Effect of *Withania somnifera* Dunal Root Extract on Behavioral Despair Model in Mice: a Possible Role for Nitric Oxide

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Abstract- *Withania somnifera* (WS) possess anti-inflammatory and antioxidant properties. WS preparations have a potential therapeutic role in the central nervous system (CNS) related disorders in animal models. In this study, the possible protective effect of acute aqueous WS root extract on behavioral despair was explored and compared with fluoxetine, an antidepressant with selective serotonin (5-HT) reuptake inhibitor activity (SSRI). Further, the probable involvement of nitric oxide (NO) determined to measure immobility time in forced swimming test (FST) and tail suspension test (TST) in male mice. Immediately after assessment of locomotor activity, the immobility time was evaluated. WS was administered intraperitoneally (200, 400 mg/kg; *i.p.*) 60 min before the behavioral tests. To assess the involvement of NO in the possible protective effect of WS, a non-specific NO synthase inhibitor, NG-L-arginine methyl ester (L-NAME, 10 mg/kg, *i.p.*) was administered 30 min before the extract administration (400 mg/kg, *i.p.*), 90 min before the tests. Acute WS extract (200, 400 mg/kg, *i.p.*) dose-dependently decreased the immobility time in FST, $P<0.05$, $P<0.001$, respectively and 400 mg/kg proved the most effective dose and this dose was comparable to fluoxetine (20 mg/kg, *i.p.* WS (400 mg/kg, *i.p.*) also lowered the immobility measure in TST ($P<0.05$). However, these effects were not related to change in locomotor activity. Moreover, L-NAME (10 mg/kg, *i.p.*) did not influence the effect of the extract on the behavioral tests. As a consequence, the immobility time was virtually constant between the group received the extract (400 mg/kg) alone, and the group received L-NAME (10 mg/kg) before the extract. It is probable that NO does not mediate this beneficial effect, and WS may affect other neurochemical systems and pathways.

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Keywords: *Withania somnifera*; Nitric oxide; Open field test; Forced swimming test; Tail suspension test

Introduction

Depression is one of the most prevalent mental disorders associated with significant disability and mortality. Depression is affected around 21% of the world's population (1). The World Health Organization (WHO) anticipates that major depression disorder (MDD) will be the second-leading cause of global disability burden by 2020 (2). Nevertheless, many anti-depressants have various drawbacks such as slow response rate, late onset and other unwanted side-effects, such as sleep disturbance, sexual dysfunction and cognitive impairment (3).

Nitric oxide (NO), a free gaseous signaling molecule, is involved in the regulation of the nervous and immune systems. It has been suggested that NO participates in depression and anxiety disorders (4). The nitric oxide synthase (NOS) enzymes are widely distributed within the mammalian brain. NOS-positive neurons are located in the hippocampus, cerebral cortex and other encephalic regions (5). The involvement of neuronal nitric oxide synthase (nNOS) in the pathophysiological mechanism of depression-like behavior in rodents was demonstrated (6, 7). Over the last two to three decades, the 'inflammatory depression hypothesis' has attracted great attention. Chronic inflammation is often associated with clinical depression (8, 9).

Inducible nitric oxide synthase (iNOS) is involved in the modulation of depressive behaviors induced by

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unpredictable chronic mild stress. Chronic stress significantly induced depressive-like behaviors in mice. The levels of iNOS mRNA expression in the cortex and nitrites in the plasma of unpredictable chronic mild stress-exposed mice were markedly increased (10).

Recently, the worldwide use and research on phototherapy in depression have gained reputation, and remarkable advances have been achieved (11). For example, several phytomedicines, such as *Hypericum perforatum* (12), *Crocus sativus* (13) and *Lavandula angustifolia* (14) have proved antidepressant activities supported by clinical evidence.

Withania somniferous (L) Dunal (WS) is an evergreen, erect, branching shrub, 30-150 cm height. W. somnifera is popularly familiar as Ashwagandha or Winter Cherry (15) and commonly known as Asgand (16). It is (family Solanaceae) (17) found throughout the drier parts of India, Baluchistan, Pakistan, Afghanistan, Sri Lanka, Congo, South Africa, Egypt, Morocco and Jordan (18). Traditional medicine practitioners in India regard WS as the "Indian Ginseng". The roots are reported to contain alkaloids, amino acids, steroids, volatile oil, starch, reducing sugars, glycosides, hentriacontane, dulcitol, withaniol, an acid (m.p. 280-283odecomp.), and a neutral compound (m.p. 294-296o). The total alkaloidal content of the Indian roots has been ranged from 0.13 to 0.31 % though much higher yields (up to 4.3%) have been recorded (19).

WS has been shown to possess anti-inflammatory property in many animal models of inflammations like carrageenan-induced inflammation, cotton pellet granuloma and adjuvant-induced arthritis (CFA) (20). WS has been evaluated for its adaptogenic activity. Its coadministration with other drugs in animals exposed to a variety of biological, physical and chemical stressors was found to offer protection against these stressors (21, 22).

Administration of WS root extract was found to reduce the severity of pentylenetetrazole (PTZ)-induced convulsions (23). WS is known to modulate the oxidative stress markers in the body. The root extract significantly reduced the lipid peroxidation (24) and increased the superoxide dismutase (SOD) and catalase activity, thus proving a free radical scavenging property (25). The phytochemicals present in WS are responsible for overcoming the excitotoxicity and oxidative damage (26, 27). The active constituents of the plant (Withaferin A, Sitoindosides VII–X) are reported to have an antioxidant activity which may contribute at least in part to the antistress, immunomodulatory, cognition facilitating, anti-inflammatory and anti-ageing properties (28). Withaferin A exhibits fairly potent anti-arthritic and anti-inflammatory activities. Anti-inflammatory activity has been attributed to biologically active steroids, of which Withaferin A is a major component (29).

The major biochemical constituents of WS are steroidal alkaloids and lactones, a class of constituents together known as withanolides (steroidal lactones with ergostane skeleton) (30). The withanolides have the structural resemblance with the active constituents present in the plant Panax ginseng known as ginsenosides (31). The withanolides have a C28 steroidal nucleus with a C9 side chain, having six membered lactone ring (32, 33). Therefore, because of this WS is named as an "Indian Ginseng" (31, 19). So far, 12 alkaloids, 35 withanoloids, and several sitoindosides have been isolated, and their structures have been elucidated (34, 35). The various alkaloids include withanine, somniferine, somnine, somniferinine, withananine, pseudo-withanine, tropine, psuedotropine, 3-α-gloyloxytropane, choline, cuscohygrine, isopelletierine, anaferine and anahydrine. Two acyl steryl glucoside viz. sitoindoside VII and sitoindoside VIII, two glycowithanoloids viz. sitoindoside IX or sitoindoside X have been isolated from the root. It has been proposed that the cholinesterase inhibitory potential along with calcium antagonistic ability could make the withanolides as possible drug candidates for further study to treat Alzheimer's disease and associated problems (36).

Administration of active principles of W. somnifera, consisting of equimolar concentrations of sitoindosidesVII-X and Withaferin A, was found to increase superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activity in rat frontal cortex and striatum. Antioxidant effect of active glyco withanolides of WS (WSG) may explain, at least in part, the anti-inflammatory, immunomodulatory, anti-stress, anti-aging and cognition-facilitating effects produced by them in experimental animals, and in clinical situations. Administration of glycowithanolides of WS was found to suppress morphine- induced inhibition of intestinal motility and to attenuate the development of tolerance to the analgesic effect of morphine in mice (37). Repeated administration of Asgand, roots of WS, in mice, attenuated the development of tolerance to the analgesic effect of morphine (38).

The acute LD50 value of WS was found to be 465 mg/kg (332–651 mg/kg) in rats and 432 mg/kg (229–626 mg/kg) in mice (39). The extract had no profound effect on CNS or autonomic nervous system in doses of

up to 250 mg/100 g of mice in toxicity studies. However, it affected spontaneous motor activity in still higher doses.

The plant preparation has anti-inflammatory (40), anti-cancer (41, 42), anti-stress, and immunomodulatory (24, 43, 44), adaptogenic (45), CNS (46, 47), endocrine (48) and cardiovascular (49) activities, respectively.

In view of above reports and regarding the antioxidant and anti-inflammatory properties of WS and its main constituents, the present work was undertaken to represent effect of WS administration on behavioral despair and also somehow clarify NO role using behavioral evaluations, forced swimming test (FST) and tail suspension test (TST), in male mice. We suggest a possible involving mechanism for acute WS and our hypothesis was that NO may be a mediator involved in this protective effect which is capable of influencing the neurotransmitter systems in the brain

Materials and Methods

Drugs

Aqueous *Withania somnifera* root extract was prepared in School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran by Mohammad Kamali Nejad. NG-L-arginine methyl ester (L-NAME) and fluoxetine were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals and experimental groups

Male NMRI mice weighing 20-27 g (Pasteur Institute, Tehran, Iran) were used throughout the study. Animals were allowed free access to food and water. All behavioral experiments were conducted during the period between 10:00 and 14:00 AM with normal room light (12- h regular light/dark cycle) and temperature (22 ± 1◦C). The mice were handled as indicated in the criteria proposed by the Guide for the Care and Use of Laboratory Animals (NIH US publication, no. 23-86, revised 1985).

All the drugs were dissolved in saline and prepared immediately before the experiments. They were injected intraperitoneally (*i.p.*). Mice were divided (128) into 16 groups of 8. Randomly, 4 groups were assigned for FST and 4 groups for TST. Control groups received only the vehicle (saline; *i.p.*). Fluoxetine (20 mg/kg, *i.p.*) was applied as a reference drug (50). To assess the antidepressant-like effect of *W. somnifera*, 4 groups were assigned as treatment groups and given (200, 400 mg/kg; *i.p.*), 60 min prior to the behavioral tests. Eight groups were determined for antagonist administration and possible involvement of NO synthesis on the antidepressant-like activity of *W. somnifera* was studied using administration of an effective dose of *W. somnifera* (400 mg/kg; *i.p.*) with a non-effective dose of L-NAME (10 mg/kg, *i.p.*) (51). L-NAME was administered 90 min before the tests. Moreover, one group received only L-NAME.

Behavioral tests

Open-field test (OFT): locomotor activity

To ensure that alterations in the duration of immobility are not resultant from the changes that occur in motor activity, the locomotor behavior was assessed in an open-field test (52). The apparatus consisted of a Plexiglas box measuring 40 × 60 × 50 cm. The floor of the cube was divided into 12 equal squares. The animals were gently placed in the left corner of the field, and the number of squares crossed with all paws counted manually.

Forced swimming test (FST)

When animals are exposed to the FST, they typically adopt an immobile posture, which is thought to reflect a state of behavioral despair or helplessness (53) and the decrease in immobility time is used as an index of antidepressant activity (54). Immediately after OFT, mice were individually placed in an open cylindrical container (diameter 10 cm, height 25 cm) containing 19 cm of water at 23 ± 1°C. Mice were allowed to swim for 6 min. The duration of immobility was recorded manually using a stopwatch during the next 4 min of the 6 min duration of the test (55). Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

Tail suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method described by Steru *et al*., (56). Briefly, mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded manually using a stopwatch during a 6 min period (57).

Statistical analysis

Statistical analysis was performed between groups by One-Way ANOVA followed by *Tukey*'s post-test (SPSS, version 18). A value of $P< 0.05$ was considered to be significant.

Results

Effect of aqueous *W. somnifera* root extract on FST and OFT in male mice

Injection (*i.p.*) of WS root extracts (200 mg/kg) and (400 mg/kg), dose-dependently reduced the immobility time in the FST in a significant manner $P<0.05$ and $P<0.001$, respectively. Moreover, the antidepressant-like effect of the extract (400 mg/kg) was comparable with fluoxetine (20 mg/kg) (Figure 1a). On the other hand, the extracts (200 mg/kg) and (400 mg/kg) exerted no significant impact on OFT (Figure 1b).

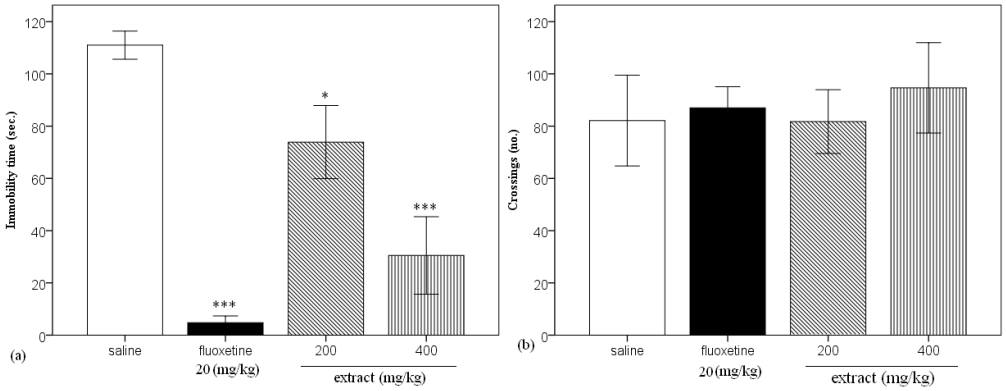

Figure 1. Effect of *Withania somnifera* root extract (200, 400 mg/kg) on FST (a) and OFT (b) in mice. * $P<0.05$ and *** $P<0.001$, significantly different from saline.

Effect of aqueous *W. somnifera* root extract on TST in male mice

Injection (*i.p.*) of WS root extract (400 mg/kg), markedly lowered the immobility time in the TST $P<0.05$. (Figure 2).

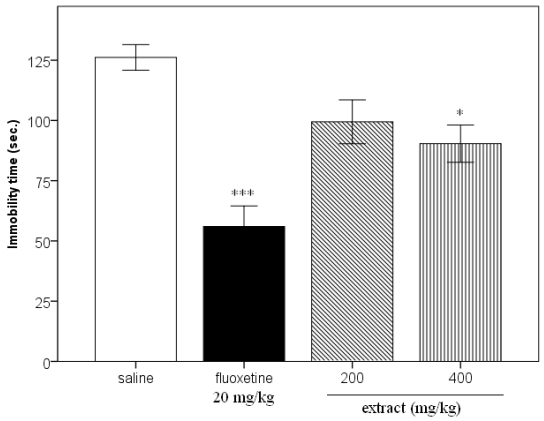

Figure 2. Effect of *Withania somnifera* root extract (200, 400 mg/kg) on TST in mice. * $P<0.05$ and *** $P<0.001$, significantly different from saline.

Effect of L-NAME, a nonspecific NOS inhibitor, on antidepressant-like effect of aqueous W. somnifera root extract on FST and OFT in male mice

Injection (*i.p.*) of a non-effective L-NAME dose (10 mg/kg) did not change the protective effect of WS root extract (400 mg/kg). The groups which received L-NAME (10 mg/kg), 30 min before the extract administration; 90 min before the tests, compared to the groups which received only the extract (400 mg/kg) showed no significant difference in the FST and TST. Also, administration of L-NAME (10 mg/kg) alone did not prove any marked difference (Figures 3, 4). On the whole, L-NAME (10 mg/kg) influenced neither the immobility times (Figs. 3a, 4) nor locomotor activity (Fig. 4) of the mice treating with the extract (400 mg/kg).

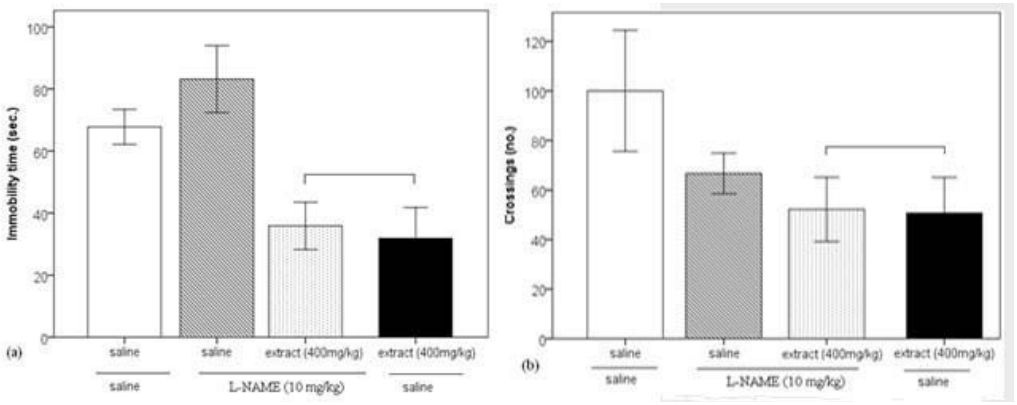

Figure 3. Effect of L-NAME (10 mg/kg) on the protective effect of *Withania somnifera* root extract (400 mg/kg) in FST (a) and OFT (b) in mice.

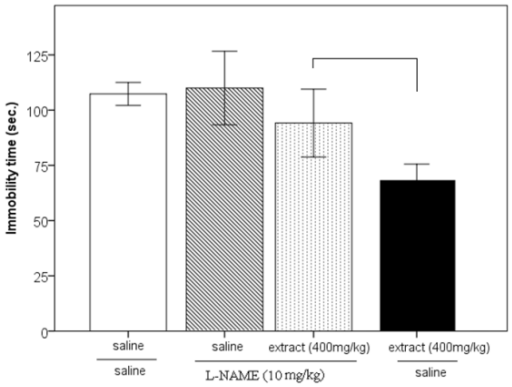

Figure 4. Effect of L-NAME (10 mg/kg) on the protective effect of *Withania somnifera* root extract (400 mg/kg) in TST in mice.

Discussion

In this study, the possible neuroprotective effect of acute aqueous WS. root extract on behavioral despair was explored. Further, the involvement of NO in this probable effect was determined to measure immobility time in forced swimming test (FST) and tail suspension test (TST) in male mice.

We showed for the first time that acute WS administration dose-dependently decreased the immobility time in FST and 400 mg/kg proved the most effective dose comparable to fluoxetine (20 mg/kg, *i.p.*). WS (400 mg/kg, *i.p.*) also lowered the immobility measure in TST thereby attenuated the behavioral despair. Fortunately, this central effect was not related to any change in locomotor activity, and the extract did not influence the generalized motor activity of the animals.

Moreover, current study indicated that L-NAME (10 mg/kg) did not influence the effect of the extract on the behavioral tests. As a result, the immobility time was virtually constant between the group received the extract (400 mg/kg) and the group received L-NAME (10 mg/kg) as well as the extract in both the tests.

Virtually consistent with our study, the anti-stressor effect of Asgand was investigated in rats using cold water swimming stress test and the drug treated animals showed better stress tolerance (44). A withanolide-free aqueous fraction isolated from the roots of WS exhibited anti-stress activity in a dose-dependent manner in mice (29).

WS preparations have been found to have a potential therapeutic role in almost every CNS related disorders. They are reported to modulate the GABAergic [(γ-amino-butyric acid (GABA)] (58, 23) or cholinergic (59) neurotransmission, accounting for various CNS related disorders (60). The active principles of WS, sitoindosides VII–X and withaferin A (glycowithanolides), have been extensively tested for antioxidant activity against the major free-radical scavenging enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) levels of frontal cortex and striatum of the rat brain. Active glycowithanolides of WS (10 or 20 mg/kg., *i.p.*) when administered chronically, an increase in all enzymes was observed (28). Recent studies have also shown the anti-Parkinson's like activity of WS, thus, possibility raises to modulate dopaminergic system in the brain (46). It is known that immobilization stress for

14 h causes 85% degeneration of the cells in the CA(2) and CA(3) subareas of the hippocampal region as compared to control rats. Pretreatment with root extract of WS significantly reduced (80%) the number of degenerating cells in both the areas, demonstrating the neuroprotective effects of the plant preparation (61).

A polyherbal medicine consisting of a standardized extract of *W. somnifera*, *Oscimum sanctum*, *Asparagus racemosus* and *Emblica Officinalis* is widely prescribed as an anti-stress formulation in the Indian system of medicine (62).

Administration of a methanolic extract of the root of the Indian ginseng, WS Dunal, prevents acquisition and expression of morphine-elicited conditioned place preference (CPP) in mice, at doses at which it fails to affect spontaneous motor activity, morphine-elicited hyperlocomotion, and spatial memory. In addition, it also demonstrated that one or more constituents of WS bind to GABAB receptors, suggesting their involvement in the observed behavioral effects. It has been proposed that the cholinesterase inhibitory potential along with calcium antagonistic ability could make the withanolides as possible drug candidates for further study to treat Alzheimer's disease and associated problems (36). It is known that immobilization stress for 14 h causes 85% degeneration of the cells (dark cells and pyknotic cells) in the CA(2) and CA(3) subareas of the hippocampal region as compared to control rats. Control rats were maintained in completely, nonstressed conditions. Pretreatment with root extract of WS significantly reduced (80%) the number of degenerating cells in both the areas, demonstrating neuroprotective effects of the plant preparation (61).

Asgand root extract showed a reduction in severity of motor seizures induced by electrical stimulation in right basolateral amygdaloid nuclear complex through bipolar electrodes. The protective effect of Asgand extract in convulsions has been reported to involve GABAergic mediation (63). The total alkaloids produced a taming and a mild depressant effect (tranquillizer-sedative type) on the CNS in several experimental animals (64). Systemic administration of Asgand root extract led to differential effects on acetylcholinesterase (ACHE) activity in basal forebrain nuclei. Slightly enhanced ACHE activity was found in the lateral septum and globus pallidus. Asgand root extract affects preferentially events in the cortical and basal forebrain cholinergic signal transduction cascade. The drug-induced increase in cortical muscarinic acetylcholine receptor capacity might partly explain the cognition-enhancing and memory-improving effects of extract from WS observed in animals and humans (59).

To conclude, exact mechanisms underlying the antidepressant action of WS cannot be clarified at the moment due to the presence of a large number of phytochemicals. The active constituents of the plant (Withaferin A, Sitoindosides VII–X) are reported to have an antioxidant activity which may contribute at least in part to the observed antidepressant-like effect. Nevertheless, the effect may be more attributed to the presence of Withaferin A as a major component and the attenuation of oxidative stress and inflammation. However, further study will be needed to extend these results by evaluating the active constituents of the plant separately rather than the whole extract. Further, examine another probably involved systems such as dopaminergic, GABAergic and cholinergic systems employing selective antagonist.

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