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Antioxidant activity of glycowithanolides from *Withania somnifera*

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Antioxidant activity of active principles of *Withania somnifera*, consisting of equimolar concentrations of sitoindosides VII-X and withaferin A, was investigated for their effects on rat brain frontal cortical and striatal concentrations of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Results were compared with effects induced by deprenyl, an agent with well documented antioxidant activity. Active glycowithanolides of *W. somnifera* (WSG) (10 and 20 mg/kg,ip), administered once daily for 21 days, induced a dose-related increase in SOD, CAT and GPX activity in frontal cortex and striatum, which was statistically significant on days 14 and 21, except with the lower dose of WSG on GPX activity, where the effect was evident only on day 21. The data were comparable to those induced by deprenyl (2 mg/kg/day, ip) with respect to SOD, CAT and GPX activities, which were evident by day 14. These findings are consistent with the therapeutic use of *W. somnifera* as an Ayurvedic *rasayana* and *medhyarasayana*. Antioxidant effect of active principles of *W. somnifera* may explain, at least in part, the reported antistress, immunomodulatory, cognition-facilitating, anti-inflammatory and anti-aging effects produced by them in experimental animals, and in clinical situations.

Withania somnifera Dunn., referred to as *Ashwagandha* in Ayurveda, finds extensive use in this system of medicine as a *rasayana* and *medhyarasayana*. *Rasayanas* are used to promote health and longevity by increasing defence against disease, arresting the aging process and revitalizing the body in debilitated conditions[1]. A subgroup of *rasayanas*, known as *medhyarasayanas*, are used to promote memory and intellect (*medhya*)[1]. Thus, *Withania somnifera* is reputed to promote physical and mental health[1], and can be classified in modern terminology as an adaptogen[2].

Clinical investigations with the *W. somnifera* root extracts indicate that it exerts significant anti-aging effect in normal healthy but aged subjects[3], has a positive effect on mental functions and memory[4], and has significant anxiolytic and antidepressant effects[4-6]. Beneficial effects of *W. somnifera* on memory and mood perturbations have a delayed onset and are usually evident after treatment for at least one month[4-6].

The likely active principles of W. somnifera are glycowithanolides (WSG), consisting of sitoindosides VII to X, and withaferin[7,8]. They have been shown to induce significant antistress[7], immunomodulatory[8,9] and cognition facilitating[10] effects. The latter effect was investigated in experimental models of Alzheimer's disease and WSG was found to reverse both the cognitive deficits and perturbed central cholinergic markers induced as a result of neurodegeneration produced by the neurotoxins[10]. *W. somnifera* has also been reported to inhibit the activity of acute phase reactants during inflammation[11] and to induce reduction in alpha-2-macrolbumin synthesis, unlike the conventional non-steroidal anti-inflammatory drugs[12].

Increased generation of oxidative free radicals, or impaired antioxidant defence mechanisms, have been implicated in the aging process, neurodegenerative conditions, including Parkinsonism and Alzheimer's disease, in chronic stress induced perturbed homeostasis, including immu-no-depression, in inflammation, diabetes mellitus, peptic ulcer and other diseased conditions[13]. Major oxidative free radical scavenging enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Deficient functioning of these enzymes leads to accumulation of toxic oxidative free radicals and consequent degenerative changes[13]. In the present

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study, the effect of subchronic (21 days) administration of WSG was investigated in two regions of rat brain, namely, frontal cortex and striatum, both being highly susceptible to oxidative free radical toxicity[14]. Deprenyl, was used as the standard antioxidant drug[15], for comparison.

Materials and Methods

Adult male Wistar rats (180-220g) were housed in colony cages, with free access to standard pellet chow and drinking water, at an ambient temperature of 25°±2°C and 45-55% RH. All drug administrations and sacrifice of the animals were done between 0900 and 1200 hrs.

An aqueous concentrate of *W. somnifera* roots was exhaustively extracted with chloroform to remove fatty materials and free withanolides. The aqueous solution was then spray-dried. Composition of sitoindosides VII-X and withaferin (collectively referred to as glycowithanolides) in spray-dried residue was determined by HPTLC (CAMAG assembly, software (C) 1990); scanner II, V. 3.14/PC/CATS Version; 3.05 lamp, deuterium, wavelength 254 nm; reflection mode; developer; (i) *n*-BuOH-AcOH-H_2O (4:1:2); (ii) $CHCl_3$-MeOH (90: 10)), using authentic markers[8]. Equimolar composition of the constituents, in an aqueous stock solution, was made by adding appropriate amounts of deficient compounds[8]. Combined formulation of *W. somnifera* glycowithanolides (WSG) was freely soluble in water and saline.

WSG (10 and 20 mg/kg) and (-)deprenyl (2mg/kg), dissolved in 0.9% saline, were administered in a volume of 1 ml/kg ip once daily for 7,14 or 21 days. Control animals received equivalent volume of vehicle through the same route. Animals were sacrificed by decapitation 1 hr after the last drug or vehicle administration on days 7, 14 or 21. Brain was removed and the frontal cortex and striatum was dissected out in each case[16]. Tissues were weighed and homogenized in 2 ml of ice-cold triple distilled water, and sonicated for 15 sec. Homogenates were then centrifuged (10.000 g, 2 min) and supernatants were used for estimation of enzyme activities.

SOD activity—Assay was based on the ability of SOD to inhibit spontaneous oxidation of adrenaline to adenochrome. Results are expressed as units (U) of SOD activity/mg protein. One unit of SOD activity induced approximately 50% inhibition of auto-oxidation of adrenaline[17].

CAT activity—Assay was based on the ability of CAT to induce disappearance of hydrogen peroxide, which was followed spectrophotometrically. One unit of CAT was defined as the amount of the enzyme required to decompose 1 μmol of peroxide per min, at 25° and *p*H 7.0[18].

GPX activity—Hydrogen peroxide was used as the substrate Sodium azide (1m*M*) was added to the reaction mixture in order to inhibit remnant CAT activity. One unit of GPX was defined as the amount of the enzyme decomposing 1 μmol H_2O_2 per min, at 25°C and *p*H 7.0[19].

Protein estimation was done by the method of Lowry *et al*[20].

Statistical analysis—Data were analysed by the Mann-Whitney U-test. *P* values lower than 0.05 were considered as statistically significant, when compared with vehicle-treated control group values.

Results

WSG (10 and 20 mg/kg, i.p,) induced a dose-related increase in SOD, CAT and GPX activities in frontal cortex and striatum of rats. The increase was, however, statistically significant only after 14 or 21 days of treatment in case of SOD and CAT, with both the doses of WSG, and in case of GPX with the higher dose of the drug. Effect of the lower dose of WSG on GPX activity was only evident after 21 days treatment. (-) Deprenyl (2 mg/kg, ip) produced increases in frontal cortical and striatal SOD, CAT and GPX, which were comparable to that induced by WSG (20 mg/kg, ip) (Tables 1-3).

Discussion

Free radical oxidative stress has been implicated in the pathogenesis of a variety of diseases, resulting usually from defective natural antioxidant defences. Potential antioxidant therapy should, therefore, include either natural antioxidant enzymes or agents which are capable of augmenting the function of these oxidative free radical scavenging enzymes[21]. By virtue of their proposed properties and clinical use in Ayurveda, *rasayanas* may provide potential therapeutic intervention against oxidative threats, both in health and disease[22]. Earlier studies from this laboratory have indicated that shilajit, an important

Table 1—Effects of *W. somnifera* glycowithanolides (WSG) and deprenyl on superoxide dismutase (SOD) activity in rat frontal cortex and striatum

Treatment (mg/kg, ip)	n	SOD activity (U/mg protein) mean ± SE					
		Frontal cortex			Striatum		
		Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Vehicle	14	13.8±1.2	14.1±2.2	12.9±1.6	16.4±2.6	16.8±2.6	17.1±1.8
WSG (10)	8	15.9±1.8	19.6±1.2[a]	21.2±0.9[b]	16.9±2.0	21.6±1.1[a]	23.6±1.2[a]
WSG (20)	8	16.6±1.9	23.4±0.8[c]	26.4±1.1[c]	18.9±1.6	24.9±1.1[b]	27.2±0.9
Deprenyl (2)	6	16.2±1.8	21.4±1.6[a]	28.2±1.1[c]	19.6±2.1	29.2±1.6[c]	33.2±1.2[c]

[a,b,c] indicate $P < 0.05$, <0.01 and <0.001, respectively, different from vehicle- treated group (Mann-Whitney U-test).

Table 2—Effects of *Withania somnifera* glycowithanolides (WSG) and deprenyl on catalase (CAT) activity in rat frontal cortex and striatum

Treatment (mg/kg, i p)	n	CAT activity (U/mg protein) mean ± SE					
		Frontal cortex			Striatum		
		Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Vehicle	14	14.6±2.6	15.2±1.9	15.8±1.6	19.2±2.2	19.8±1.6	20.2±1.4
WSG (10)	8	16.9±1.8	19.4±1.2[a]	23.4±1.2[b]	21.4±1.6	24.9±1.3[a]	29.9±1.8[b]
WSG (20)	8	17.8±1.9	23.4±1.6[b]	29.8±1.2[c]	24.6±2.0	30.6±1.4[c]	34.8±1.6[c]
Deprenyl (2)	6	16.8±1.6	19.6±1.8[a]	26.8±1.6[b]	23.4±1.9	26.4±1.9[a]	38.4±1.2[c]

[a,b,c] indicate $P < 0.05$, <0.01 and <0.001, respectively, different from vehicle- treated group (Mann-Whitney U-test).

Table 3—Effects of *W. somnifera* glycowithanolides (WSG) and deprenyl on glutathione peroxidase (GPX) activity in rat frontal cortex and striatum

Treatment (mg/kg, i p)	n	GPX activity (U/mg protein) mean ± SE					
		Frontal cortex			Striatum		
		Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Vehicle	14	0.052 ± 0.01	0.049 ± 0.009	0.056 ± 0.012	0.076 ± 0.011	0.074 ± 0.008	0.072 ± 0.012
WSG (10)	8	0.056 ± 0.008	0.064 ±0.012	0.084[a] ± 0.01	0.088 ± 0.012	0.098 ± 0.014	0.11[a] ± 0.016
WSG (20)	8	0.062 ± 0.009	0.076[a] ± 0.01	0.092[b] ± 0.009	0.084 ± 0.008	0.098[a] ± 0.009	0.14[a] ± 0.012
Deprenyl (2)	6	0.064 ± 0.009	0.072[a] ± 0.007	0.088[b] ± 0.006	0.092 ± 0.011	0.099[a] ± 0.012	0.12[b] ± 0.009

[a, b, c] indicate $P < 0.05$, <0.01 and <0.001, respectively, different from vehicle-treated group (Mann-Whitney U-test).

Ayurvedic *rasayana*, augments antioxidant activity in experimental animals[23-26].

In the present study, the active principles (WSG) of *W.somnifera*, were found to increase the cortical and striatal concentrations of the antioxidant enzymes, SOD, CAT and GPX. Most abundant oxidative free radicals generated in living cells are superoxide anions ($O_2^{\cdot}$-) and derivatives, particularly the highly reactive and damaging hydroxyl radical, which appears to act via peroxidation of membrane lipids. Superoxide is inactivated by SOD, the only enzyme known to use a free radical as a substrate. However, the free radical scavenging activity of SOD is effective only when it is followed by increases in CAT or GPX activity, since SOD generates hydrogen peroxide as a metabolite which is more toxic than oxygen radicals and has to be removed by CAT or GPX. Thus, a concomitant increase in CAT and / or GPX activity is essential if a beneficial effect from increase in SOD activity is to be expected[27-28]. Choice of the brain areas selected for this

study was based on evidence that they are highly vulnerable to oxidative stress induced damage[14].

Deprenyl, in the dose used in this study, has been shown to increase SOD and CAT activity in several brain areas, including the corpus striatum[15]. However, its effect on brain GPX activity remains controversial[15]. Deprenyl has been reported to arrest the progression of Parkinson's disease[29] and to retard the process of aging leading to dramatic increase in life-span in experimental animals[30]. In the present study, deprenyl was found to increase SOD, CAT and GPX activity only after subchronic administration, similar to that noted with WSG. Earlier studies have also shown that the free radical scavenging activity of deprenyl has a delayed onset, ranging from 2 to 3 weeks[15,23]. Likewise, studies have indicated that the antistress[7], immunomodulatory [8,9] and cognition-facilitating[10] actions of WSG are evident only after subchronic administration, ranging from 1 to 3 weeks.

The present findings indicate that increase in the oxidative free radical scavenging activity of WSG may be responsible, at least in part, for the antistress, immunomodulatory, nootropic, anti-inflammatory and anti-aging effects of *W. somnifera* and its active principles, as reported for drugs with anti-oxidative stress functions[21].

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