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Comparative Behavioral Activity of Methanolic and Aqueous *Withania somnifera* Root Extracts in Stressed Rats

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In the present study, the effect of *Withania somnifera* extracts prepared by two different methods on behavioral parameters assessed using open field exploratory behavior, behavioral despair and passive avoidance tests were compared in young and old stressed Wistar rats. Stress was induced on the animals by giving 30 intermittent shocks for 3 s consecutively for 7 d. *W. Somnifera* extracts prepared with 50% methanol and solvent containing water, *ghee* and honey were administered orally as fine suspension during the shock period. The results revealed that stress produced depression anxiety and retention deficit in young and old rats. Administration of *W. Somnifera* methanolic extract 250 mg/kg during shock period in young and old rats attenuated the stress-induced depression and enhanced memory. *W. Somnifera* traditional extract 250 mg/kg produced memory enhancement in both control and stressed young and old rats. Both the *W. Somnifera* extracts failed to reverse the stress-induced anxiety. It can be concluded that in comparison to methanolic extract, traditional extract was found to be more active in memory enhancement than anxiolytic and antidepressant activity.

Roots of *Withania somnifera* (WS) Dunal (Solanaceae), most commonly known as *Ashwagandha*, has been used as a valuable drug in Ayurveda and Unani as adaptogen, nervine tonic, sedative, antiinflammatory and cognitive enhancer. The major constituent of the plant withanolides were found to be responsible for the pharmacological activities[1]. The methanolic extract of WS produced anticonvulsant activity by acting through GABA receptors[2], and ethanolic extract produced antitumor activity[3]. It has been observed that in traditional usage, herbs having CNS action were found to have better activity when administered along with *ghee* or honey[4]. However proper pharmacological validation for this traditional extract preparation is not available. Being the extract prepared with *ghee* and honey have shown better CNS activity, in the present study, WS extract prepared with the mixture of water:honey:*ghee* (*W. Somnifera* traditional extract WST) was evaluated for anxiolytic, antidepressant and cognitive function in stressed rats and the effect was compared with methanolic extract of WS (WSM). Since Ashwagandha is known for its adaptogenic activity, stress model was selected. Further, stress has varied response on aging; effect of these extracts in one year old rats was also studied.

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MATERIALS AND METHODS

Induction of stress:

Wistar rats of either sex both young (7 w, 75-80 g) and old (12 mo, 300-350 g) were used for the study. Animals were procured from the Central Animal House of the Institute. All animal experimental protocols have been cleared by the Institutional Animals Ethics Committee. Rats were housed in a group of 5-6, in colony cages at an ambient temperature of 25±2° and 45-55% relative humidity with 12 h light/dark cycle. They had free access to pellet chow (Brook Bond, Lipton, India) and water *ad libitum*. Repeated inescapable shock was used to induce stress in rats. Foot shocks

were given in a one compartment box made of plexi glass. Shock schedule was as follows: rats were placed individually on foot shock chamber and 30 intermittent shock (0.2 mA) for 3 s of every minute for 30 min period daily for 7 d was given to produce chronic unpredictable stress. Control rats were placed in the test chamber but received no shocks. Rats were exposed only once to every experiment. Dried WS traditional extract prepared using the vehicle consisting of water:ghee:honey in the proportion of 60:7.5:32.5 (WST; 250 mg/kg) 50% methanolic extract (WSM; 250 mg/kg) and diazepam (0.5 mg/kg) were prepared as fine suspension in 0.3% CMC and administered orally during the shock period[5].

Behavioral assessment; Open field exploratory behavior test:

An open field apparatus similar to that of Bronstein[6] was used to study the open field exploratory behavior of rats. It was made of plywood and consists of a square (61x61cm) with high walls (61 cm). The entire apparatus was painted black except for 6 mm white lines that divided the floor into 16 squares. The open field was lighted by a 100 W bulb focusing onto the field from a height of about 100 cm from the floor. The entire room, except the open field, was kept dark during the experiment. Each animal was centrally placed in the test apparatus for 5 min and the following behavioral aspects of anxiety were recorded. Ambulation, which was measured in terms of the number of squares crossed by the animal. Rearing was measured by counting the number of times the animal stood on its hind limbs. Self grooming was the number of times the animal made these responses such as grooming of the face, licking/washing and scratching the various parts of the body and defecation which is the number of fecal boli excreted during the period and immobility period.

Behavioral despair test:

The rat was placed in a chamber (45x20 cm) containing 25 cm water (25±2°), so that the rat could not touch the bottom of the cylinder with its hind limb or tail, or climb over the edge of the chamber. Two swim sessions were conducted, an initial 15 min pre test, followed by a 5 min test 24 h later. The period of immobility (remained floating in water without struggling and making only those movements necessary to keep its head above water) during 5 min test period was noted[7].

Passive avoidance step through behavioral test:

The step through passive avoidance behavior was evaluated by following the procedure of King and Glasser[8]. The light dark box apparatus was fabricated locally. It had two walls of transparent plexi glass. It was divided into two equal compartments (30x25x30) by a plexi glass wall with a 10x10 cm opening in the center. A guillotine door between the two compartments controlled the opening. The light compartment was painted white and it was illuminated by a 15 w lamp. The interior of the dark chamber was painted black and had a ceiling. Each compartment had a copper grid floor. To ensure electrical separation, there was a 1.5 cm gap between the two floors in the light dark box at the opening between the two chambers. On day 1, rat was placed in the white box and the time taken to enter into the dark box was noted. As soon as the rat entered into the dark box guillotine door was closed and an electric shock (0.5 mA, 3 s) was delivered. Then rat was replaced to its home cage. On the following day (24 h retention interval) each rat was again placed in the white box and was given a 5 min inhibition period. Latency to step through into the dark chamber was recorded. Electric shock was not delivered on day 2. If the animals remained in the white box for 5 min test period, the maximum score of 300 s was assigned. On day 9 (after a gap of one week) latency to step through was again recorded to test the retention of the passive avoidance learning. The retention scores were obtained for each animal by calculating the inflexion ratio by the formula, Inflexion ratio=L_1-L_0/L_0,, where, L_0 is the initial step through latency in s, and L_1 is the step through latency in s after 24 h or 1 w retention intervals.

Statistical analysis:

Data were expressed as mean±SE and were subjected to non-parametric Kruskal Wallis test followed by Mann Whitney U test and 95% probability level was considered as test of significance.

RESULTS

Open field exploratory behavior test:

Stressed young and old rats exhibited decreased ambulation, increased grooming, immobility and fecal excretion in the open field exploratory behavior indicates anxiety in these animals. Administration of WSM and WST extracts failed to reverse the stress induced anxiety. In control old rats WSM and WST (250 mg/kg) significantly increased the ambulation, rearing and decreased immobility period indicate anxiolytic activity of these extract. In control young rats WS extracts did not produce significant effect on the exploratory behavior (Table 1).

TABLE 1: COMPARATIVE EFFECT OF *WITHANIA SOMNIFERA* EXTRACTS ON OPEN FIELD AND BEHAVIORAL DESPAIR TEST.

Group	Ambulation	Rearing	Grooming	Immobility period	Fecal pellets	Swim test Immobility (s)
Non-stressed						
Young	55.2±5.5	14.0±1.4	4.7±1.1	37.0±5.3	1.0±0.3	84.3±5.2
DZP (0.5)	79.3±5.3[a]	21.5±1.8[a]	6.5±1.0	28.5±1.6	4.2±0.8[a]	95.5±10.5
WSM (250)	53.6±6.3	6.1±1.7[a]	11.8±0.8[a]	45.0±3.9	2.8±1.4	45.2±5.2[a]
WST (250)	60.8±4.3	8.6 ± 1.3[a]	6.3±2.3	30.5± 5.3	3.0±1.6	60.6±9.6
Old	38.2±3.3	6.5±0.7	5.5±0.6	77.5±14.2	2.8±1.7	98.6±15.2
DZP (0.5)	49.8±5.7[a]	13.6±2.3[a]	3.8±0.6	46.3±4.2[a]	5.9±0.6[a]	110.5±7.5
WSM (250)	62.3±9.2[a]	11.5±3.3[a]	5.0±3.3	45.3±4.1[a]	0.0±0.0	58.6±5.9[a]
WST (250)	50.6±5.6[a]	9.3±1.2	4.2±0.9	40.6±5.9[a]	3.1±0.6	78.3±10.6
Stressed						
Young	39.6±5.5	16.7±4.7	11.0±1.0	98.8±2.8	3.25±1.18	145.3±9.6
DZP (0.5)	48.2±3.6[a]	10.1±1.8	6.3±0.8[a]	40.8±2.5[a]	0.86±0.12[a]	86.2±4.6[a]
WSM (250)	39.7±2.9	8.2±1.1[a]	10.2±1.1	91.0±12.0	2.25±0.48	60.2±7.6[a]
WST (250)	36.2±4.4	9.5±0.6[a]	12.2±2.0	97.7±15.3	3.75±1.10	105±12.6
Old	28.5±2.6	11.7±1.1	9.7±1.1	176±19.1	4.75±2.13	141±15.3
DZP (0.5)	39.6±4.2[a]	8.2±1.2[a]	7.2±0.3	125±17.5[a]	3.00±0.82	70.2±5.3[a]
WSM (250)	22.0±6.7	5.2±2.2[a]	2.2±0.2[a]	163±22.3	4.25±0.48	79.5±10.0[a]
WST (250)	29.2±13.0	4.7±1.4[a]	4.0±0.9[a]	212±19.9	4.25±1.75	112±14.3

N=6 in each group. Superscripts a denotes statistical significance in comparison to vehicle treated groups at $p \leq 0.05$ (Mann Whitney, 'U' test).

Behavioral despair test:

Stress significantly elevated the immobility period in swim test denotes development of depression. Treatment of WSM (250 mg/kg) during stress period significantly reduced the immobility period in both young and old stressed animals. Interestingly, the level of antidepressant activity of WSM in stressed young and old rats was found to be significantly more in comparison to WST. Further, in control young and old rats, WSM produced significant antidepressant activity where as WST failed to show this activity (Table 1).

Passive avoidance step through behavioral test:

Retention deficit noticed in the step through passive avoidance response in the stressed young and old rats was found to be significantly reversed by WSM and WST (250 mg/kg) treatment as indicated by retention scores on day 2 (IF1) and day 9 (IF2). Interestingly, WST treated rats exhibited significantly increased retention scores in comparison to WSM treated rats indicate better efficacy of WST on memory in stressed condition. Similar findings were observed in control animals (Table 2).

DISCUSSION

The results revealed that, 30 min intermittent shock for 7 d produced anxiety, depression, decreased or no alteration in 24 h and 1 w retention of learned passive avoidance step through behavior. The observed behavioral alteration may be due to stress[5,9]. This may be attributed to elevated catecholamines, serotonin or altered hormonal levels and down regulation of GABA receptors[10]. The adaptogenic activity of WS was assessed mainly in immobilization stress and cold stress models. Induction of stress with intermittent shock is a validated model to produce stress. Both WSM and WST did not attenuate the observed anxiety in young and old stressed rats. Earlier reports indicate anxiogenic activity of WS in elevated plus maze in rats[9]. Contradictory findings were also reported[11]. The observed anxiety in control animals may be due to the presence of excitatory amino acids like aspartate, glycine and glutamate in WS[12] and it might have augmented the observed anxiety in stressed animals? Even though reports indicate direct action of WS on GABA[2], no inhibitory activity was observed in stressed animals[13]. Hence, WS may produce mild or no

TABLE 2: COMPARATIVE EFFECT OF *WITHANIA SOMNIFERA* EXTRACTS ON PASSIVE AVOIDANCE STEP THROUGH BEHAVIOR TEST.

Groups	IF_1	IF_2
Non-stressed		
Young	0.70±0.08	0.56±0.08
DZP (0.5)	0.36±0.01	0.67±0.05
WSM (250)	0.26±0.31	1.25±0.96[a]
WST (250)	0.65±0.24	2.79±0.33[a]
Old	0.37±0.009	0.05±0.004
DZP (0.5)	0.57±0.06	0.38±0.10
WSM (250)	1.02±0.36[a]	0.32±0.11[a]
WST (250)	1.37±0.78[a]	1.98±0.45[a]
Stressed		
Young	0.51±0.01	0.16±0.09
DZP (0.5)	0.39±0.08	0.22±0.05
WSM (250)	2.34±0.06[a]	0.57±0.32[a]
WST (250)	5.59±1.05[a]	4.18±0.96[a]
Old	0.11±0.16	0.14±0.04
DZP (0.5)	0.09±0.006	0.4±0.03
WSM (250)	2.06±1.01[a]	2.53±0.97[a]
WST (250)	3.65±0.99[a]	4.18±1.68[a]

N= 6 animals in each group. Superscript a denotes statistical significance in comparison to vehicle treated groups at $p \leq 0.05$ (Mann Whitney 'U' test).

anxiolytic activity in both stressed/non-stressed animals[9].

Administration of WSM during shock period in young and old rats attenuated the stress induced alterations as indicated by decreased immobility period in behavioral despair test and impaired memory in step through behavioural response. Similar findings were made by Archana and Namasivayam[14]. Aqueous methanolic extract of WS with the combination of sitoindosides also exhibited similar property in mice subjected to swim test[1,15], which supports our present findings with WSM extracts. Whereas WST reversed only the observed memory deficit in stressed young and old rats and failed to produce any effect in swim test. The selective activity of WST on memory is not clear, however it may be due to the difference in solvents used in extraction. The usage of ghee and honey might extracted more lipid soluble active principles of WS, which would have facilitated lipid solubility and transport of the active principles? In WSM, the solvents used being polar in nature, the active principles like withanolides, withaferin A would have present, which contributed the antidepressant activity also. Interestingly, WST in stressed animals exhibited better memory in comparison to respective non-stressed groups. Treatment of WS in humans/animals produced enhancement of memory by increasing cortical muscarinic receptors[13] or by direct action of WS on GABA receptors[9]. It can be concluded that, extract of *Withania somnifera* produced adaptogenic effect and attenuated the stress induced depression and augmented memory. WST produced activity selectively on memory, which was novel; complete physiochemical analysis will delineate the observed differential activity between WST and WSM extracts

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