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Assessment of Oxidative Stress Effect of *Withania somnifera* in Rats after 28 Days Repeated Oral Treatment

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Abstract

Objective: *Withania somnifera*, a well-known medicinal plant, proved to have a remarkably broad range of therapeutic properties. However, studies evaluating its toxic effects are very scarce. A 28 day repeated oral toxicity study was conducted to evaluate oxidative stress inducing effect, if any, to ascertain its safe use. **Method:** The present study was designed following the Organization for Economic Cooperation and Development guideline 407. *W. somnifera* root extract was given daily for 28 days through oral route at 30, 300, and 1000 mg/kg dose levels. **Results:** The results revealed that treated rats exhibited no mortality and toxic symptoms throughout the study period. It was also observed that there was no significant difference in body, organ weights, and feed, water intake in treated rats when compared to control. *W. somnifera* root extract treatment also brought no significant dose-related changes in lipid peroxidation, reduced Glutathione (GSH) content, superoxide dismutase, catalase, GSH-S transferase, and GSH peroxidase enzymes activity in liver, kidney, and brain of treated rats. **Conclusion:** It can be concluded from the study that 28 days repeated oral treatment of *W. somnifera* root extract found to be devoid of any toxic symptoms and oxidative stress effect.

Keywords: Antioxidant Enzymes, Oxidative Stress, Rat, *Withania somnifera* Root Powder

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1. Introduction

Plants products have been extensively used for the treatment of human diseases from ancient times. Besides allopathic, herbal medicines have also been used and become part of human life, especially in India (Kuete, 2014). The increased usage of plant medicines is because of their abundance, low cost, safety, and cultural attachment. *Withania somnifera* (L) Dunal, commonly called Ashwagandha, an Indian Ayurvedic medicinal plant, has been widely used as medicine and dietary supplement (Prabu *et al.,* 2013). This plant is known to possess vast biologically active components among them with anolides are potential active constituents, which are present at higher concentrations in roots (Khedgikar *et al.,* 2013). Ashwagandha extracts have shown to possess anticancer (McKenna et al., 2015; Wadegaonkar and Wadegaonkar, 2013; Yang *et al.,* 2013), adaptogenic/ antistress (Singh *et al.,* 2011), anti-inflammatory (Chandra *et al.,* 2012), antihypertensive, hypoglycemic (Gorelick *et al.,* 2015), hypolipidemic, neuroprotective, (Prakash *et al.,* 2013; Ahmad *et al.,* 2015) antioxidant (Gupta *et al.*, 2003), cardioprotective (Dhuley, 2000), antiarthritic (Khan *et al.,* 2015), and antibacterial (Bisht and Rawat, 2014) properties.

Medicinal properties of Ashwagandha are established to a great extent; however studies on its toxicity are very few in the literature. Prabhu *et al.* (2013) demonstrated acute and subacute toxicity study of hydro alcoholic extract of *W. somnifera* root in Wistar rats. In another study, the acute and chronic study was conducted in rats with hydro alcoholic extract of *W. somnifera* root (Sahni *et al.,* 2014). The toxicity of medicinal plant practice may result due to inherent toxic phytochemicals, adulteration of medicines, impurities such as chemicals and heavy metals and poor quality control of herbal products (Kuete,

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2014). Hence, a detailed safety evaluation of *W. somnifera* extracts is essential.

Oxidative reactions of various biological functions of aerobic organisms are a continuous source of potentially cytotoxic Reactive Oxygen Species (ROS). Under physiological conditions, a balance is maintained between ROS and antioxidant system (Vaziri *et al.,* 2000). However, xenobiotics when entering the body, either increases ROS generation or weaken antioxidant system that leads to increased ROS activity, which damage lipids, DNA, and protein leading to cellular injury called oxidative stress (Kuete, 2014). Antioxidant enzymes activity varies depending on the oxidant/antioxidant equilibrium in the cells. Hence, these parameters are considered as the sensitive markers in toxicity testing. Therefore, in the present study, oxidative stress effect of *W. somnifera* root extract in rat after 28 days repeated oral treatment was evaluated by estimating oxidative stress biomarkers such as Lipid Peroxidation (LPO), reduced Glutathione (GSH) content and antioxidant enzyme levels in liver, kidney, and brain.

2. Materials and Methods

2.1 Materials

All the reagents used in the study were of analytical grade and purchased from Sigma, USA. Ashwagandha root extract was provided by Ixoreal Biomed Pvt. Ltd., Hyderabad, India.

2.2 Animals and Treatment

Female albino Wistar rats of 8–10 week old were procured from the National Institute of Nutrition, Hyderabad, India. The animals were housed in a controlled environment of 22°C temperature, 50-60 % humidity, and 12 h light/dark cycle. Feed and drinking water were provided *ad libitum*. Prior to the experiments, animals were acclimatized to laboratory conditions for 7 days. The animal experiments were approved by the Institutional Animal Ethics Committee.

The 28 day repeated oral toxicity study of *W. somnifera* root extract was conducted in female albino Wistar rats following the Organization for Economic Cooperation and Development (OECD) guideline 407 (OECD, 2008). Fresh dilutions of *W. somnifera* root extract were prepared in drinking water every day. Three experimental groups (5 rats/group) were treated with *W. somnifera* root extract each at 30, 300, and 1000 mg/kg body weight (b.w) doses. Control group was treated with drinking water. The rats were treated once a day for 28 days. The symptoms and mortality in rats were observed daily. The rats were sacrificed after 28 days of treatment. Liver, kidney, and brain were collected, perfused with cold Potassium Chloride (KCl) buffer (1.15% KCl containing 0.5 mM ethylenediaminetetraacetic acid). Tissues were homogenized in Potassium Phosphate Buffer (KPB) (KPB; 0.1M, pH 7.4) and centrifuged at 15,000 rpm for 30 min. The clear supernatant was collected and stored in 85°C deep freezer (Thermo Scientific, USA). The samples were analyzed within 1 month.

2.3 Lipid Peroxidation

Malondialdehyde (MDA), a LPO biomarker in tissue homogenate, was measured according to the method described by Wills (1969) with some modifications. Briefly, 0.5 ml of tissue homogenate and 2 ml of 0.375% thiobarbituric acid +15% trichloro acetic acid reagents were taken in a tube, boiled in water bath for 30 min and cooled. The absorbance was measured at 532 nm using a spectrophotometer (Spectramax Plus, Molecular Devices, USA).

2.4 Reduced Glutathione

The GSH content was measured using the method of Jollow *et al*. (1974). Equal volumes of tissue homogenate and 4% sulfosalicylic acid were mixed, kept in ice for 1 h, and centrifuged at 10,000 rpm for 10 min. 1 ml supernatant, 0.4 ml DTNB (4 mg/ml) and 1.6 ml KPB (0.1 M, pH 7.4) were mixed in a tube. The yellow color developed was read at 412 nm.

2.5 Superoxide Dismutase

Superoxide Dismutase (SOD) activity was estimated in tissue supernatant using the method of Marklund and Marklund (1974). The 3 ml assay mixture contained 50 mM tris–HCl buffer (pH 8.2) containing 1 mM DTPA, 45 µl of 10 mM pyrogallol, and 100 µl tissue supernatant. The rate of inhibition of pyrogallol auto oxidation after the addition of enzyme extract was recorded at 420 nm.

2.6 Catalase

The Catalase (CAT) activity was assayed using the spectrophotometric method of Aebi (1984). Briefly, 3 ml assay mixture contained 0.063% H_2O_2 in 0.1 M KPB pH

7.4 and tissue supernatant. The decrease in absorbance was recorded for 5 min at 240 nm. The enzyme activity was expressed as milli moles of H_2O_2 decomposed per minute per milligram protein using a molar extinction coefficient of 43.6 M/cm.

2.7 Glutathione Peroxidase

The GSH Peroxidase (GPx) activity was measured using the procedure of Paglia and Valentine (1967). The enzyme assay was performed by adding 750 ml KPB, 60 ml 2.25 mM NADPH, 15 µl GSH reductase (7.1 µl/ml), and 25 µl GSH (11.52 mg/ml) in the cuvette. The enzymatic reaction was initiated by adding 100 µl supernatant and 100 µl hydrogen peroxide (1.5 mM). The decrease in absorbance at 340 nm was observed for 5 min in the spectrophotometer.

2.8 Glutathione Reductase

The GSH Reductase (GR) activity was assayed using the method of Carlberg and Mannervik (1985). In a 3 ml reaction mixture, 50 mM KPB, 1.0 mM oxidized GSH, 0.15 mM β-NADPH, and 0.01% bovine serum albumin was taken, and 100 µl tissue supernatant was added. The change in the absorbance was recorded at 340 nm.

2.9 Glutathione S-Transferase

The GSH-S Transferase (GST) activity was determined according to the method of Habig *et al.,* (1967). 3 ml reaction mixture consisted of 2.7 ml KPB, 100 µl GSH (75 mM), 100 µl 2,4-dinitrochlorobenzene (30 mM), and 100 µl supernatant. The change in absorbance was recorded at 340 nm for 5 min.

2.10 Statistical Analysis

The data were presented as mean±standard deviation. The *P* values were calculated in GraphPad prism by one-way ANOVA followed by a Dunnett's multiple comparison test. The value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Body and Organ Weights

Figure 1 represents the body and organs weights of the treated and control rats. Significant differences in body and organ weights were not observed between control and treated rats. Similarly, feed and water intake (data not shown) were also not changed in treated rats when compared to controls. Behavioral changes and mortality were not observed in treated, and control rats throughout the study.

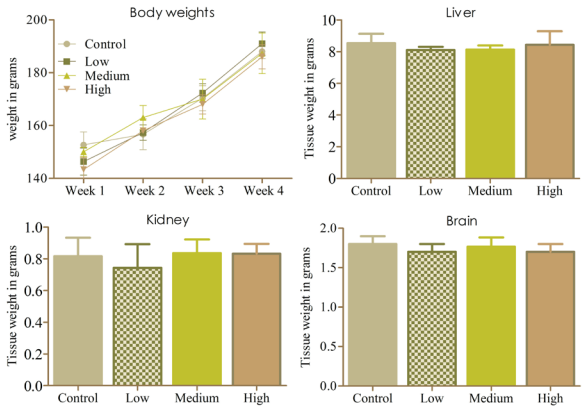


Figure 1. Body and organ weights of control and treated rats after 28 days of repeated oral treatment with Ashwagandha root powder. Each value represents the mean ± standard deviation; $n = 5$ rats. $^*P < 0.05$ when compared with controls.

3.2 Lipid Peroxidation

Measurement of LPO was used to investigate the process of cellular damage induced by free radicals. The levels of LPO end product, MDA, in control and *W. somnifera* root extract treated rats are presented in Figure 2. *W. somnifera* root extract treatment did not show any significant change in MDA levels in all the tissues of treated rats at all the dose levels.

3.3 Reduced Glutathione

Changes in GSH levels can be monitored as an indication of disturbance in oxidant/antioxidant homeostasis. In the present study, GSH levels of treated and control rats were measured and shown in Figure 2. *W. somnifera* root extract treatment at 30, 300, and 1000 mg/kg dose levels did not bring any significant change in GSH content in the treated rat organs.

3.4 Superoxide Dismutase

Figure 3 represents the activity of the SOD in various tissues of the experimental animals. The present study reveals that *W. somnifera* root extract administration repeatedly for 28 days did not cause any significant change

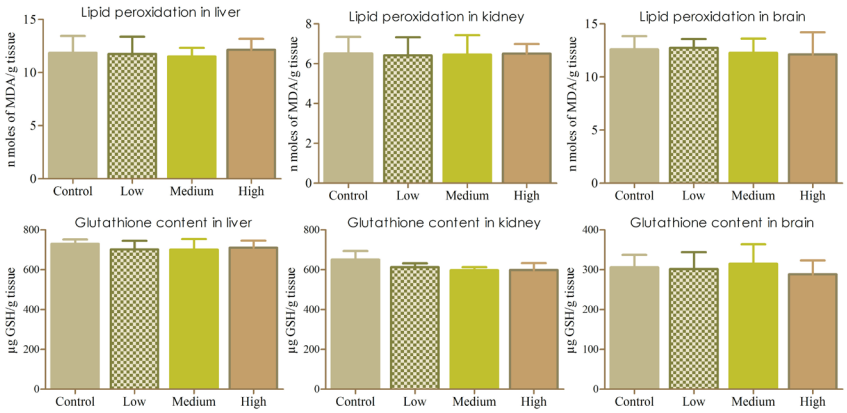


Figure 2. Levels of malondialdehyde, a lipid peroxidation end product and concentration of glutathione in various organs of rats after 28 days of repeated oral treatment with Ashwagandha root powder. Each value represents the mean ± standard deviation; $n = 5$ rats. *$P < 0.05$ when compared with controls.

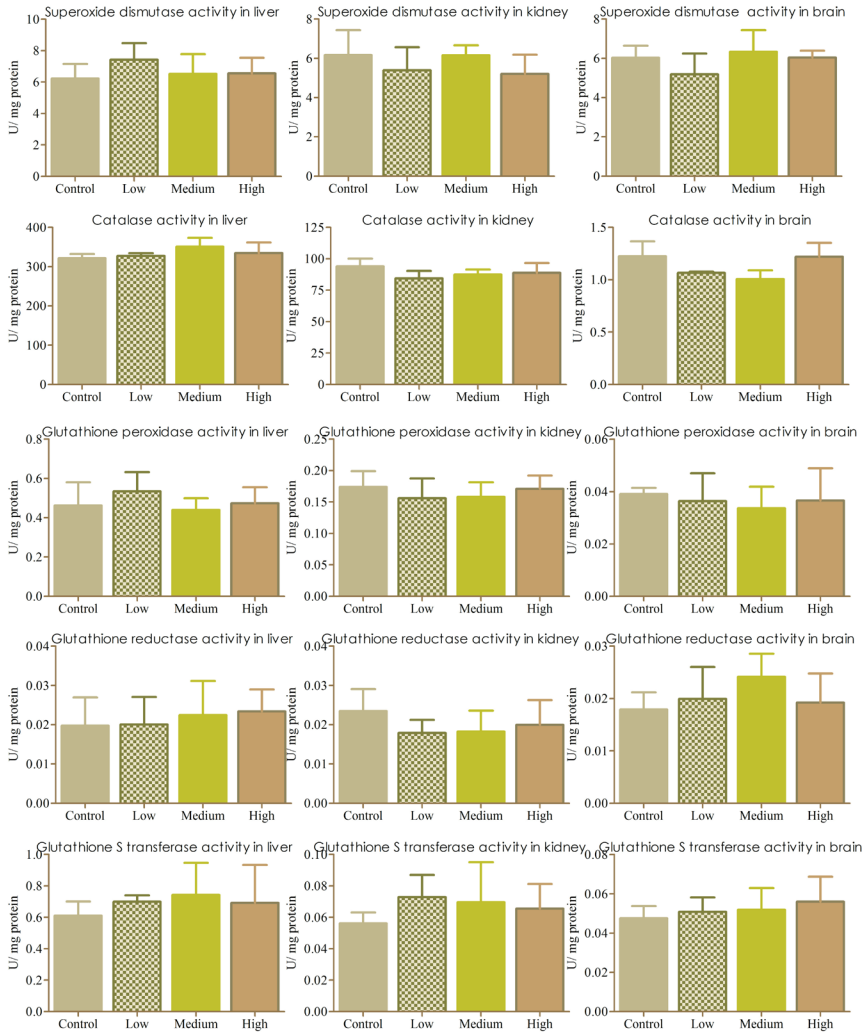


Figure 3. Activity of antioxidant enzymes in various organs of rats treated with Ashwagandha root powder after 28 days repeated oral treatment. Each value represents the mean ± standard deviation; $n = 5$ rats. *$P < 0.05$ when compared with controls.

in SOD activity in liver, kidney, and brain of treated rats at all dose levels.

3.5 Catalase

The CAT activity was estimated in control and treated rats, and the results are presented in Figure 3. *W. somnifera* root extract treatment at 30, 300, and 1000 mg/kg doses brought no significant alteration in the CAT activity in the liver, kidney and brain of treated rats.

3.6 Glutathione Peroxide

Effect of *W. somnifera* root extract on GPx activity after oral treatment for 28 days in various tissues is presented in Figure 3. *W. somnifera* root extract treatment at three dose levels caused no significant change in GPx activity in liver, kidney, and brain of treated rats.

3.7 Glutathione Reductase

The GR activity was estimated in control and *W. somnifera* root extract treated rats and presented in Figure 3. Repeated oral treatment of rats with *W. somnifera* root extract for 28 days showed no significant change in the GR activity at three dose levels.

3.8 Glutathione S-Transferase

The GST activity was estimated in various tissues of control and treated rats and presented in Figure 3. The study revealed that *W. somnifera* root extract treatment at all the doses showed no significant dose-related change in GST activity was observed in treated rat organs.

4. Discussion

Herbal medicines are generally complex mixtures of numerous bioactive compounds which may differ in the mechanisms of action, in their dose-response relationships and in the synergistic effects (Wills and Asha, 2012). Hence, toxicity studies are carried out on drugs to identify potential health hazards before their use. OECD test guideline 407 is intended to investigate effects on a very broad variety of xenobiotics. This guideline suggests that high dose has to be chosen with the objective of bringing toxic effects, but not death, medium doses have to be selected for establishing dose response with no observed adverse effects at the lowest dose level. It provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time.

The changes in body, organ weights, and food, water intake serves as sensitive signs of toxicological effects of any drugs treatment in rats (Ping *et al.,* 2013). In the present study, 28 days repeated oral treatment of *W. somnifera* root extract at 30, 300, and 1000 mg/kg doses showed no significant changes in body and organs weight of rats. Similarly, the treated rats did not show any significant change in food and water intake when compared with the controls.

Oxidative stress biomarkers were analyzed in liver, kidney and brain of treated rats to evaluate possible oxidative stress inducing the effect of the *W. somnifera* root extract. MDA, a LPO end product is generated *in vivo* via peroxidation of polyunsaturated fatty acids (Ho *et al.,* 2013). In the present study, *W. somnifera* root extract at 30, 300, and 1000 mg/kg dose levels showed no significant change in LPO in all the treated rat organs. Using nucleophilic and reducing property, GSH reacts with oxidizing molecules before they damage cellular components. GSH levels were also not changed significantly in the treated rat organs. Both LPO and GSH are in general considered to be the best biomarkers of oxidative stress. No change in LPO and GSH signifies that *W. somnifera* root extract treatment did not result any ROS in the treated rats.

It is well established that SOD detoxifies O_2^- to less toxic hydrogen peroxide which is further reduced to H_2O by both CAT and GPx (Prabhakar *et al.,* 2012). In addition, GSH reductase reproduces GSH from oxidized GSH to maintain cellular GSH levels (Schülke *et al.*, 2012). The treatment of *W. somnifera* root extract did not show any significant change in the activities of these antioxidant enzymes. On the other hand, GST involves primarily in the metabolism of foreign chemicals such as carcinogens, pollutants, drugs as well as potential endogenous reactive compounds. In the current study, GST activity was also unchanged. On the whole, 28 day repeated oral treatment of *W. somnifera* root extract had brought no significant change in body/organ weights, feed/water intake, and oxidative stress biomarkers in all the organs of treated rats. Which imply that *W. somnifera* root extract did not elicit ROS generation and thus devoid of oxidative stress-related toxicity in treated rats hence may considered as safe for repeated long-term usage for the medicinal purpose.

Few previous studies are in line with the current

study results. Acute and subacute oral toxicity study of *W. somnifera* root extract in rats demonstrated no toxic signs/mortality, body weights, organ weights, hematobiochemical parameters, and gross histopathological lesions (Prabu *et al.,* 2013). Similarly in another study, acute and chronic treatment of *W. somnifera* root extract to rats showed initial excitement, mild depression, dullness, decreased respiration, and reduced spontaneous motor activity but no mortality in any group (Sahni *et al.,* 2014).

5. Acknowledgments

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