Ameliorative Potential of Aqueous Root Extract of *Withania somnifera* Against Paracetamol Induced Liver Damage in Mice

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**ABSTRACT**

Paracetamol (also known as Acetaminophen) is a cheap but effective analgesic with few side-effects. However, outside of the therapeutic window, the liver’s ability to process the toxic metabolites of paracetamol is exceeded and hepatotoxicity may result in the process. The present study was undertaken exhaustively to investigate hepatoprotective activity of *Withania somnifera* root extract against paracetamol induced hepatotoxicity, in mice. Activities of liver markers enzymes Glutamate-oxaloacetate Transaminase (AST), Glutamate-pyruvate Transaminase (ALT), Alkaline Phosphatase (ALP), Bilirubin and total protein were estimated in serum, whereas, lipid peroxidation and antioxidant status (Superoxide dismutase, Catalase, Glutathione Peroxidase and Reduced glutathione content) were estimated in liver homogenate. Paracetamol (500 mg kg⁻¹ b.wt.) induced hepatotoxicity was established by a significant increase in serum AST, ALT, ALP activity and bilirubin level accompanied by significant decrease in total serum protein level. Paracetamol hepatotoxicity was evident by an increase in lipid peroxidation, reduction of reduced glutathione content, Catalase, Glutathione reductase and Glutathione peroxide activity in liver. Treatment of *Withania somnifera* root extract (500 mg kg⁻¹ b.wt.) notably decreased AST, ALT, ALP, Bilirubin and Total Protein content in PCR compared to control. In addition *Withania somnifera* root extract significantly reduced hepatic lipid peroxidation, enhanced antioxidant enzyme activities and GSH levels in the liver of mice.

**Key words:** Antioxidant activity, bio-markers, hepatotoxicity, paracetamol, *Withania somnifera*


**INTRODUCTION**

Paracetamol (PCM) is widely prescribed and cheap to buy over-the-counter, making it a common drug taken in overdose. Paracetamol can cause serious or fatal adverse effects at around 150 mg kg⁻¹ for most adults. Acute overdoses of paracetamol can cause potentially fatal liver damage and, in rare individuals, a normal dose can do the same; the risk is heightened by alcohol consumption. Paracetamol hepatotoxicity has been attributed to the formation of toxic metabolites, when a part of paracetamol is activated by cytochrome P-450 to a highly reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI). NAPQI therefore remains in its toxic form in the liver and reacts with cellular membrane molecules, resulting in wide spread hepatocyte damage and death, leading to acute hepatic necrosis.

Many medicinal plants i.e., *Curcuma longa, Lantana camara, Ocimum sanctum* and *Aloe vera* are rich sources of natural antioxidants i.e., curcuminoids, oleocanthal, ursolic acid and barbamol, that can protect from oxidative stress, thus playing an important role in chemoprevention of diseases. *Withania somnifera* (L.) Dunal, also known as Ashwagandha, Indian ginseng, Winter cherry, Ajagandha Kanaja in Hindi and Amukkara in Tamil is a plant in the *Solanaceae* or nightshade family. *Withania somnifera* has been used for centuries in Ayurvedic medicine. Current research supports its polypharmaceutical use, confirming antioxidant, anti-inflammatory, immune-modulating and antistress properties in the whole plant extract and several separate constituents. Preliminary studies have found various constituents of ashwagandha exhibit a variety of therapeutic effects with little or no associated toxicity. *Withania somnifera* root has been considered to be the most active for therapeutic purposes. Root contains maximum amount of Alkaloids viz., Nicotine, Sominine, Sommiferin, Sommiferinine, Withanine, Withanonine, Pseudo withanine, Tropin, Withanolides etc. Antihepatoprotection of *Withania somnifera* extracts on carbon tetrachloride-induced hepatotoxicity has been established.

However, despite the observation of diverse medicinal activities attributed to this plant, no biochemical studies have been carried out to explore the role of *W. somnifera* on paracetamol induced hepatotoxicity in mice. An attempt has been made in the
present study to elucidate the effects of oral feeding of aqueous root extract of *Withania somnifera* on paracetamol (PCM) induced liver damage in mice.

**MATERIALS AND METHODS**

**Plant material, extraction and HPTLC quantification of *Withania somnifera***: The whole plant material of *Withania somnifera* was collected from Lovely Professional University campus, Punjab, India. The authenticity of the species was confirmed by a taxonomist. After collection the plant material was carefully washed and separated into root, stem and leaf and dried at 50°C.

**Processing and extraction of withanolides (withaferine and withanolide A)**: The root part of the *Withania somnifera* was shade-dried and powdered in a grinder. To extract authentic withaferine A and withanolide A, the powdered root material (1.0 g) of *Withania somnifera* and 20 mL distilled water were added to a 50 mL conical flask. The contents were rapidly heated to reflux for one hour. After cooling, the aqueous extract was transferred to 50 mL conical flask. Aqueous extract was filtered and evaporated to dryness with the help of rotavapour.

**HPTLC quantification of withanolides**: The content of withaferine A and withanolide A were determined by the modified method developed by Srivastava. Standard Withanolide A and Withaferine A and the root samples were spotted on precoated silica gel 60 F254 TLC plates (10 x 10 cm; with 0.25 mm layer thickness: Merck KGaA; 1.05554. 0007) as bands 6 mm wide, with 10 mm distance between tracks, by means of Linomat-5 automatic sample applicator (with nitrogen flow) equipped with a 100 μL Hamilton syringe. HPTLC of *W. somnifera* aqueous root extracts was performed by using solvent system with dichloromethane-methanol-acetone-hexane (7.5:0.5:0.5:0.5 v/v) as a mobile phase. Upon separation, quantitative evaluation of these withanolides was performed in the absorption reflection mode at 235 nm. The method was validated for precision, reproducibility and accuracy. On the basis of *R* sub c values of 0.58 and 0.68 for withaferine A and withanolide A, respectively, were identified. On the basis of linear calibration curves for all withanolides in the range of 0.2-1.2 μg, an average recovery of withaferine A and withanolide A was 98 and 98%, respectively. The method is very simple, precise, specific, sensitive, accurate and economical for rapidly validating the aqueous root extract of *W. somnifera* (Ashwagandha).

**Experimental animals**: Female Swiss Albino mice weighing approximately 25 ± 5 g were procured from the animal house of NIPER, Mohali, Punjab, India. The study was approved from Institutional Animal Ethical Committee (IAEC) (Registration no 954/ac/06). They were housed in air-conditioned room with temperature maintained at 25 ± 3°C and fed with commercial pelleted feed from Hindustan Lever Ltd. and water was made freely available.

**Experimental protocol**: Animals were divided into three groups of six animals each.

- **Group I**: Control: The animals were given 1.00 mL of saline (0.9% NaCl) through oral gavages once in a day for 6 days/week for 28 days
- **Group II**: PCM: The animals were intoxicated with PCM (600 mg kg⁻¹ b.wt.) through oral gavages once in a day for 6 days/week for 28 days
- **Group III**: PCM and root extract of *Withania somnifera*: This group were given PCM (as in Group II) and after 60 min of the dose, animals were given root extract (500 mg kg⁻¹ b.wt.) for 6 days/week for 28 days

**Collection of serum and vital organs**: After 28 days, the animals were weighed and sacrificed by cervical dislocation under light anesthesia. Blood samples were collected from each animal in clear sterile centrifuge tube without anticoagulant for obtaining serum for determination of ALT, AST, ALP, Bilirubin and serum total protein. The tubes were left in a big beaker to facilitate coagulation at room temperature. After 1 h the tubes were centrifuged at 2500 rpm for 10 min to pellet the coagulant mass. Supernatant which is serum, was siphoned off carefully in micro centrifuge tubes with the help of pipettes and was stored at 4°C until assayed. Liver was quickly excised from the mice and whole liver was washed with normal cold saline (pH 7.4). The washed liver was then kept in ice cold conditions. The liver was removed and cleansed from blood and extraneous tissue. Part of the liver was fixed with 10% buffered formal saline for 1 week and further processed for histopathological studies. Tissues were weighed for 10% homogenate and individually chopped into pieces with the help of forceps and homogenized using homogenizer in ice cold 0.1 M Phosphate buffer (pH 7.4) containing 0.15 M KCl for the estimation of different enzymes and biomolecules. Aliquot of homogenate was centrifuged at 9000 rpm to obtain supernatant (S9 fraction) for the estimation of various enzymes.

**Biochemical assays**: The activities of alanine and aspartate aminotransferases in serum were estimated by Reitman and the activity of alkaline phosphatase was measured according to the method of Bower. Bilirubin
level in serum was measured according to Jendrassik\(^{12}\). Lipid peroxidation was measured by the method of Utley\(^{13}\). Malondialdehyde, formed as an end product served as an index of oxidative stress. Catalase activity was measured according to the method of Sinha\(^{14}\). 0.1 mL of liver homogenate was mixed with 1.0 mL of 0.01 M phosphate buffer (pH 7.4) where one unit represents 1.0 µmol of H₂O₂ consumed/min/mg protein. Hepatic reduced glutathione level was determined by the method of Ellman\(^{15}\). The GSH contents were calculated using GSH as standard and expressed as mg g⁻¹ tissue. Glutathione peroxidase (GPx) was assayed by the method of Rotruck\(^{16}\). Based on the reaction between glutathione remaining after the action of GPx and 5, 5'-dithiobis-(2-nitrobenzoic acid) to form a complex that absorbs maximally at 410 nm. Glutathione Reductase (GR) that utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form was assayed by the method of Tietze\(^{17}\). Protein content was determined by the method of Lowry\(^{18}\), using bovine serum albumin as standard.

**Statistical analysis:** Resulting data were represented as Mean±SD. Statistical data was analyzed by student's t-test. The p<0.05 was considered statistically significant.

**RESULTS**

Administration of paracetamol to mice by oral route caused liver damage as indicated by a significant increase in serum enzyme ALT, AST, ALP activity and bilirubin level compared to control mice (Table 1). Elevated levels of these enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. Co-administration of mice with aqueous root extract of *Withania somnifera* remarkably (p<0.001) restored paracetamol induced elevated serum levels of ALT, AST, and bilirubin towards normal values, respectively.

The obtained results indicated a high degree of protection against the hepatotoxic effect of paracetamol. The serum total protein level was significantly (p<0.001) declined in paracetamol treated group. Treatment with the aqueous root extract of *Withania somnifera* could significantly (p<0.001) restored serum total protein level towards their control value.

The levels of TBARS as an index of lipid peroxidation, a degradative process of membranous lipids, in liver tissue of paracetamol treated mice were significantly (p<0.001) elevated when compared to control animals (Table 2). Lipid peroxidation level was restored towards their normal value by treatment with *Withania somnifera* root extract on paracetamol induced toxicity. Reduced glutathione level was depleted significantly (p<0.001) in the liver tissue of paracetamol treated mice compared to control group. Treatment with the aqueous root extract of *W. somnifera* had significantly improved level of GSH in liver tissue. Likewise, the decreased activities of catalase, glutathione reductase, glutathione peroxidase and SOD (data not shown) as a result of the treatment with paracetamol were also restored by the aqueous root extract of *Withania somnifera* (p<0.001).

The results obtained from histopathological studies were in agreement with the biochemical studies. Exposure of paracetamol produced extensive vascular degenerative changes and centrilobular necrosis in hepatocytes (Fig. 1, 2). Treatment with PCM and *Withania somnifera* root extract shows normal architecture with mild hepatocyte degeneration comparable to control (Fig. 3). All these results indicate a hepatoprotective potential of the extract against paracetamol induced liver damage through its antioxidant potential, however, further studies are required to elucidate the molecular mechanisms involved in order to support the clinical use of the plant extract.

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<th>Table 1: Effect of PCM and PCM + <em>W. somnifera</em> on ALT, AST, bilirubin, ALP and serum total protein</th>
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Values are Mean ± SD of 6 animals in each group. *p<0.001 compared to control. **p<0.001 compared to PCM.

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<th>Table 2: Effect of PCM and PCM + <em>W. somnifera</em> on lipid peroxidation, reduced glutathione, catalase, glutathione reductase and glutathione peroxidase</th>
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Values are Mean ± SD of 5 animals in each group. *p<0.001 compared to control. **p<0.001 compared to PCM.
**DISCUSSION**

The liver damage is largely assessed by rise of serum enzyme levels such as ALT and AST. The observed rise in the serum levels of alanine transaminase, aspartate transaminase and alkaline phosphatase in paracetamol treated mice has been ascribed to the damaged structural integrity of the liver. The administration of root extract of *Withania somnifera* has diminished the increased serum marker enzymes AST, ALT, ALP and bilirubin level. Reduction of serum albumin in paracetamol treated mice may be due to formation of protein adduct. Hydrogen peroxide a harmful by-product of many normal metabolic processes is quickly converted into to H$_2$O and O$_2$ by Chellakoti.

The reduction in the activity of this enzyme may result in number of harmful effects due to accumulation of highly toxic metabolites and hydrogen peroxide in paracetamol administered mice. In addition, a metabolite of paracetamol NAPQI can increase the formation of reactive oxygen and nitrogen species such as superoxide anion, hydrogen radical, hydrogen peroxide, nitric oxide and peroxynitrite, respectively. Excess levels of reactive oxygen and nitrogen species can attack biological molecules which can lead to lipid peroxidation, nitration of tyrosine and depletion of the antioxidant enzymes resulting in oxidative stress Hinson. The present study also demonstrates that paracetamol overdose resulted in a decrease in the SOD and CAT activities, when compared with control group. It could be due to inactivation of the antioxidant enzymes. Malondialdehyde (MDA), one of the end products in the lipid peroxidation process, increases with oxidative stress. The increase in MDA level observed in liver suggests enhanced lipid peroxidation and tissue damage. A massive decrease in lipid peroxidation in liver tissue of root extract treated groups indicate that *Withania somnifera* possess antioxidative properties. Glutathione (GSH) is known to be an endogenous antioxidant, preventing damage to important cellular components caused by reactive oxygen species. The principal toxic metabolite of paracetamol NAPQI is initially detoxified by conjugation with reduced glutathione (GSH) to form mercapturic acid. However, when the rate of NAPQI formation exceeds the rate of detoxification by GSH (reduced glutathione), it oxidize tissue macromolecules such as lipid or -SH group of proteins. In this present study, the decreased level of glutathione has been observed in PCM treated mice, whereas, its level was significantly increased in *Withania somnifera* root extract treated PCM induced mice. Catalase, glutathione peroxidase and glutathione reductase are thought to be the fundamental antioxidant enzymes, for the direct
elimination of reactive oxygen species. SOD is known to convert \( \text{O}_2^\cdot \) to \( \text{H}_2\text{O}_2 \) and the resulting \( \text{H}_2\text{O}_2 \) is removed by Catalase and glutathione peroxidase\(^{26}\). Glutathione reductase reduces glutathione disulfide (GSSG) to the reduced form of glutathione (GSH) which is an important cellular antioxidant\(^{26}\). The activity of glutathione reductase is used as indicator for oxidative stress. In the present study, Withania somnifera root extract treatment was observed to exhibit hepatoprotective effect as demonstrated by enhanced activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and significant increase in reduced glutathione content and decreased lipid peroxidation.

CONCLUSION
This study signifies that Withania somnifera root extract shows hepatoprotective effect through its antioxidant potential on Paracetamol induced liver damage in mice. However, further studies are required to elucidate the molecular mechanisms involved in order to support the clinical use of the plant extract.

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