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## Ameliorative effect of *Withania somnifera* and *Allium sativum* on ochratoxin a induced neurotoxicity in Wistar rats

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**Abstract**

We have evaluated the ameliorative effects ethanolic extracts of *Allium sativum* bulb (20 mg/kg, bw) and *Withania somnifera* root (100 mg/kg, bw) through oral gavage on Ochratoxin A (OTA)-induced toxicity in rats. The protective effects were assessed through estimation of antioxidants defensive systems and histopathological alteration in rat brain after OTA (2 mg/kg) was given in feed to rats. A total of 120 rats were randomly divided into eight groups of 15 animals each, for 9 weeks and 5 animals each were sacrificed at 3, 6 and 9 weeks intervals. The brain was isolated, blotted free of blood and processed for biochemical and histopathological analysis. OTA cause significant increase of lipid peroxidation level and decrease of total and reduced glutathione levels. Activities of superoxide dismutase, catalase and glutathione reductase were significantly decrease in OTA treated rats with histological alteration such as neuronal degeneration, increased perineuronal spaces and oedema of neuropil. After alcoholic extracts administration, the activities of enzymatic and non enzymatic antioxidants levels were significantly increased and histopathological changes were significantly decreased. These results suggest that OTA may cause oxidative damage to brain and alcoholic extracts of *W. somnifera* and *A. sativum* may protect the OTA-mediated neurotoxicity by its ability to prevent lipid peroxidation and replenishing the GSH levels.

**Keywords:** Ochratoxin A, oxidative stress, *Withania somnifera*, *Allium sativum*, brain, rats

**Introduction**

The mycotoxin ochratoxin A (OTA) is a secondary fungal metabolite produced by *Aspergillus ochraceus* and *Penicillium verrucosum*. The kidney is the main target tissue of OTA toxicity. However, recent findings indicate that OTA may, to some extent, also affect the neuronal system [1]. Studies in rodents suggest that OTA crosses the blood brain barrier and accumulates in the brain as time and concentration dependent manner [2]. It has been hypothesized that low level exposure of OTA may exert delayed neurotoxic effects which may in turn contribute to the development of neurodegenerative disorders [2]. Interestingly, OTA has also been shown to cause widespread oxidative stress as measured by an increase in lipid peroxidation and DNA damages in mice brain [1]. However, the exact underlying molecular mechanisms for OTA neurotoxicity are not clear. But studies in peripheral organs and tissues reveals that OTA has complex mechanisms of action include inhibition of protein synthesis, mitochondrial impairment, oxidative stress and DNA damage [3].

*Allium sativum* has been playing one of the most important dietary and medicinal roles in human beings for centuries. It is considered to be one of the best disease-preventive foods, based on its potential and varied effects [4]. A wide array of therapeutic effects, such as hypolipidaemic, antiatherosclerotic, hypoglycaemic, anticoagulant, antihypertensive, antimicrobial, antidote (for heavy metal poisoning), immune enhancement, and hepatoprotective, has been reported [5, 6]. Further, health benefits of garlic as a neuroprotective agent are beginning to emerge [7] in which oxidative stress plays a role [8].

*Withania somnifera* is widely used in Ayurvedic medicine, the traditional medical system of India. It is known to augment defense against diseases, arrest aging, revitalize the body in debilitated condition, increase the capability of the individual to resist adverse environmental factors and create a sense of mental wellbeing [9]. Clinical trials and animal research support the use of *Withania* for treatment of anxiety, cognitive and neurological disorders, senile dementia, Alzheimer's and Parkinson's disease [10]. The active principles of *Withania*, consisting of sitoindosides VII-X, and withaferin-A have been shown to exhibit significant anti-stress and antioxidant effect in rat brain frontal cortex and striatum [11]. Bhattacharya *et al.*, [12] reported that antioxidant activity of *Withania somnifera* in chronic foot shock stress-

induced changes in rat brain, where *Withania* root extract cause dose-dependent decrease of LPO level and enhanced the activities of catalase and glutathione peroxidase. In the present study, we evaluated the ameliorative efficacy of ethanolic extracts of *A. sativum* and *W. somnifera* individually and in combination, on OTA-induced brain Wistar rats.

## Materials and Methods

### Experiment Animals

The study was conducted in apparently healthy adult male Wistar rats (21 days) procured from the Laboratory Animals Resources Section of the Institute. All animals were housed in polypropylene cages with chopped wheat straw as the bedding material. All the procedures, conducted on the experimental animals were duly approved by the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Rats were given formulated feed (tested to be free of aflatoxin B<sub>1</sub> and ochratoxin-A) obtained from Feed Technology Unit of IVRI, and distilled water *ad libitum* throughout the experiment. Prior to experiment, all the animals were kept in the laboratory conditions for a period of 7 days or more for acclimatization.

### OTA production, Purification and Analysis

Details regarding OTA production, purification, analysis have been given in our earlier report [13].

### Preparation of Alcoholic Extract of the Plant Materials

Authenticated plant materials (bulbs of *A. sativum* and roots of *W. somnifera*) were procured from authorized local Ayurvedic medical shop, Bareilly and processed plant materials (freshly crushed bulbs of *A. sativum*, shade dried and powdered roots of *W. somnifera*) were then subjected to estimation of OTA to rule out OTA contamination, if any, and were extracted with 70% ethanol. The prepared ethanolic extracts were stored at 4°C, and before administration, extracts were dissolved in Tween 80 in such a way that the final concentration of the later should not exceeds 1% and then were administered to rats by oral gavage at the dose of 20 mg/kg bw for *A. sativum* bulb extract (ABE) and 100 mg/kg bw for *W. somnifera* root extract (WRE).

### Experimental design

Male Wistar rats (21 days of age) were divided randomly into eight groups, 15 animals each. The rats were housed in an animal facility at 22±1°C with 12-h light–dark cycle, controlled humidity and circulation of air. Then these groups were treated as follows: group I served as control; group animals in group II were given OTA @ 2 ppm in diet *ad lib* for a maximum period of 9 weeks.; group III, ethanolic extract of *Allium sativum*; group IV, ethanolic extract of *Withania somnifera*; group V, and VI were given OTA along with ethanolic extract of *Allium sativum* and *Withania somnifera* alone respectively; group VII was given both extract simultaneously with OTA; group VIII, like group VII along with activated charcoal. OTA (2 ppm) and AC (0.5%) were administered through feed and extracts through oral gavage. Animals in different groups were sacrificed (5 rats) under light chloroform anesthesia at each interval (3 wks) and

brains were removed and homogenized in ice cold phosphate buffer solution (pH 7.4).

### Assessment of Oxidative Stress

A 200 mg of liver sample was taken in 2 ml of ice-cold PBS. Another 200 mg of sample was separately taken in 2 ml of 0.02 M EDTA for GSH estimation. The homogenate (10%) prepared with homogenizer (IKA, Germany) under ice-cold condition was centrifuged for 10 min at 3000 rpm and the supernatant was stored at -20 °C until assay. LPO was evaluated in terms of malondialdehyde (MDA) production [14]. Total (T-SH), protein bound (PB-SH) thiol groups and reduced glutathione (GSH) in the tissue homogenate were determined as per the method of Sedlak and Lindsay [15]. The molar extinction coefficient of 13100 at 412 nm was used for the determination of thiol contents and the values were expressed in mmol/g of wet tissue. The PB-SH was calculated by subtracting the NP-SH (GSH) from the T-SH [15].

### Assessment of antioxidants enzyme activities

Superoxide dismutase (SOD) activity was measured following the method of Madesh and Balasubramanian [16]. One unit of SOD activity was defined as the quantity of enzyme that inhibited pyrogallol autooxidation by 50% under the given experimental conditions. Catalase activity was estimated by measuring the breakdown of hydrogen peroxide in the reaction mixture using a spectrophotometer at the wave length of 240 nm and the values were expressed in mmol H<sub>2</sub>O<sub>2</sub> utilized/mg of protein [17]. The enzyme *Glutathione reductase (GR) activity* was assayed by the method of Goldberg and Spooner [18]. The activity has been expressed as μmol NADPH utilized/min/mg protein. Protein concentrations were determined by the method of Lowry *et al.* [19] calibrated with bovine serum albumin.

### Histological Examination

Tissues for histological examination were taken from liver and fixed in 10% neutral buffered formalin. The fixed tissues were processed for paraffin embedding, sectioned at 4-5 μ thickness and stained with haematoxylin-eosin.

### Statistical analysis

Data have been expressed as mean ± SEM. Statistical analysis of data was performed using SPSS 11.0.1 software. Data were analyzed by ANOVA and means were compared with Duncan's multiple comparison *post-hoc* test. A value of  $p < 0.05$  was considered statistically significant.

## Results

### Effect of WRE and ABE on lipid peroxidation

The MDA concentrations, an end product indicative of the degree of lipid peroxidation and thiols status in brain are shown in Table 1. MDA levels in brain showed increasing trend starting from 3 weeks onwards in the OTA fed groups as compared to control. Amelioration groups revealed significantly lower MDA levels. Brain thiol (T-SH, GSH and PB-SH) levels in OTA-exposed group significantly decreased at 6 and 9 weeks as compared with those of control values respectively. WRE alone and /or ABE, significantly elevated thiol status than that in group treated with OTA.

**Table 1:** Ameliorative effect of alcoholic extract of WRE and ABE on OTA-induced Lipid peroxidation and thiol pool (Means  $\pm$  S.E) in brain of male rats for 9 weeks

Parameters	Interval	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII
LPO	3wk	19.71 $\pm$ 3.28 <sup>b</sup>	32.59 $\pm$ 4.03 <sup>aB</sup>	22.31 $\pm$ 2.59	21.85 $\pm$ 1.95 <sup>b</sup>	29.11 $\pm$ 3.47 <sup>B</sup>	26.51 $\pm$ 4.82 <sup>B</sup>	25.37 $\pm$ 2.35 <sup>B</sup>	22.65 $\pm$ 2.22 <sup>B</sup>
	6wk	22.85 $\pm$ 2.69 <sup>d</sup>	56.91 $\pm$ 6.41 <sup>aA</sup>	23.85 $\pm$ 2.32 <sup>d</sup>	22.77 $\pm$ 2.16 <sup>d</sup>	50.50 $\pm$ 4.97 <sup>abA</sup>	45.35 $\pm$ 7.76 <sup>abcA</sup>	40.70 $\pm$ 6.25 <sup>bcA</sup>	32.66 $\pm$ 3.18 <sup>cd</sup>
	9wk	23.31 $\pm$ 3.61 <sup>d</sup>	70.91 $\pm$ 8.46 <sup>aA</sup>	20.71 $\pm$ 2.90 <sup>d</sup>	22.98 $\pm$ 2.06 <sup>d</sup>	62.91 $\pm$ 10.04 <sup>abA</sup>	50.50 $\pm$ 4.97 <sup>bcA</sup>	47.39 $\pm$ 5.09 <sup>bcA</sup>	39.15 $\pm$ 5.61 <sup>cdA</sup>
TSH	3wk	0.182 $\pm$ 0.015	0.155 $\pm$ 0.015	0.176 $\pm$ 0.015	0.186 $\pm$ 0.017	0.161 $\pm$ 0.007	0.167 $\pm$ 0.008	0.166 $\pm$ 0.011	0.162 $\pm$ 0.018
	6wk	0.178 $\pm$ 0.016 <sup>a</sup>	0.121 $\pm$ 0.011 <sup>c</sup>	0.171 $\pm$ 0.016 <sup>ab</sup>	0.18 $\pm$ 0.017 <sup>a</sup>	0.132 $\pm$ 0.014 <sup>bc</sup>	0.142 $\pm$ 0.009	0.141 $\pm$ 0.011	0.167 $\pm$ 0.009 <sup>ab</sup>
	9wk	0.172 $\pm$ 0.020 <sup>a</sup>	0.086 $\pm$ 0.013 <sup>c</sup>	0.166 $\pm$ 0.025 <sup>a</sup>	0.173 $\pm$ 0.019 <sup>a</sup>	0.106 $\pm$ 0.015 <sup>bc</sup>	0.138 $\pm$ 0.009 <sup>ab</sup>	0.151 $\pm$ 0.010 <sup>ab</sup>	0.154 $\pm$ 0.019 <sup>ab</sup>
GSH	3wk	0.118 $\pm$ 0.012	0.100 $\pm$ 0.011 <sup>A</sup>	0.114 $\pm$ 0.007	0.120 $\pm$ 0.013	0.104 $\pm$ 0.014	0.108 $\pm$ 0.015	0.106 $\pm$ 0.013	0.100 $\pm$ 0.011
	6wk	0.116 $\pm$ 0.012 <sup>a</sup>	0.070 $\pm$ 0.010 <sup>b</sup>	0.110 $\pm$ 0.015	0.116 $\pm$ 0.017 <sup>a</sup>	0.078 $\pm$ 0.012	0.086 $\pm$ 0.010	0.090 $\pm$ 0.012	0.098 $\pm$ 0.014
	9wk	0.112 $\pm$ 0.012 <sup>a</sup>	0.054 $\pm$ 0.010 <sup>cB</sup>	0.108 $\pm$ 0.019 <sup>ab</sup>	0.110 $\pm$ 0.012 <sup>a</sup>	0.068 $\pm$ 0.013 <sup>bc</sup>	0.090 $\pm$ 0.011	0.094 $\pm$ 0.008 <sup>ab</sup>	0.102 $\pm$ 0.012 <sup>ab</sup>
PBSH	3wk	0.064 $\pm$ 0.007	0.055 $\pm$ 0.009	0.062 $\pm$ 0.009	0.066 $\pm$ 0.009	0.057 $\pm$ 0.010	0.059 $\pm$ 0.007	0.06 $\pm$ 0.009	0.062 $\pm$ 0.009
	6wk	0.062 $\pm$ 0.007	0.051 $\pm$ 0.006	0.061 $\pm$ 0.008	0.064 $\pm$ 0.009	0.054 $\pm$ 0.008	0.056 $\pm$ 0.009	0.051 $\pm$ 0.006	0.059 $\pm$ 0.008
	9wk	0.06 $\pm$ 0.009 <sup>a</sup>	0.032 $\pm$ 0.007 <sup>b</sup>	0.058 $\pm$ 0.007	0.063 $\pm$ 0.007 <sup>a</sup>	0.038 $\pm$ 0.009	0.048 $\pm$ 0.010	0.057 $\pm$ 0.009	0.052 $\pm$ 0.009

LPO: Lipid peroxidation (nM MDA/g); MDA: Malondialdehyde; T-SH: Total thiol (mM/g); GSH: Reduced glutathione (mM/g); PB-SH: Protein bound thiol/sulphydryl (mM/g). Means bearing at least one common superscript small letters do not differ significantly between groups and capital letters do not differ significantly within groups ( $P < 0.05$ ). Group I (Control), Group II (OTA), Group III (ABE), Group IV (WRE), Group V (ABE+OTA), Group VI (WRE+OTA), Group VII (ABE+ WRE+OTA), Group VIII (ABE+ WRE+AC+OTA)

### Effect of WRE and ABE on antioxidants enzymes activities in brain

The values of various antioxidants enzymes in brain are presented in Table 2. The activities of antioxidant enzymes SOD, Catalase and Glutathione reductase were significantly diminished in the OTA-intoxicated group. Treatment with

neither ABE alone nor WRE alone did not allow the significant increase in antioxidant enzymes activities in brain as compared to OTA-treated rats. However, combination of both alcoholic extract and alcoholic extract with activated charcoal significantly increased the activities of antioxidant enzymes (SOD, CAT and GR) than OTA treated group.

**Table 2:** Activities different enzymatic antioxidants in brain of male rats co-exposed to OTA and alcoholic extract of WRE and ABE for 9 weeks

Parameters	Interval	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII
SOD	3wk	10.57 $\pm$ 0.62	9.45 $\pm$ 0.70 <sup>A</sup>	10.67 $\pm$ 0.95	11.31 $\pm$ 0.86	9.58 $\pm$ 0.63 <sup>A</sup>	9.93 $\pm$ 1.57	10.31 $\pm$ 1.03	10.75 $\pm$ 1.03
	6wk	10.51 $\pm$ 1.00 <sup>a</sup>	7.35 $\pm$ 0.84 <sup>b</sup>	10.04 $\pm$ 0.83	10.91 $\pm$ 0.67 <sup>a</sup>	7.96 $\pm$ 1.04	8.16 $\pm$ 1.04	8.60 $\pm$ 1.04	9.03 $\pm$ 0.72
	9wk	9.94 $\pm$ 0.62 <sup>a</sup>	5.49 $\pm$ 0.75 <sup>dB</sup>	9.71 $\pm$ 0.88 <sup>ab</sup>	10.10 $\pm$ 0.72 <sup>a</sup>	6.45 $\pm$ 0.71 <sup>cdB</sup>	7.19 $\pm$ 0.99 <sup>bcd</sup>	8.288 $\pm$ 0.98 <sup>abc</sup>	8.85 $\pm$ 1.21 <sup>abc</sup>
CAT	3wk	69.83 $\pm$ 5.57	60.51 $\pm$ 6.46	68.91 $\pm$ 4.74	70.71 $\pm$ 5.08	62.70 $\pm$ 4.89	64.89 $\pm$ 4.71	65.19 $\pm$ 4.51	67.55 $\pm$ 4.07
	6wk	69.11 $\pm$ 6.43 <sup>a</sup>	52.71 $\pm$ 6.49 <sup>b</sup>	67.97 $\pm$ 5.18 <sup>a</sup>	68.91 $\pm$ 4.64 <sup>a</sup>	55.75 $\pm$ 5.16	59.74 $\pm$ 5.24	61.25 $\pm$ 4.08	65.13 $\pm$ 5.14 <sup>a</sup>
	9wk	68.51 $\pm$ 4.40 <sup>a</sup>	45.61 $\pm$ 4.94 <sup>c</sup>	67.07 $\pm$ 4.94 <sup>a</sup>	67.97 $\pm$ 5.47 <sup>a</sup>	48.84 $\pm$ 5.15 <sup>bc</sup>	54.36 $\pm$ 3.43	59.88 $\pm$ 4.25 <sup>ab</sup>	62.31 $\pm$ 3.43 <sup>ab</sup>
GR	3wk	90.74 $\pm$ 9.15	79.15 $\pm$ 4.44 <sup>A</sup>	89.94 $\pm$ 9.66	91.14 $\pm$ 8.92	82.15 $\pm$ 4.22 <sup>A</sup>	84.14 $\pm$ 4.99	85.35 $\pm$ 6.47	87.14 $\pm$ 6.36
	6wk	90.16 $\pm$ 6.32 <sup>a</sup>	63.33 $\pm$ 4.72 <sup>bB</sup>	89.06 $\pm$ 7.12 <sup>a</sup>	90.56 $\pm$ 6.06 <sup>a</sup>	69.33 $\pm$ 4.77	77.56 $\pm$ 8.43	78.73 $\pm$ 6.74	84.76 $\pm$ 8.32 <sup>a</sup>
	9wk	89.83 $\pm$ 6.09 <sup>a</sup>	53.12 $\pm$ 4.19 <sup>dB</sup>	88.87 $\pm$ 6.45 <sup>a</sup>	89.23 $\pm$ 6.52 <sup>a</sup>	60.12 $\pm$ 4.95 <sup>cdB</sup>	68.63 $\pm$ 5.50 <sup>bcd</sup>	71.12 $\pm$ 4.47 <sup>bc</sup>	78.83 $\pm$ 5.98 <sup>ab</sup>

SOD: Superoxide dismutase (units/mg protein); CAT: Catalase (mmol H<sub>2</sub>O<sub>2</sub> utilized/min/mg protein); GR: Glutathione reductase ( $\mu$ mol NADPH utilized/min/mg protein). Means bearing at least one common superscript small letters do not differ significantly between groups and capital letters do not differ significantly within groups ( $P < 0.05$ ). Group I (Control), Group II (OTA), Group III (ABE), Group IV (WRE), Group V (ABE+OTA), Group VI (WRE+OTA), Group VII (ABE+ WRE+OTA), Group VIII (ABE+ WRE+AC+OTA)

### Effect of WRE and ABE on histopathology

No histopathological changes were found in brain of control rats. At 6 weeks onwards, however, blood vessels in the meninges and brain were variably congested with occasional perivascular cuffing. Shrinkage of neurons with increase perineuronal and perivascular spaces in cerebral cortex and loosening of cells at the interface of molecular and granular layer with thin cell density in granular layer and degeneration of purkinje cells in cerebellar folia were observed in three out of five cases. At 9 weeks, the brain changes were more prominent which included dilatation and engorgement of meningeal blood vessels, neuronal degeneration and shrinkage with occasional neuropil oedema. ABE treatments were shown less severe lesions with moderate degree of oedema of the neuropil, neuronal shrinkage with increased perineuronal and perivascular spaces at 6 weeks and mild congestion of meningeal blood vessels with WRE treatment. Alcoholic extract along with activated charcoal treated rats remained normal at all intervals.

### Discussion

In the present study, OTA induce oxidative stress in rat brain, showing increase of LPO and decreased antioxidants defense

levels. Furthermore, this change in oxidative stress indices was accompanied by significant histopathological changes in OTA treated rats suggesting the OTA-mediated oxidative damages in brain. Oxidative stress is believed to be an important mechanism of OTA-induced toxicity [20]. An increased lipid peroxidation level was observed during OTA treatment. Lipid peroxidation is a reaction of oxidative degeneration of polyunsaturated fatty acids. The increase in lipid peroxides due to the administration of OTA is supported by previous studies (Sava *et al.*, 2006a) as well. The significance of OTA-induced LPO on cellular damage was further evaluated by histopathological changes in brain where, neuronal degeneration, increased perineuronal spaces and oedema of neuropil were consistent with previous findings [21]. The edematous changes might be due to vascular damages, provoked by OTA. The wide spread distribution of OTA in the body [22] and its ability to pass through blood-brain barrier [1] further suggested a direct toxic effect of OTA on brain. Coexposures to alcoholic extracts with OTA prevent LPO on cerebral tissue by lowering MDA levels.

High reactive oxidants and low thiol proteins can result in damage to cellular macromolecules and subsequent cytotoxicity. GSH is an important antioxidant system in

cerebral tissue due to their ability to non-enzymatically neutralize ROS [23]. In the present study decrease of TSH and GSH in time dependent manner and it is believed as one of the immediate responses to oxidative stress [24]. Despite the depletion in a cumulative dose-dependent of total glutathione, the proportion of reduced glutathione relative to total glutathione remained relatively constant in each region with a trend towards an increase following chronic infusion with the highest dose of OTA were shown by Sava *et al.*, [2]. Restoration of thiols status by combination of alcoholic extracts suggesting that OTA is forming reversible bonds with thiols groups. Therefore, it is possible that may inactivate the OTA and, thus, promote its cellular excretion.

Oxidative stress results from imbalance between free radical generation and antioxidant defense system. Effects of OTA on antioxidant enzymes observed by several research group but meager in brain tissue. In the present study OTA cause decrease the activities of SOD, CAT and GR in brain. Depletion of antioxidant defense by inhibition of the Nrf-2 regulatory pathway may be a novel and probable mechanism, by which OTA may increase oxidative stress [25].

Treatment with neither ABE alone nor WRE alone did not allow the significant increase in antioxidant enzymes activities in brain as compared to OTA-treated rats. Either combination of both alcoholic extracts treatment or extracts with activated charcoal (AC) were found to increase the brain thiol antioxidant (TSH, GSH and PBTH) and antioxidant enzymes (SOD, CAT and GR) compared to OTA treated rats.

### Conclusion

The present results revealed that cerebral oxidative stress by OTA is due to decrease GSH, thiol levels and suppression of antioxidant enzyme activities accompanied by enhance LPO. The reversal of these biomarkers for oxidative stress by alcoholic extracts of *W. somnifera* and *A. sativum* is beneficial actions against OTA-induced toxicity in brain. Further studies should be directed toward testing the useful of these extracts in treatment and prevention of neurotoxic processes.

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