamol+WSE/excluded/I-100	5.68±0.12*	110.76±2.10*	80.64±1.60**	54.23±1.99*	1.80±0.04**	5.92±0.08**
Paracetamol+WSE/excluded/II-100	5.92±0.16	117.16±1.38	92.58±1.22	60.36±2.14	1.97±0.05	5.67±0.12
Paracetamol+silymarin	3.49±0.08**	58.49±1.80**	50.21±1.29**	46.89±1.58**	0.66±0.04**	6.50±0.08**

Data represented as Mean ± SEM; for 6 rats.

[#] $p < 0.05$; ^{##} $p < 0.01$; in comparison to Group 1 (normal control) rats treated with vehicle.

* $p < 0.05$; ** $p < 0.01$; in comparison to Group 2 (negative control) rats paracetamol induced hepatotoxic and treated with vehicle.

WSE: Metal ions weakly and strongly conjugated with withasteroids and oligosaccharides.

WSE/excluded-I: Metal ions strongly conjugated with withasteroids and oligosaccharides.

WSE/excluded-II: Metal ions-depleted withasteroids and oligosaccharides.

Effects of WSE fractions at higher dose on hepatic parameters in paracetamol intoxicated rats- Administration of WSE and WSE/excluded-I at higher dose (200 mg/kg, p.o.) inhibited augmentation in SGOT, SGPT, alkaline phosphatase, total bilirubin and caused concomitant decrease in total protein content due to paracetamol induced hepatotoxicity, in a dose dependant manner. WSE/excluded-I fraction exhibited a better trend in effects, in these parameters, compared to those of WSE. The effects of WSE/excluded-II fraction at higher dose (200 mg/kg, p.o.) were less pronounced than WSE and WSE/excluded-I. The results are incorporated in Table 2.

Table 2: Effects of WSE fractions on hepatic parameters in paracetamol intoxicated rats at higher dose

Groups	Liver weight and Hepatic Parameters					
	Liver Weight (gm/ 100 g b.w)	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Total Bilirubin	Total Protein (mg/dl)
Paracetamol+WSE-200	4.08±0.06**	79.45±2.04**	68.39±2.78**	43.55±1.15**	1.39±0.05**	6.53±0.08**
Paracetamol+WSE/excluded/I-200	3.98±0.09**	79.50±1.90**	66.43±2.32**	42.87±1.27**	1.35±0.07**	6.57±0.09**
Paracetamol+WSE/excluded/II-200	5.77±0.12*	110.54±1.72*	90.56±1.06*	50.98±1.51**	1.88±0.04**	5.96±0.11**

Data represented as Mean ± SEM; for 6 rats.

$p < 0.05$; ## $p < 0.01$; in comparison to Group 1 (normal control) rats treated with vehicle.

* $p < 0.05$; ** $p < 0.01$; in comparison to Group 2 (negative control) rats paracetamol induced hepatotoxic and treated with vehicle.

Antioxidant effect of WSE fractions on the liver of paracetamol intoxicated rats- The results of antioxidant effects of WSE and WSE/excluded-I (100 mg/kg, p.o.) on rat liver tissue, lipid peroxidation, glutathione, and antioxidant enzyme levels, such as SOD and CAT, are incorporated in Table 4. 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 WSE and WSE/excluded-I (100 mg/kg, p.o.) significantly attenuated the levels of MDA, extent of decrease of glutathione, SOD and CAT. The effects of WSE and WSE/excluded-I (100 mg/kg, p.o.) were compared with those of a standard drug, silymarin. The results are incorporated in Table 3.

Table 3: Antioxidant effects of WSE fractions on liver functions in paracetamol intoxicated rats

Groups	Liver Antioxidant Parameters			
	Lipid Peroxidation (nm MDA/mg Protein)	GSH (µg/mg Protein)	SOD (Units/mg Liver Protein)	CAT (Units/mg Liver Protein)
Normal control	0.82±0.02	5.34±0.06	89.36±1.44	373.5±4.43
Paracetamol control	2.85±0.08 ^{##}	1.68±0.05 ^{##}	53.48±1.02 ^{##}	309.5±3.39 ^{##}
Paracetamol+WSE-100	2.29±0.07 ^{**}	2.67±0.06 ^{**}	63.60±1.62 ^{**}	331.4±2.88 [*]
Paracetamol+WSE/excluded/I-100	2.26±0.06 ^{**}	2.63±0.05 ^{**}	63.04±1.58 ^{**}	330.7±3.02 [*]
Paracetamol+WSE/excluded/II-100	2.62±0.05	1.86±0.03	57.05±0.83	316.6±1.15
Normal control	1.10±0.05 ^{**}	4.47±0.08 ^{**}	87.44±1.09 ^{**}	351.6±2.37 ^{**}

Data represented as Mean ± SEM; 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; in comparison to Group 2 (negative control) rats paracetamol induced hepatotoxic and treated with vehicle.

Antioxidant effect of WSE fractions on the liver of paracetamol intoxicated rats- Administration of WSE and WSE/excluded-I at higher dose (200 mg/kg, p.o.) attenuated the changes in antioxidant levels (MDA, SOD, CAT, GSH) occurred due to paracetamol induced hepatotoxicity, in a dose dependant manner. WSE/excluded-I fraction exhibited a better trend in effects, in these parameters, compared to those of WSE. Furthermore, the effects of WSE/excluded-II fraction at higher dose were less pronounced than WSE and WSE/excluded-I. The results are incorporated in Table 4.

Table 4: Antioxidant effects of WSE fractions on liver functions in paracetamol intoxicated rats at higher dose

Groups	Liver Antioxidant Parameters			
	Lipid Peroxidation (nm MDA/mg Protein)	GSH (μ g/mg Protein)	SOD (Units/mg Liver Protein)	CAT (Units/mg Liver Protein)
Paracetamol+WSE-200	1.53 \pm 0.07**	3.05 \pm 0.12**	72.43 \pm 1.04**	346.5 \pm 2.30**
Paracetamol+WSE/excluded/I- 200	1.46 \pm 0.05**	3.01 \pm 0.11**	71.64 \pm 0.87**	342.1 \pm 2.46**
Paracetamol+WSE/excluded/II- 200	2.17 \pm 0.06**	2.19 \pm 0.07**	66.48 \pm 0.73**	329.8 \pm 1.09**

Data represented as Mean \pm SEM; 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; in comparison to Group 2 (negative control) rats paracetamol induced hepatotoxic and treated with vehicle.

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) [28]. 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 [29]. 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 [30]. In the present study, it was found that WSE and WSE/excluded-I significantly improved all the abovementioned hepatic biochemical parameters in paracetamol induced hepatotoxic rats. These findings suggested the hepatoprotective effects of WSE and WSE/excluded-I. This was further substantiated by the improvement of total protein content of serum and liver weights of paracetamol intoxicated rats. The hepatoprotective effects of WSE/excluded-I were found to be marginally better than those of WSE. In contrast, WSE/excluded-II fraction, drastically depleted of conjugated metal ions, on similar treatment elicited only marginal effect indicating significant contribution of metal ions conjugation to withasteroids to confer different types of hepatoprotective effects.

Lipid peroxidation has been postulated to be the destructive process of liver injury due to paracetamol administration [31]. 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 WSE and WSE/excluded-I significantly attenuated the MDA level. Hence, it may be postulated that, the hepatoprotective action of the test compounds was due, at least partly, to their antioxidant effect. Here also, the hepatoprotective effects of WSE/excluded-I were found to be better than those of WSE. WSE/excluded-II (100mg/kg, p.o.), as predicted, did not produce any significant hepatoprotective effect.

SOD is an most important enzyme in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide [32, 33]. In the present study, it was observed that, WSE and WSE/excluded-I significantly inhibited the decrease in the hepatic SOD activity of the paracetamol intoxicated rats. The effect was marginal in case of WSE/excluded-II fraction.

Catalase is another 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 [34]. In the present study, it was observed that, WSE and WSE/excluded-I significantly

augmented the hepatic catalase activity of the paracetamol intoxicated rats. The effect was marginal in case of WSE/excluded-II fraction.

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 [35]. In the present study, the decreased level of GSH was associated with enhanced lipid peroxidation in paracetamol treated rats. Administration of WSE and WSE/excluded-I significantly inhibited the decrease in the level of glutathione. In case of WSE/excluded-II fraction (100mg/kg, p.o.) the effects were only marginal and statistically insignificant, suggesting the important role of metal ions conjugation to withasteroids for eliciting these bioactivities.

The hepatoprotective activities of WSE and WSE/excluded-I fractions exhibited dose-dependant relationship. Thus, administration of the two test compounds at a higher dose (200 mg/kg, p.o.) elicited better response than those elicited by 100 mg/kg, p.o. of each drug. The effect of WSE/excluded-II fraction at higher dose (200 mg/kg, p.o.), though significant, was less pronounced than those of WSE and WSE/excluded-I.

In conclusion, WSE and WSE/excluded-I, exhibited potent hepatoprotective and antioxidant effects in paracetamol induced hepatotoxic rats. Based on the above findings it is postulated that metal ions in conjugation with the withasteroids have a profound bearing on the true bioactive principles of the adaptogenic *Withania somnifera* standardized extract(s) [15, 18].

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References

1. Ward FM, Daly MJ. Hepatic disease, chapter 14 in clinical pharmacy and therapeutics. Walker B., Edwards C., 2nd edition, Harcourt Brace and company limited, Churchill Livingstone, London 1999: 195.
2. Shahani S. Evaluation of hepatoprotective efficacy of APCL-A poly herbal formulation *in vivo* in rats. Indian Drugs 1999; 36: 628-631.
3. Subramonium A, Pushpangadan P. Development of phytomedicine for liver diseases. Indian Journal of Pharmacology 1999; 31: 166-175.
4. Wendel A, Feurensteins S, Konz KH. Acute paracetamol intoxication of starved mice leads to lipid peroxidation *in vivo*. Biochemical Pharmacology 1987; 28: 2051-2053.
5. Recknagel RO. A new direction in the study of carbon tetrachloride hepatotoxicity. Life Sciences 1983; 33: 401-408.

6. Jyoti TM, Prabhu K, Lakshminarasu S, Ramachandra SS. Hepatoprotective and antioxidant activity of *Euphorbia antiquorum*. Pharmacognosy Magazine 2008; 4: 127-133.
7. Chrungoo VJ, Singh K, Singh J. Differential biochemical response of freshly isolated rat hepatocytes to paracetamol, carbontetrochloride and D- galactosamine toxicity. Indian J Exp Biol 1997; 35: 603-610.
8. Hinson JA, Pohl M, Gillete JR. Acetaminophen induced hepatotoxicity. Life Sci. 1981; 29: 107-116.
9. Prescott LF, Wright N, Roscoe P, Brown SS. Plasma paracetamol half life and hepatic necrosis in patients with paracetamol overdose. Lancet I: 519-522.
10. Dahlin D, Miwa G, Lu A, Nelson S. N-acetyl-*p*-benzoquinoneimine: A cytochrome P-450- mediated oxidation product of acetaminophen. Proc Natl Acad Sci 1984; 81: 1327-1331.
11. Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. Biochem Biophys Acta 1979; 582: 67-78.
12. Gupta AK, Chitme H, Dass SK, Misra N. Hepatoprotective activity of *Rauwolfia serpentina* rhizome in paracetamol intoxicated rats. J Pharmacol Toxicol 2006; 1: 82-88.
13. Chattopadhyay RR. Possible mechanism of hepatoprotective activity of *Azadirachta indica* leaf extract: part II. J Ethnopharmacol 2003; 89: 217-219.
14. Chatterjee TK. Medicinal plants with hepatoprotective properties, in Herbal Opinions, third ed. Books & Allied (P) Ltd., Calcutta 2000; 135.
15. Ghosal S, Srivastava RS, Bhattacharya SK, Upadhyay SN, Jaiswal AK, Chattopadhyay U. Immunomodulatory and CNS effects of sitoindosides IX and X, two new glycowithanolides from *Withania somnifera*. Phytother Res 1989; 3: 201-206.
16. Gupta AK, Ganguly P, Majumder UK, Ghosal S. Antioxidant and other beneficent effects of free and conjugated phytosteroids of *Solanum* and *Withania*. J Indian Chem Soc 2008; 85: 1227-1238.
17. Bhattacharya A, Ramanathan M, Ghosal S, Bhattacharya SK. Effect of *Withania somnifera* Glycowithanolides on Iron-induced Hepatotoxicity in Rats. Phytother Res 2000; 14: 568-570.
18. Ghosal S. In pursuit of standardization of Ayurvedic drugs. Acad Ind Med 1986; 1: 1-13.
19. Bergmeyer HU, Scheibe P, Wahlefeld AW. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin Chem 1978; 24: 58-61.
20. King J. The hydrolases-acid and alkaline phosphatase. Practical Clinical Enzymology, Van, D (ed.), London: Nostrand company Ltd; 1965 :191-208.
21. Malloy HJ, Evelyn KA. The determination of bilirubin with the photoelectric colorimeter. J Biol Chem 1937; 119: 481.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin- phenol reagent. J Biol Chem 1951; 193: 265-275.
23. Ohkawa H, Onishi N, Yagi K. Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.
24. Ellman GL. Tissue sulphhydryl groups. Arch Biochem Biophys 1959; 82: 70-77.

25. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Ind J Biochem Biophys* 1984; 21: 131-132.
26. Aebi H. Catalase. In: Bergmeyer HV, editor. *Methods in enzymatic analysis*. Vol 2, New York: Academic press; 1974: 674-684.
27. Ganguly P, Gupta AK, Majumder UK, Ghosal S. Withasteroid metal ion conjugates: their natural occurrence in *Withania somnifera* and effects on cold-restraint stress in mice. *Pharmacologyonline* 2009 (in press).
28. Vermeulen NPE, Bessems JGM, Van de Streat R. Molecular aspects of paracetamol induced hepatotoxicity and its mechanism based prevention. *Drug Metab Rev* 1992; 24: 367-407.
29. Willianson EM, Okpako DT, Evans FJ. Selection, preparation and pharmacological evaluation of plant material, John Wiley, England, 1996.
30. Moss DW, Butterworth PJ. *Enzymology and Medicine*. Pitman Medical, London, 1974: 139.
31. Muriel P, Garciapina T, Perez-Alvarez V, Murelle M. Silymarin protect against paracetamol-induced lipid peroxidation and liver damage. *J Appl Toxicol* 1992; 12: 439-442.
32. Curtis SJ, Moritz M, Snodgrass PJ. Serum enzymes derived from liver cell fraction and the response to carbon tetrachloride intoxication in rats. *Gastroenterology* 1972; 62: 84-92.
33. Korsrud GO, Grice HG, Goodman RK, Knipfel SH, Mc Laughlan JM. Sensitivity of several enzymes for the detection of thioacetamide, nitrosamine and diethanolamine induced liver damage in rats. *Toxicol Appl Pharmacol* 1973; 26: 299-313.
34. Chance B, Green Stein DS, Roughton RJW, The mechanism of catalase action 1-steady state analysis. *Arch biochem Biophys* 1952; 37: 301-339.
35. Prakash J, Gupta SK, Kochupillai V, Singh N, Gupta YK, Joshi S. Chemopreventive activity of *Withania somnifera* in experimentally induced fibrosarcoma tumors in Swiss albino mice. *Phytother Res* 2001; 15: 240-244.