Withania somnifera (ASHWAGANDHA) ATTENUATES ANTIOXIDANT DEFENSE IN AGED SPINAL CORD AND INHIBITS COPPER INDUCED LIPID PEROXIDATION AND PROTEIN OXIDATIVE MODIFICATIONS

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SUMMARY

Withania somnifera is classified in Ayurveda, the ancient Indian system of medicine, as a rasayana, a group of plant-derived drugs which promote physical and mental health, augment resistance of the body against disease and diverse adverse environmental factors, revitalize the body in debilitated conditions and increase longevity. We investigated the effects of *Withania somnifera* on copper-induced lipid peroxidation and antioxidant enzymes in aging spinal cord of Wistar rats. The activity of glutathione peroxidase (GPx) decreased significantly in the spinal cord from adult to aged mice. Treatment with *Withania somnifera* successfully attenuated GPx activity and inhibited lipid peroxidation in a dose dependent manner. *Withania somnifera* inhibited both the lipid peroxidation and protein oxidative modification induced by copper. These effects were similar to those of

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superoxide dismutase and mannitol. The results indicate the therapeutic potential of *Withania somnifera* in aging and copper-induced pathophysiological conditions.

KEY WORDS

Withania somnifera, Ashwagandha, aging, glutathione peroxidase, spinal cord, rats

INTRODUCTION

Under normal conditions, free radicals and their quenching enzymes, pro-oxidants and antioxidants, remain in perfect homeostasis /1/. Disturbance of this balance contributes to deficits associated with aging and neurological disorders such as Alzheimer's disease, Parkinson's disease, stroke, etc. /2-8/. Various studies have shown a surge in free radical induced oxidative damage in the brain of aged animals but only a few such studies have been done on the spinal cord /9/. A specific impairment in the glutathione redox system in the form of accumulation of reduced glutathione (GSH) at the dendrites of motor neurons has been observed /10/. Since GSH acts as substrate for glutathione peroxidase (GPx), the accumulation of GSH at the dendrite might be due to the lowered GPx activity in the spinal cord of aged animals. One of the aims of the present study was to investigate age-associated changes in GPx activity in the spinal cord of aged rats.

The progressive accumulation of copper in hepatocytes of humans and animals may lead to hepatocellular necrosis /11/ and Wilson's disease /12-14/. Copper-induced peroxidation has also been implicated in other degenerative diseases, including Alzheimer's disease, amyloid lateral sclerosis and cardiovascular disorders /15-24/.

W*ithania somnifera* (Ashwagandha), a subtropical undershrub, belongs to plants grouped under rasayana in Ayuevedic medicine /25/. An inhibitory effect of *Withania somnifera* on lead-induced lipid peroxidation has been established in the kidney /26-27/. It protects against cadmium-induced hepatotoxicity, nephrotoxicity /28/ and inhibits iron-induced lipid peroxidation /29/. Clinical investigations have shown the positive effects of Ashwagandha in Parkinson's disease /30/. We investigated the effects of *Withania somnifera* on

GPx activity and lipid peroxidation in the spinal cord of rats. The effects of *Withania* on lipid peroxidation and protein oxidative modification by copper were also investigated.

MATERIALS AND METHODS

Materials

Withania somnifera extract was supplied by Lakshmi Natural Products Pvt. Ltd., Bombay. Bovine serum albumin (BSA), butylated hydroxytoluene (BHT), $CuCl_2$, 2,4-dinitrophenylhydrazine,5-dithio-bis(2-ni-trobenzoic) acid (DTNB), ethylenediaminete-triacetic acid disodium salt (EDTA), mannitol, lecithin (from egg yolk), mannitol, thiobarbituric acid (TBA), trichloroacetic acid (TCA), urea and superoxide dismutase (SOD) were purchased from Sigma Chemical Co., USA.

Male albino rats of 3 months, 6 months and 24 months were used to study the age dependent variations in GPx activity. All experimental procedures were in accordance with the guidelines for animal care of Kurukshetra University. 24 month-old animals were grouped into four groups of five animals each; one group served as control and the other three groups were injected (i.p.) with 50 mg/kg body weight, 100 mg/kg body wt and 150 mg/kg body wt of *Withania somnifera* for 1 month. After completion of the experiment, animals were sacrificed and the spinal cord was removed, quickly homogenized, and GPx activity /31/ and MDA content /32/ were quantified.

Inhibitory test on lecithin peroxidation

Lecithin peroxidation (LPO) induced by copper was determined by the following method. The test sample was added to the reaction mixture containing 2.5 mM lecithin and 250 mM $CuCl_2$ in 50 mM Tris-HCl buffer (pH 7.4) in a total volume of 1 ml. The mixture was incubated at 37°C for 15 min. Next, 2 ml of TBA reagent, which contained 0.37% TBA, 15% TCA, 0.04% BHT and 2% ethanol, was added to the reaction mixture. This mixture was heated at 100°C for 15 min. The solution was centrifuged at 3,000 rpm for 10 min. Absorbance of the supernatant at 535 nm was determined as malondialdehyde (MDA) /32/.

The inhibitory ratio of the test sample was evaluated by the following equation: Inhibitory ratio (%) = [MDA formed in absence of test sample – MDA formed in presence of test sample] / [MDA formed in absence of test sample] x 100.

Inhibitory test on protein oxidative modification

Albumin oxidative modification by copper was determined by the following method. The test sample was added to the reaction mixture containing albumin (10 mg/ml) and 100 mM $CuCl_2$ in 50 mM Tris-HCl buffer (pH 7.4) in a total volume 0.3 ml. The mixture was incubated at 37°C for 2 h. Next, 1.6 ml of 0.125 M phosphate buffer (pH 8.0) containing 12.5 mM ETDA and 10.0 M urea, and 0.1 ml of 50 mM phosphate buffer (pH 7.0) containing 10 mM DTNB, were added to the reaction mixture. This solution was allowed to stand at room temperature for 5 min. Absorbance was read at 412 nm as cysteine-SH residue /33/.

The inhibitory ratio of the test sample was evaluated by the following equation: Inhibitory ratio = [cysteine-SH residue in presence of test sample – cysteine-SH residue in absence of test sample] / [cysteine-SH residue before incubation – cysteine-SH residue in absence of test sample] x 100.

Calculation of 50% inhibitory concentration (IC_{50})

The inhibitory ratio was plotted against the log concentration, and these dose response data were used to calculate IC_{50} values using IC_{50} calculator (a web based program of BD Biosciences Discovery Labware).

Statistical analysis

Results were expressed as means ± SEM and were analyzed by χ^2 test and one-way ANOVA followed by Duncan's multiple range test to detect intergroup differences using the computer software 'Stata-7' (Stata Corporation, Texas, USA).

RESULTS

The activity of GPx did not change much from 3 months to 6 months but it decreased significantly from 6 months to 24 months of age (Fig. 1). Treatment with 100 mg/kg and 150 mg/kg body weight Ashwagandha increased GPx activity (Fig. 2) and reduced lipid peroxidation significantly (Fig. 3). However, 50 mg/kg body weight of Ashwagandha was not effective.

The inhibitory ratio of Ashwagandha on LPO (Fig. 4) was as high as 77.2 ± 4.4% at a concentration of 45 μg/ml and increased in a concentration dependent manner ($p < 0.05$).

The inhibitory ratios of mannitol and superoxide dismutase were 84.5 ± 5.8 % and 91.3 ± 6.5 % at a concentration of 25 x 10^2 μM and 25 x 10^2 U/ml, respectively.

The inhibitory ratio of Ashwagandha on protein oxidation increased significantly in a concentration dependent manner ($p < 0.01$) (Fig. 5).

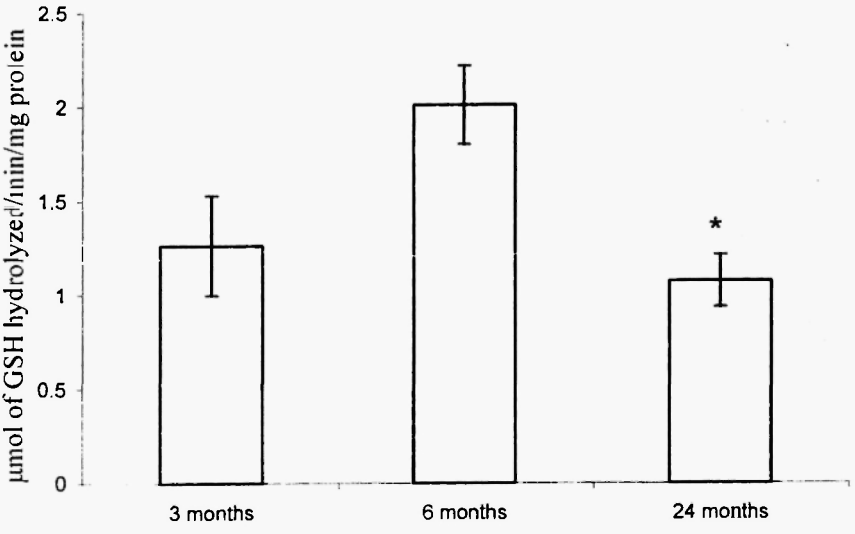

Fig. 1: Age dependent variations in glutathione peroxidase activity (enzyme unit is defined as μmol of reduced glutathione (GSH) hydrolyzed/ min/mg protein, n = 4, error bars represent SEM, * significantly different from 6 month old rats, F = 5.55, $p < 0.05$).

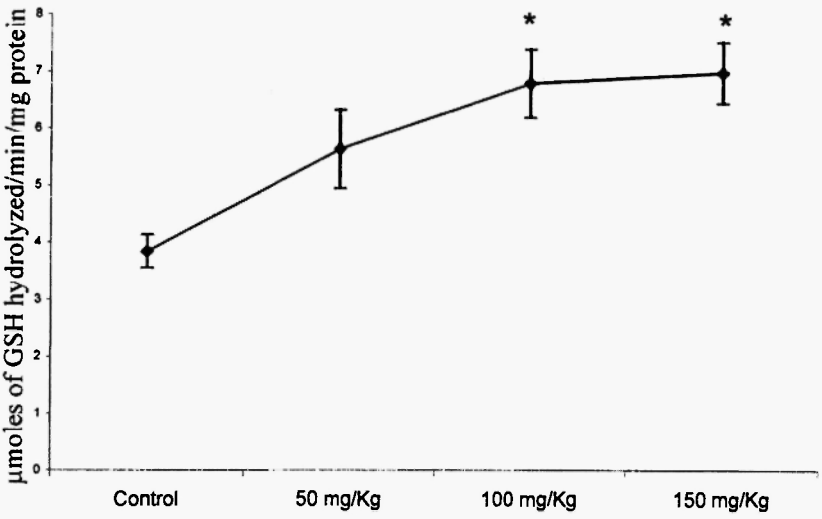

Fig. 2: Effect of Ashwagandha on glutathione peroxidase activity (enzyme unit is defined as µmol of reduced glutathione (GSH) hydrolyzed/min/mg protein, n = 5, error bars represent SEM, * significantly different from control, F = 7.120, p <0.01).

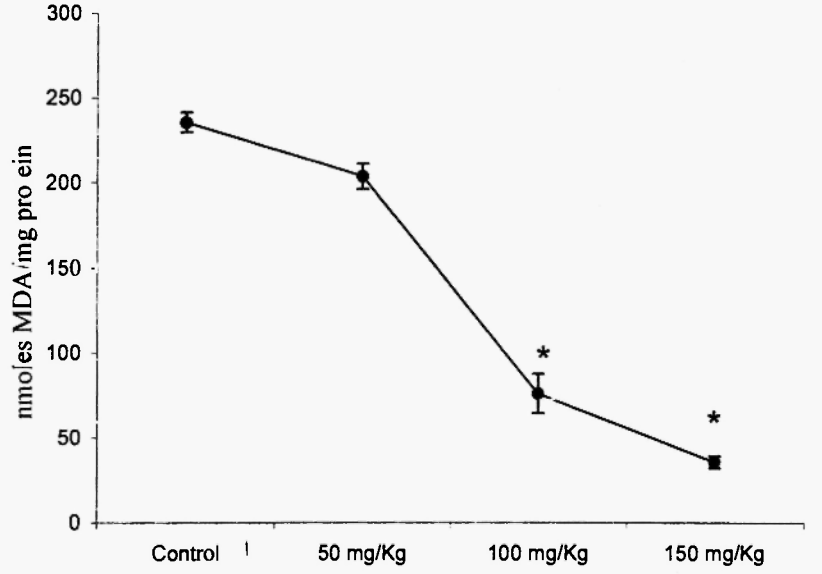

Fig. 3: Effect of Ashwagandha on lipid peroxidation (data represent nmoles of MDA/mg protein, n = 5, error bars represent SEM, * significantly different from control, F = 157.943, p <0.0001).

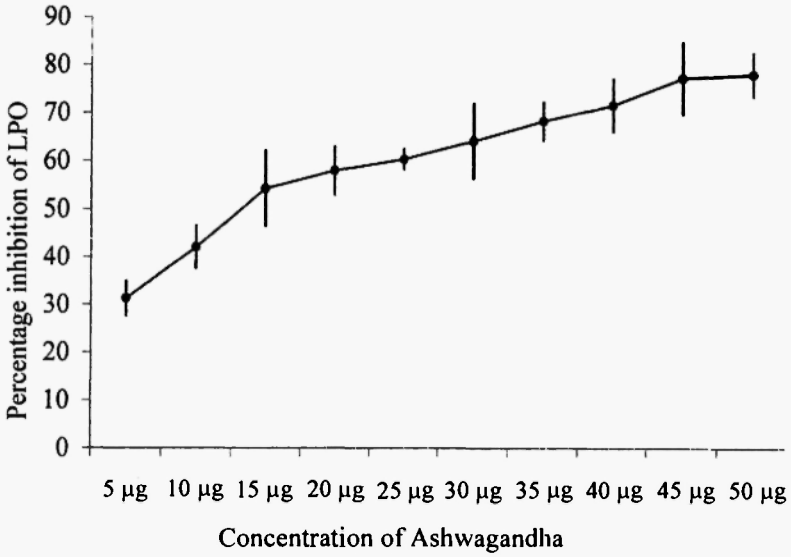

Fig. 4: Effect of Ashwagandha on percentage inhibition of lipid peroxidase (LPO) (χ^2 = 19.571, $p < 0.05$).

DISCUSSION

The present study revealed an age-dependent loss in GPx activity in rat spinal cord, which is in accordance with previous studies in guinea-pig spinal cord /9/. *Withania somnifera* is known to have several medicinal properties, including sedative, hypotensive, anti-aging, anti-inflammatory, immunomodulatory, bradycardiac and antiperoxidative effects /27,28/. Treatment with *Withania somnifera* inhibited LPO and increased GPx and catalase activities in stressed rats /34/. In the present investigation, treatment with *Withania* increased GPx activity in a dose dependent manner. The increase in GPx protects against peroxidative damage in aged animals, as evident by lowered MDA production in the spinal cords of Ashwagandha-treated rats. GPx can also limit the accumulation of peroxides by catalyzing the conversion of peroxidated lipids into alcohol, and this also contributed to the lower endogenous TBARS accumulation after treatment with *Withania*.

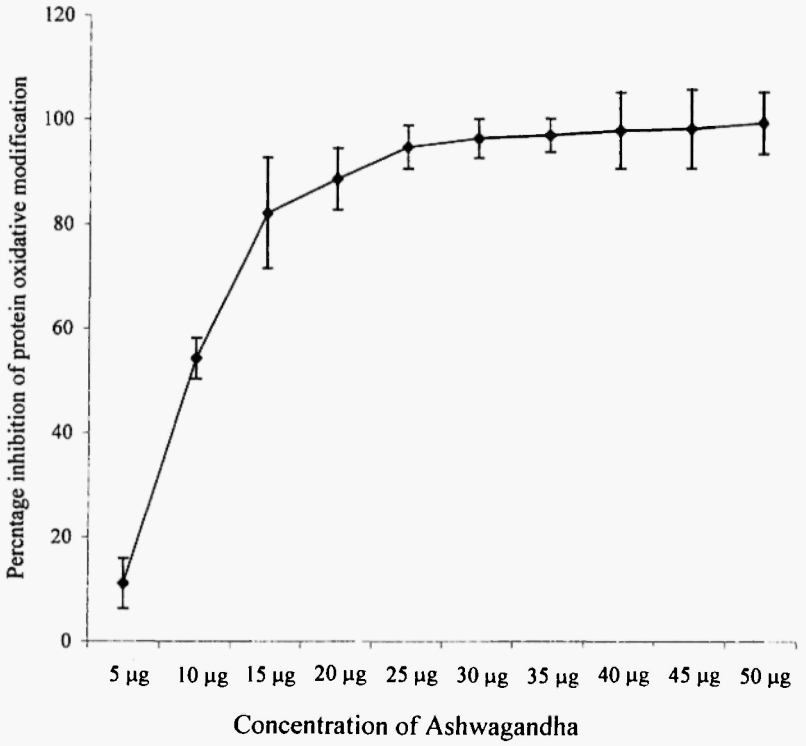

Fig. 5: Effect of Ashwagandha on protein oxidative modification by copper (χ^2 = 21.820, p <0.01).

The GPx redox system plays an important role in motor neuron survival in aged organisms /10/ and aging-related degeneration may, at least in part, be explained by a changed cellular redox status with decreased antioxidant capacity and/or increased oxidative stress /35/. The increased GPx activity after treatment with Ashwagandha might be of added significance in the aging spinal cord because GPx has a higher K_m for H_2O_2 than catalase /36/ and its activity has been positively correlated with SOD activity /37/; there are also reports of changes in glutathione redox status in Parkinson's and Alzheimer's diseases /38,39/.

Free radicals induced by copper damage neurons in Alzheimer's disease and produce lipid peroxidation in the erythrocyte membrane

and protein oxidation /17-19,29-30/. Increased free serum copper ions catalyze free radical reactions and result in hepatic dysfunction and hemolytic anemia in Wilson's disease /11-16/. Free copper ions in the biological system lead to non-enzymatic $OH^{\cdot}$ generation ($Cu^{+} + H_2O_2 = Cu^{2+} + OH^{\cdot}$). The $OH^{\cdot}$ and the oxidative substrate (glutamyl radicals) may trigger oxidative injury to cellular lipids and proteins /40/. In the present investigation, *Withania somnifera* successfully inhibited the lipid peroxidation and oxidative protein modification induced by copper, and these effects were similar to that of SOD and mannitol. Another herb, *Astragali* radix (commonly used in oriental medicine), has also shown a similar type of inhibitory effect on lipid peroxidation and oxidative protein modification induced by copper /41/. Taken together with these findings, the present study suggests that *Withania somnifera* is likely to have anti-aging and antioxidative activities on copper-induced oxidative stress, and its therapeutic potential against copper-mediated pathological conditions should be further explored.

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REFERENCES

1. Vohra BPS, Hui X. Taurine protects against carbon tetrachloride toxicity in the cultured neurons and in vivo. Arch Physiol Biochem 2001; 109: 90-94.
2. Michiels C, Raes M, Toussaint O, Remacle J. Importance of Se-glutathione peroxidase, catalase and Cu-Zn-SOD for cell survival against oxidative stress. Free Rad Biol Med 1994; 17: 235-248.
3. Halliwell B, Gutteridge MC. Oxygen radicals and the nervous system. Trends Neurosci 1985; 8: 22-34.
4. Cebbalos I, Javoy-Agid F, Delacourte A, De-Fossez A, Nicole A, Sinet P. Parkinson's disease and Alzheimer's disease: neurodegenerative disorders due to brain antioxidant system deficiency. In: Emerit I, Packer L, Auclair C, eds. Antioxidants in Therapy and Preventive Medicine. New York: Plenum Press, 1994; 493-498.
5. Makar TK, Cooper AJ, Tpfel-Grehl B, Thaler HT, Blass JP. Carnitine, carnitine acetyltransferase, and glutathione in Alzheimer brain. Neurochem Res 1995; 20: 705-711.

6. Nagy IZ. On the true role of oxygen free radicals in the living state, aging, and degenerative disorders. Ann NY Acad Sci 2001; 928: 187-199.
7. Droge W. Free radicals in the physiological control of cell function. Physiol Rev 2002; 82: 47-95.
8. Wickens AP. Ageing and the free radical theory. Respir Physiol 2001; 128: 379-391.
9. Vohra BP, Sharma SP, Kansal VK. Maharishi Amrit Kalash rejuvenates ageing central nervous system's antioxidant defence system: an in vivo study. Pharmacol Res 1999; 40: 497-502.
10. Ramirez-Leon V, Kullberg S, Hjelle OP, Ottersen OP, Ulfhake B. Increased glutathione levels in neurochemically identified fibre systems in the aged rat lumbar motor nuclei. Eur J Neurosci 1999; 11: 2935-2948.
11. Sternlieb I. Copper and the liver. Gastroenterology 1980; 78: 1615-1628.
12. Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DW. The Wilson disease gene is a putative copper transporting P-type ATPase similar to Menkes gene. Nat Genet 1993; 5: 327-337.
13. Hussain PA, Raja K, Amstad PA, Sawyer M, Trudel LJ, Wogan GN, Hofseth LJ, Shields GS, Billiar TR, Trautwein C, Hohler T, Galle PR, Phillips DH, Markin R, Marrogi AJ, Harris CC. Increased p53 mutation load in nontumorous human liver of Wilson disease and hemochromatosis: oxyradical overload diseases. Proc Natl Acad Sci USA 2000; 97: 12770-12775.
14. Klein D, Lichtmannegger J, Heinzmann U, Muller-Hocker J, Michaelsen S, Summer KH. Association of copper to metallothionein in hepatic lysosomes of Long Evans cinnamon (LEC) rats during the development of hepatitis. Eur J Clin Invest 1998; 28: 302-310.
15. Tachon P. Ferric and cupric ions requirement for single strand breakage by H_2O_2. Free Rad Res Commun 1989; 7: 1-10.
16. Meyers BM, Prendergast FG, Holman R, Kuntz SM, LaRusso NF. Alterations in hepatocyte lysosomes in experimental hepatic copper overload in rats. Gastroenterology 1993; 105: 1814-1823.
17. Multhaup G, Schlicksupp A, Hesse L, Beher D, Ruppert T, Masters CL, Beyreuther K. The amyloid precursor protein of Alzheimer's disease in the reduction of copper (II) to copper (I). Science 1996; 271: 1406-1409.
18. Multhaup G, Ruppert T, Schlicksupp A, Hesse L, Bill E, Pipkorn R, Masters CL, Beyreuther K. Copper-binding amyloid precursor protein undergoes a site-specific fragmentation in the reduction of hydrogen peroxide. Biochemistry 1998; 37: 7224-7230.
19. White AR, Multhaup G, Maher F, Bellingham S, Camakaris J, Hui Z, Bush AI, Beyreuther K, Masters CL, Cappai R. The Alzheimer's disease amyloid precursor protein modulates copper-induced toxicity and oxidative stress in primary neuronal cultures. J Neurosci 1999; 19: 9170-9179.
20. Yim MB, Kang JH, Yim HS, Kwak HS, Chock PB, Stadtman ER. A gain of function of an amyotrophic lateral sclerosis-associated Cu,Zn superoxide dismutase mutant: an enhancement of free radical formation due to a decrease in K_m for hydrogen peroxide. Proc Natl Acad Sci USA 1996; 93: 5709-5714.

21. Brown DR, Qin K, Herms JW, Madlung A, Manson J, Strome R, Fraser PE, Kruck T, Bohlens A, Schultz-Schaeffer W, Giese A, Westaway D, Kretzschmar HA. The cellular prion protein binds copper in vivo. Nature 1997; 390: 684-687.
22. Viles JH, Cohen FE, Prusiner SB, Goodin DB, Wright PE, Dyson HJ. Copper binding to the prion protein: structural implications of four identical cooperative binding sites. Proc Natl Acad Sci USA 1999; 96: 2042-2047.
23. Chan PC, Peller OG, Kesner L. Copper(II)-catalyzed lipid peroxidation in liposomes and erythrocyte membranes. Lipids 1982; 17: 331-337.
24. Parinandi NL, Zwizinski CW, Schmid HH. Free radical-induced alteration of myocardial membrane protein. Arch Biochem Biophys 1991; 289: 118-123.
25. Shivarajan VV, Balachanderan I. Ayurvedic Drugs and their Plant Sources. New Delhi-Bombay-Calcutta: Oxford and IBH Publication Co. Pvt. Ltd., 1994; 65.
26. Akbarsha MA, Vijenderakumar S, Kadalmani B, Girija R, Faridha A. Curative property of *Withania somnifera* Dunal root in the context of carbendazime-induced histopathological changes in liver and kidney of rat. Phytomedicine 2000; 7: 499-507.
27. Mishra LC, Singh BB, Dagenais S. Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha). Altern Med 2000; 5: 334-346.
28. Panda S, Kar A. Protective role of Aswagandha in cadmium induced hepato-toxicity and nephrotoxicity in male mouse. Curr Sci 1997; 72: 546-547.
29. Scartezzini P, Speroni E. Review on some plants of Indian traditional medicine with antioxidant activity. J Ethnopharmacol 2000; 71: 23-43.
30. Nagashayana N, Sankarankutty P, Namopoothiri MR, Mohan PK, Mohankumar KP. Association of L-DOPA with recovery following Ayurvedic medication in Parkinson's disease. J Neurol Sci 2000; 176: 124-127.
31. Anderson BB, Carandina G, Lucci M, Perry GM, Vullo C. Red-cell GSH regeneration and glutathione reductase activity in G6PD variants in the Ferrara area. Br J Haematol 1987; 67: 459-466.
32. Burge JA, Aust SD. Microsomal lipid peroxidation. Meth Enzymol 1988; 52: 302-310.
33. Ellman GL. Tissue sulfhydryl group. Arch Biochem Biophys 1959; 82: 70-77.
34. Bhattacharya A, Ghosal S, Bhattacharya SK. Antioxidant effect of *Withania somnifera* glycowihanolides in chronic footshock stress-induced perturbations of oxidative free radical scavenging enzymes and lipid peroxidation in rat frontal cortex and striatum. J Ethnopharmacol 2001; 74: 1-6.
35. Butterfield DA, Howard BJ, LaFontaine MA. Brain oxidative stress in animal models of accelerated aging and the age-related neurodegenerative disorders, Alzheimer's disease and Huntington's disease. Curr Med Chem 2001; 8: 815-828.
36. Christpherson BO. Formation of monohydroxypolynic fatty acid from lipid peroxides by glutathione peroxidase in the adult brain. J Neurochem 1968; 35: 1013-1024.

37. Bhuyan KC, Bhuyan DK. Superoxide dismutase of the eye: relative functions of superoxide dismutase and catalase in protecting the ocular lens from oxidative damage. Biochim Biophys Acta 1978; 542: 28-38.
38. Perry TL, Godin DV, Hansen S. Parkinson's disease: a disorder due to nigral glutathione deficiency? Neurosci Lett 1982; 33: 305-310.
39. Adams JD Jr, Klaidman LK, Odunze IN, Shen HC, Miller CA. Alzheimer's and Parkinson's disease. Brain levels of glutathione, glutathione disulfide, and vitamin E. Mol Chem Neuropathol 1991; 14: 213-226.
40. Toda S, Shirataki Y. Inhibitory effects of *Astragali* radix, a crude drug in Oriental medicines, on lipid peroxidation and protein oxidative modification by copper. J Ethnopharmacol 1999; 68: 331-333.