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Ameliorative Activity of *Withania somnifera* Root Extract on Paraquat-induced Oxidative Stress in Mice

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ABSTRACT

Paraquat (PQ), an oxidative stress inducing substance, is a herbicide which is very toxic to the animals and humans. A number of antioxidant treatments against PQ-induced oxidative stress have been tried but unfortunately, none of them has been proven to be enormously effective. In this context, attentions are needed to explore the antioxidants from the natural resources. Thus, the antioxidant activity of *Withania somnifera* (Ws) root extract on paraquat (PQ) induced oxidative stress in mice was investigated. The doses of 100, 250 and 500 mg kg⁻¹ of Ws were given orally on alternate day for 30 days. PQ (3.5 mg kg⁻¹) was injected intraperitoneally on day 15th of start of the study. On day 30th antioxidant activity of Ws was evaluated by measuring lipid peroxides (MDA) and GSH contents as well as by measuring the activities of antioxidant enzymes; superoxide dismutase (SOD) and catalase (CAT) in the blood of mice. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, creatinine and urea levels were also estimated to investigate the protective effects of Ws on liver and kidney functions. Ws remarkably alleviated the over production of MDA and improved GSH level in PQ intoxicated mice blood dose-dependently. In addition, SOD and CAT activities were improved significantly by Ws treatment. Ws had also considerably alleviated the elevated AST and ALT activities as well as the total bilirubin, creatinine and urea levels in a dose dependent manner. Ws protected the mice against paraquat-induced oxidative stress mainly through alleviating the over production of MDA and modulating the lowered GSH level, SOD and CAT activities. Thus, Ws may constitute an alternative sole or adjunct remedy against paraquat-induced oxidative stress.

Key words: Antioxidant, lipid peroxidation, oxidative stress, paraquat, *Withania somnifera*

INTRODUCTION

Ayurveda, the Indian traditional system of medicine uses many herbal extracts to cure a variety of diseases including states of oxidative stress. *Withania somnifera* (Ashwagandha or Indian Ginseng) family Solanaceae, is an Indian traditional medicine, used for over 4000 years in Indian ayurvedic medicine. It grows in India, Africa and the Mediterranean countries. *W. somnifera* has multifaceted medicinal properties including antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory and antibacterial (Sundaram *et al.*, 2011). The root extract contains soluble proteins, total amino acids, reducing sugars, non-reducing sugars, crude fibers (Khanna *et al.*, 2006) and

steroidal lactones with ergostane which includes withanone, withaferin, withanolides, withasomidienone, withanolide C and alkanoids (about 0.2%). The root could induce antioxidants, glutathione (GSH) and glutathione peroxidase (GPX) and therefore has been used to treat various disease conditions including stress, anxiety, insomnia arthritis and neurodegeneration (Spelman *et al.*, 2006). The roots are also used in constipation, senile debility, general debility, nervous exhaustion, loss of memory, loss of muscular energy and spermatorrhoea (Harikrishnan *et al.*, 2008). Currently, *W. somnifera* root extract (Ws) is used as a dietary supplement throughout the world including United States. Since many of ashwagandha uses have not been scientifically validated, skepticism can naturally be expected when presented with a herb purportedly useful in so many ailments.

Paraquat (PQ), an oxidative stress inducing substance, is a herbicide which is very toxic to the animals and humans. PQ belongs to the class of redox cycling compounds capable of inducing mitochondrial damage, increase Reactive Oxygen Species (ROS) production and causes oxidative stress (Castello *et al.*, 2007). The toxic effects of PQ in animals have been attributed to the generation of superoxide radicals after reduction of PQ by intracellular oxidases and amplified generation of further ROS (Newstead, 1996). Clinicians have tried a number of antioxidant treatments against PQ poisoning including controlled hypoxia, superoxide dismutase, Vitamins C, N-acetylcysteine, desferrioxamine, nitrous oxide, tocotrienols and α -tocopherol (Asmadi *et al.*, 2005). Unfortunately, none of these have been proven to be effective (Eddleston *et al.*, 2003). The fatality rate resulting from PQ intoxication is still very high due to the lack of effective treatments (Dinis-Oliveira *et al.*, 2008). Antioxidant and protective effect of the natural plant products against PQ-induced oxidative stress has been demonstrated by previous scientific workers (Gamal El-Din *et al.*, 2005).

The compromised antioxidant defense and increased peroxidation products in blood are indicative for the oxidative stress (Dimri *et al.*, 2008). Measurement of lipid peroxidation in terms of malondialdehyde (MDA), reduced glutathione (GSH) level, superoxide dismutase (SOD) and catalase (CAT) activities in biological samples are widely used to determine the state of oxidative stress. Considering these, the present study was intended to evaluate antioxidant activities of *Withania somnifera* root extract on paraquat-induced oxidative stress in mice.

MATERIALS AND METHODS

Chemicals: Paraquat (1-1' dimethyl-4, 4'-dipyridilium dichloride), tris cacodylic acid, nitro blue tetrazolium (98%), pyrogallol (>98%) and thiobarbituric acid (99%) were obtained from Sigma Chemicals, USA. Sulphuric acid (98%), hydrochloric acid (36%) excelsar grade and pyridine (99%), 1-butanol (0.99%), 5, 5-dithiobis-(2-nitro-benzoic acid) (DTNB), sodium tungstate solution (0.3 M) extrapure grade were obtained from Qualigens chemicals, India. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, creatinine and urea assay kits were purchased from Span Diagnostics, Surat (India).

Plant extract: Roots of *Withania somnifera* were freshly harvested from two year old plants and duly authenticated from Central Herbarium (Botanical Survey of India, Government of India, Howrah-03) (voucher no. CHN/I (174)/2007/TechII/104). Thoroughly washed roots were dried in shade and powdered. The powder was subjected to methanol (70%) extraction under reflux and was concentrated under reduced pressure. Finally, *W. somnifera* root extract (Ws) was suspended in normal saline and stored in refrigerator until the use.

Animals: Eighty male Swiss albino mice (IVRI strain), weighing 28-32 g were obtained from the Laboratory Animal Resource Section, IVRI, Izatnagar. All mice were maintained under standard laboratory conditions (27±1°C temperature; 12:12 h light/dark and 50-60% humidity) and quarantined for 7 days prior to the start of study. Standard rodent chow and tap water were provided *ad libitum* to the experimental animals. The experiment performed was in full compliance with Institutional Animal Ethics Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted under the directives of Ministry of Social Justice and Empowerment, Government of India.

Safety study of Ws and dose optimization of PQ: Thirty mice were randomly divided into six groups (I-VI, n = 5). After quarantine of 7 days, all mice of the groups (I-III) were given Ws orally at doses 100, 250 and 500 mg kg⁻¹, respectively, on alternate days for 30 days. Whereas, PQ was injected intraperitoneally to the mice of groups IV-VI at doses 3.5, 7.0 and 10 mg kg⁻¹, respectively, as per Prasad *et al.* (2007). On day 30th, all animals were humanized by decapitation under anesthesia. Blood samples were collected by cardiac puncture in for serum biochemistry. Mortality and any adverse events were recorded throughout the study period.

Experimental protocol: Fifty mice were randomly divided into five groups (I-V, n = 10). All mice (III-V) were given Ws at doses 100, 250 and 500 mg kg⁻¹ (suspended in normal saline) by gavages on alternate day for 30 days. Based on dose optimization study, on day 15th of the experiment paraquat (3.5 mg kg⁻¹) was injected intraperitoneally to all mice, except group I. On day 30th, all animals were humanized by decapitation under anesthesia. Blood samples were collected by cardiac puncture and used for assays of oxidative stress and serum biochemistry.

Assays of oxidative stress: Blood samples collected for assays of oxidative stress were centrifuged at 200x g for 10 min to harvest the erythrocytes. Erythrocytes were washed thrice with normal saline solution and finally, 10% haemolysate was prepared by adding chilled distilled water. For estimation of reduced glutathione, RBC suspension was prepared by adding equal volume of erythrocytes and normal saline solution. Haemolysate and RBC suspension were kept at 70°C and used for antioxidant assay within 6 h. Haemoglobin concentration was estimated by cyanomethaemoglobin method (Vankampen and Zinglstra, 1961).

Lipid peroxidation (LPO) assay: The concentration of malonyldialdehyde (MDA), marker of lipid peroxidation, was estimated in haemolysate by the method of Placer *et al.* (1966). Lipid peroxidation was calculated on the basis of molar extinction coefficient of MDA (1.56×10⁵) and expressed in terms of μmol of MDA/mg of Hb.

Reduced glutathione (GSH) assay: The concentration of reduced glutathione in RBC suspension was estimated by 5, 5-dithiobis-(2-nitro- benzoic acid) (DTNB) method as per the procedure of Prins and Loos (1969). Reduced glutathione concentration in the test sample was calculated by employing the molar extinction coefficient of DTNB-GSH conjugate 13600/M/Xcm.

Superoxide dismutase (SOD) assay: SOD activity in haemolysate was measured by using nitro blue tetrazolium as a substrate after suitable dilution as described by Masayasu and Hiroshi (1979). One unit of SOD activity was defined as the amount of enzyme that inhibited auto-oxidation by 50% under the given experimental condition and the values were expressed as unit mg⁻¹ of haemoglobin.

Catalase (CAT) assay: Catalase activity in haemolysate was estimated by using H_2O_2 as a substrate as per the method of Bergmayer (1983). One unit of activity is equal to mmol of H_2O_2 degraded per minute and is expressed as units mg^{-1} of haemoglobin.

Serum biochemistry: Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and levels of serum urea, creatinine and total bilirubin were estimated by using kits as per manufacturer protocols. Body weight of each animal was recorded at the intervals of seven days.

Statistical analysis: All data were expressed as Mean \pm S.E. The one way ANOVA analysis was performed to assess data differences among various groups. A p -value <0.05 was considered statistically significant.

RESULTS

Safety of Ws and dose optimization of PQ: Ws treatment was found to be safe at all the given dose regimens. No adverse events were observed during safety study. The body weight gain was recorded in a dose-dependent manner. The amounts of AST, ALT, total bilirubin, urea and creatinine in serum were found within the normal reference range. For dose optimization of PQ, survival rate of mice was recorded. PQ (10 mg kg^{-1}) intoxicated mice revealed severe signs of toxicity and 80% mortality was recorded within 30 days. PQ intoxication resulted to mortality of 60 and 20% at doses 7.0 and 3.5 mg kg^{-1} , respectively. All serum biochemistry panels were found to be elevated in a dose dependent manner (3.5 , 7.0 and 10 mg kg^{-1}).

Effect of Ws on LPO: Blood lipid peroxidation was assessed by measuring content of thiobarbituric acid-reactive substance (MDA). The formation of MDA is considered as a biomarker of oxidant. After PQ intoxication, blood MDA content was significantly elevated ($p<0.01$) in comparison to the respective control. Ws has significantly alleviated the elevated blood MDA content compared to the respective control ($p<0.01$). The reduction of MDA content was found to be in a dose-dependent manner (100 , 250 and 500 mg kg^{-1} ; Table 1).

Effect of Ws on antioxidants: In order to investigate the effect of Ws on improvement antioxidants, level of potential antioxidant GSH and activities of SOD and CAT were measured. Level of GSH as well as activities of SOD and CAT were found to be significantly lowered in PQ intoxicated mice blood in comparison to the respective control ($p<0.01$). Ws significantly enhanced the reduced GSH content and activities of SOD and CAT in comparison to the respective control ($p<0.01$). Improvements of the GSH content as well as SOD and CAT activities were found to be dose-dependent (100 , 250 and 500 mg kg^{-1} ; Table 1).

Effect of Ws on serum biochemistry panels and adverse events: The activities of AST and ALT and levels of total bilirubin, serum urea and creatinine were estimated to evaluate protective effect of Ws on liver and kidney dysfunctions. PQ intoxicated mice revealed significantly increased AST and ALT activities ($p<0.01$). Levels of total bilirubin, serum urea and creatinine were also significantly elevated in comparison to the respective control ($p<0.01$). Ws significantly alleviated these biochemical panels in comparison to the respective control ($p<0.01$) (Table 2). In addition, 40% mortality was recorded in PQ intoxicated mice. Whereas, in Ws treated mice only 10%, 10 and 0%

Table 1: Effect of Ws on LPO and GSH levels, SOD and CAT activity in PQ administered mice blood

Group	Particular	Dose (mg kg ⁻¹)	LPO (nmMDA g ⁻¹ Hb)	GSH (µmDTNB conjugated g ⁻¹ Hb)	SOD (unit mg ⁻¹ Hb)	CAT (unit mg ⁻¹ Hb)
I (n = 10)	Vehicle	NA	70.50±2.75	4.80±0.12	30.80±0.95	280.80±4.9
II (n = 6)	PQ	3.5 i.p.	105.33±2.9*	2.27±0.16*	15.40±0.23*	177.33±3.71*
III (n = 9)	PQ + Ws	100 p.o.	84.12±1.81**	3.80±0.20**	21.48±0.44**	235.20±2.12**
IV (n = 9)	PQ + Ws	250 p.o.	78.50±1.46**	4.14±0.17**	25.20±0.42**	257.00±3.15**
V (n = 10)	PQ + Ws	500 p.o.	74.75±1.76**	4.63±0.14**	28.50±0.50**	268.00±3.35**

Values are expressed as Mean±SEM. *p<0.01 vs. group I, **p<0.01 vs. group II

Table 2: Effect of Ws on Serum creatinine, serum urea, ALT, AST and Total bilirubin in PQ administered mice blood

Group	Particular	Dose (mg kg ⁻¹)	Creatinine (mg dL ⁻¹)	Serum urea (mg dL ⁻¹)	ALT (IU L ⁻¹)	AST (IU L ⁻¹)	Total bilirubin (mg%)
I (n = 10)	Vehicle	NA	0.71±0.02	23.6±1.31	42.3±0.4	22.4±0.5	1.42±0.11
II (n = 6)	PQ	3.5 i.p.	4.22±0.10*	100.4±2.45*	230.0±2.1*	250.0±2.6*	5.31±0.22*
III (n = 9)	PQ + Ws	100 p.o.	1.43±0.11**	39.0±1.41**	68.1±3.2**	77.6±3.23**	2.9±0.15**
IV (n = 9)	PQ + Ws	250 p.o.	1.37±0.12**	35.0±1.51**	56.1±2.1**	64.9±3.34**	2.91±0.23**
V (n = 10)	PQ + Ws	500 p.o.	1.23±0.14**	34.0±1.32**	55.6±1.4**	60.2±2.19**	1.92±0.13**

Values are expressed as Mean± SEM. *p<0.01 vs. group I, **p<0.01 vs. group II

mortalities were recorded (100, 250 and 500 mg kg⁻¹). After, PQ intoxication all mice revealed adverse events such as, dullness, apathy and polypnea, reduction in food and water intake and loss of body weight during first week. Among the Ws treated mice adverse events were ameliorated and gain in body weight was recorded from 2nd week onwards of PQ intoxication.

DISCUSSION

The observed safety was with 100% survival rate, no adverse events and unaltered serum biochemical panels revealed at the given dose of Ws (100, 250 and 500 mg kg⁻¹). Dose optimization for PQ indicates that the dose rate of 3.5 mg kg⁻¹ induces toxicity with higher survival rate over the other doses (7.5 and 10 mg kg⁻¹). Therefore, the dose rate of 3.5 mg kg⁻¹ was preferred for the present experiment to induce oxidative stress.

When ROS generation exceeds the antioxidant defense, the free radicals can interact with endogenous macromolecules and alter the cellular functions (Muthukumaran *et al.*, 2008). Animal body counteracts against ill effects of free radicals via antioxidant defense system, comprising of antioxidant enzymes, like SOD, CAT, glutathione peroxidase and glutathione reductase; endogenous antioxidants, such as GSH and ubiquinone and nutritional antioxidants such as vitamins C and E, zinc, folate, selenium and carotenoids. PQ belongs to the class of redox cycling compounds capable of inducing mitochondrial damage, increased ROS production and oxidative stress (Castello *et al.*, 2007). The imbalance between increased production of free radicals and decreased antioxidant capacity, results in a persistent lipid peroxidation (LPO). MDA is a breakdown product that is frequently quantified as a measure of lipid hydroperoxides, leading to term lipid peroxidation. MDA assay has been found to be one of the better predictor of oxidative damage and often shown excellent correlation with other markers, such as isoprostanes which are considered to be the most reliable markers of lipid peroxidation (Morrow, 2000).

In the present study, elevated level of MDA in PQ intoxicated mouse erythrocytes indicates that PQ can induce oxidative damage. PQ induces elevation in levels of MDA and protein carbonyls, as well as DNA fragmentation, indicating oxidative damage to major cellular components (Yang and

Tiffany, 2005). Remarkable reduction in the elevated MDA content by Ws treatment indicates the potential antioxidant activity of Ws against PQ-induced stress. GSH is the prominent endogenous antioxidant in mammalian cells and its GSH homeostasis relies on the activities of a number of antioxidant enzymes, including SOD, CAT and glutathione reductase (Webb *et al.*, 2008). SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism. Current study revealed, lowered level of GSH and reduced activities of SOD and CAT in the PQ intoxicated mice. Ws treatment significantly improved the lowered GSH content and reduced SOD and CAT activities towards normalcy.

W. somnifera root extracts have been shown to be effective in treating various disorders by inducing GSH and GPx (Singh *et al.*, 2008). Present study indicates the potential protective activity of Ws against PQ induced oxidative stress. Elevated levels of biochemical panels such as serum urea, creatinine, total bilirubin and activities of AST and ALT indicate liver and kidney dysfunctions in PQ intoxicated mice. This may be attributed to the over production of ROS in PQ intoxicated mice. PQ has been linked to lung, liver and kidney toxicity in humans (Dinis-Oliveira *et al.*, 2008). Raina *et al.* (2008) reported elevated levels of serum urea, creatinine, total bilirubin and activities of SGOT, SGPT and ALP in PQ poisoned human patients. PQ-Induced remarkable increase in activities of tissue AST and ALT has also been reported in the gill, kidney, muscle, liver, spleen, intestine and brain of the experimental fish (Kori-Siakpere *et al.*, 2007). In the present studies, Ws treatment has remarkably alleviated the elevated levels of these biochemical panels. This may be a consequence of antioxidant activity of Ws, conferring protection against the cytotoxic deed of PQ. Ws may be a potential sole or adjuvant promising treatment against PQ poisoning.

CONCLUSION

As a conclusion, paraquat can induce oxidative imbalance in the blood of mouse by elevating MDA content as well as by reducing GSH content and antioxidant enzymes activities. *W. somnifera* root extract has a potential protective effect against paraquat-induced oxidative stress. Thus, *W. somnifera* may constitute a sole or adjuvant potential remedy against paraquat poisoning.

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