National Journal of Basic Medical Sciences ORIGINAL ARTICLE Volume - III, Issue-2

PRELIMINARY STUDIES ON ANXIOLYTIC ACTIVITIES OF WITHANIA SOMNIFERA (ASHWAGANDHA) FAT EXTRACT IN EXPERIMENTAL ANIMAL MODELS

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ABSTRACT

Background; Anxiety remains the most common psychiatric disorder affecting man. Therapy of anxiety has been a difficult task since ages. Conventional Anxiolytic drugs cause undesirable adverse effects. Study on Indigenous medicinal drugs can throw light on development of newer, safer, well tolerated, economical and naturally accessible remedies. The present study evaluated the anxiolytic like effects of the fat extract of *Withania somnifera in mice.*

Materials and Methods : fat extract of *Withania somnifera(WS) was* evaluated in various paradigms - elevated plus maze test (EPMT), light and dark box (L and DT) and open field test (OFT) to establish anxiolytic activity of the extract. In addition, motor coordination was also assessed by rota rod test (RRT). Diazepam 1 mg/kg served as a standard anxiolytic drug, administered orally.

Results: The fat extract of *WS* (100 and 200 mg/kg, p.o.) significantly increased the percentage of time spent and number of entries in open arm in EPMT. In L and DT, the extract produced significant increase in time spent, number of crossing and decrease in the duration of immobility in light box. In OFT, the extract showed significant increase in number of rearings and square crossed, all of which are demonstrations of exploratory behavior. Furthermore, the extract produced skeletal muscle relaxant effect assessed by RRT.

Conclusion: The results suggest that the extract has anxiolytic – like activity. It may be worthwhile isolating the constituents for further study

Keywords: Anxiolytic, elevated plus maze, open field, rota rod, Withania *somnifera*

INTRODUCTION

Anxiety is a normal emotional behaviour. When it is severe and/or chronic, however, it becomes pathological and can precipitate or aggravate cardiovascular and psychiatric disorders. The disorder is associated with significant disability (including educational and occupational) which has a negative impact on the quality of life. [1] Although many drugs are available in allopathic medicine to treat anxiety disorders, they produce various systemic side effects or exhibit tolerance upon chronic use. In ayurvedic medicine, many plant products have been claimed to be free from side effects and less toxic than synthetic drugs.[2] Pharmacotherapeutic approaches for the management of anxiety disorders include psychotropic drugs, but these agents are limited by their side-effect profile, the need for dietary precautions, and drug interactions. [3] Regular use of benzodiazepines causes deterioration of cognitive functioning, addiction, psychomotor impairment, confusion, aggression, excitement, anterograde amnesia, physical dependence, and tolerance. [4] This necessitates the search for newer, better-tolerated, and more efficacious therapeutic agents, for better management of anxiety. As medicinal plants offer a huge potential for new pharmacological agents, the assessment of herbal medicines for use as alternative / complementary therapies is justified. Much has gone on, progressively, in the search for novel pharmacotherapy from medicinal plants, for psychiatric illnesses, over the past decade. This is reflected in the large number of herbal medicines whose psychotherapeutic potential has been assessed in a variety of animal models. [5] Various types of herbal medicines have been used as anxiolytic agents in different part of the world, such as *Citrus aurantium* from Brazil-Indians, Afro-Brazilians and Caboclosroots of kava plant from the topical pacific region[6], and the saponin-

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containing fraction of leaves of *Albizia lebbeck* from India are all known to have anxiolytic effects. [7] The major obstruction in the application of herbal medicine into medical practice is the lack of sufficient scientific and clinical data and better understanding of efficacy and safety of the herbal products.

In Ayurveda, the roots of Withania somnifera (Ashwagandha) is believed to possess aphrodisiac, sedative, rejuvenative and life prolonging properties. It is traditionally used to treat the following symptoms and conditions, although there are few scientific studies on the health benefits of Ashwagandha.[8,9] Although Ashwagandha has been shown to be effective in the treatment of chronic fatigue, nervous exhaustion, memory loss and neurodegenerative disorders only a few studies are there with the evidence of health benefits of Ashwagandha.[10]

Withania somnifera grows as a short shrub (35-75 cm) with a central stem from which branch extend radially in a star- like pattern (stellate) and covered with a dense matte of wooly hairs (tomentose). The flowers are small and green, while the ripe fruit is orange-red and has milk-coagulating properties. The plant also has long brown tuberous roots that are used for medicinal purposes. It is cultivated in many of the drier regions of India such as Manasa, Neemuch, and Jawad tehsils of the Mandsaur District of Madhya Pradesh, Punjab, Sind and Rajasthan.[8]

Properties of AGG can be studied with various extracts, however the traditional usage of herbs have shown that its CNS activity will be better when administered along with ghee or honey.[11] Hence, the present study in order to see the effect of Ashwagandha, its grutha (fat) extract was considered. Specific objectives of the study were: a) to evaluate the **Anxiolytic** activity of WS, b) to compare the **Anxiolytic** activity of WS with the Standard drug Diazepam.in experimental mice.

MATERIAL AND METHODS

Preparation of test drug: Ashwagandha roots were obtained from Govt. Ayurveda Medical College, Mysore and authenticated. A mixture of Ashwagandha root paste, ghee and water in the ratio of 1:4:16 was prepared and boiled till the water component evaporated. Ghee portion was then filtered & collected in an air-tight container.

Test dose: A pilot study was conducted with different doses (10 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg, and 200 mg/kg) to assess the appropriate dose for the study. The anxiolytic activity were observed at the dose of 100 mg/kg and 200 mg/kg of body weight and hence the same doses were used in the study.

Chemicals: Diazepam, Withania somnifera, Normal saline and other chemicals were of analytical grade.

Drugs and Chemicals

The standard anxiolytic drug, diazepam tablets (Calmpose ® 5 mg, Ranbaxy Laboratories Limited Navi-Mumbai, India B-No. 1700593) were used as a standard drug. Diazepam was suspended in 0.5% of carboxymethyl cellulose in distilled water. Two different doses (100 and 200 mg/kg), a fat extract of *Withania somnifera* was suspended in 1% gum acacia solution. Each drug solution was prepared freshly just before the administration. Drugs and vehicles were administered orally.

Animals: Swiss albino mice weighing around 25 g – 30 g of either sex were obtained from Central animal facility of JSS Medical College, Mysore. Animals were maintained under standard laboratory conditions at an ambient temperature of 25±1°C. Animals had free access to food and water with a natural light and dark cycle. Animals were acclimatized for at least 5 days before behavioral experiments. The study protocol was approved by Institutional Animal Ethics Committee (IAEC) of the college and the experiments were carried out as per CPCSEA guidelines.

Behavioral Assessment of Anxiolytic Activity

Treatment schedule : The anxiolytic activity was examined by using the elevated plus maze (EPM) test, open field test (OFT), light and dark test (L and DT), foot shock induced aggression test (FSIAT) and motor coordination test assessed by rota rod test (RRT). The animals were divided into four groups, with each group consisting of six male mice. Group 1 received vehicle (normal saline); group 2 received diazepam (1 mg/kg); groups 3 and 4 received *WS* extract (100 and 200 mg/kg).

Elevated plus maze test

The EPMT apparatus consisted of four arms elevated 30 cm above the floor, with each arm positioned at 90° relative to the adjacent arms. Two of the arms were

enclosed with high walls (30 X 7 X 20 cm), and the other arms were connected via a central area (7 X 7 cm) to form a plus sign. The maze floor and the walls of enclosed arms were painted black. The room was illuminated with a 40-W lamp at the central platform. The animals were treated with vehicle, extract and diazepam orally, 60 min prior to the test. The experiment was performed between 0900 and 1400 hours, and the mice became accustomed to the dimly lit experimental laboratory for 30 min prior to behavioral testing. Each mouse was individually placed on the central platform facing toward an open arm. The frequency and duration of entries into the open and closed arms were observed for 5 min. An entry was counted when all four paws of the mouse entered an open or closed arm. Subsequently, the percentage of time spent (duration) in the open arms [100 X open/ (open + enclosed)] and percentage of the number of open arm entries (frequency, 100 X open/total entries) were calculated for each animal. The apparatus was thoroughly cleaned after each trial. [12]

Open field test

The apparatus consisted of a wooden box (60 X 60 X 60 cm). The arena of the open field was divided into 16 squares (15 X 15 cm): the four inner squares in the center and 12 squares in the periphery along the walls. The experimental room was a sound attenuated, dark room. The open field arena was illuminated with a 40-W lamp, focusing on the field from a height of about 75-100 cm. After 60 min of oral treatment with vehicle, diazepam (1 mg/kg) and *N. alba* extract (100 and 200 mg/kg), animals were placed individually in one of the corner squares and number of rearings, assisted rearings and number of squares crossed were observed for 5 min. [13]

Light and dark test

The L and DT apparatus consisted of open top wooden box. Two distinct chambers, a black chamber (25 cm long X 35 cm wide X 35 cm deep), painted black and made dark by covering its top with black plywood, and a bright chamber (25 cm long X 35 cm wide X 35 cm deep), painted white and brightly illuminated with 40-W white light source, were placed 25 cm above the open box. The two chambers were connected through a small open doorway, (7.5 cm long X 5 cm wide) situated on the floor level at the center of the partition. The mice were placed individually in center of the light box after 60 min of oral treatments and observed for 5 min. [14]

Motor coordination test by rota rod

Rota rod apparatus consisted of a base platform and an iron rod of 3 cm diameter and 30 cm length, with a non-slippery surface. This rod was divided into three equal sections by two disks, thus enabling three mice to walk on the rod at the same time at the speed of 32 rpm. Intervals between the mounting of the animal on the rod and falling off of it were recorded as the performance time. The training of mice in RRT was given 20 times at 5-15 min intervals. Thereafter, three mice were randomly selected to determine the retention of the walking technique. Other animals that performed on the rod for more than 10 sec were used for assaying the drug effects. The remaining animals showing poor results or the ones that did not reach the criterion (10 sec) were excluded from the experiments. After the administration of the standard drug and extract, the performance time was measured at 15-min time intervals for 90 min for 300 sec (cut-off time). For the assay of each dose, 24 trained mice were used, and repetitions of the drug test were never made with the same animal. [15]

Statistical Analysis

All the results were expressed as mean ± SEM and the data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's "*t*" *test. A* P value of <0.05 was considered as the level of significance.

RESULTS

Elevated Plus Maze

Diazepam treated mice showed significant increase ($P < 0.05$) in the number of open arm entries, time spent in open arms and the number of rears in the open arm. They showed a reduction in the time spent in closed arm .***Withania somnifera*** extract treated mice exhibited significant increase ($P < 0.05$) in the number of open arm entries (100 and 200 mg/kg), time spent in open arm, percentile ratio of open arm to total arm entries, the number of total arm entries, and the number of rears in the open arm entries, but decrease in time spent in closed arm [Table 1].

Open Field Test

In the OFT, diazepam treated mice showed significant increase ($P < 0.05$) in the number of rearings, number of squares crossed and number of assisted rearings during 5 min interval of test as compared to vehicle treated groups. ***Withania somnifera*** extract treated rats (100 and 200 mg/kg) also produced significant increase in the number of rearings ($P < 0.05$), number of assisted rearings and number of squares crossed ($P < 0.01$) [Table 2].

Light and Dark Box

Treatment with diazepam significantly increased the time spent ($P < 0.001$) in light box as well as the number of crossings ($P < 0.05$) between the light and dark boxes, whereas the time spent in dark box ($P < 0.001$) and duration of immobility ($P < 0.01$) were significantly reduced. ***Withania somnifera*** treated mice also showed significant increase ($P < 0.001$) in the time spent in light box and the number of crossings between light and dark boxes. However, the time spent in dark box ($P < 0.01$) and duration of immobility were significantly reduced ($P < 0.05$) as compared to the vehicle treated group [Table 3].

Rota Rod Test

In this test, ***Withania somnifera*** (100 and 200 mg/kg) significantly reduced the time spent by the animals on revolving rod when compared to control ($P < 0.05$). The standard drug diazepam also showed significant effect when compared to control ($P < 0.01$). Low dose of drug (100 mg/kg) did not show any significant effect at 30 and 45 min time intervals [Table 4].

Table 1: Effect of Withania Somnifera fat extract on behavior of mice in elevated plus maize test

Treatment (mg/kg, p.o.)	No of entries Open arm		Time spent sec Closed arm		%OAE% Open arm	TSOA sec Closed arm
Vehicle	6±0.67	20± 1.51	33.67± 6.77	200.2± 13.74	22±2.84	14.08± 3.02
Diazepam(1)	11.5± 1.18[b]	13.33± 0.98[b]	66.67± 7.44[a]	108±5.85[c]	46.61± 2.72[c]	38.16± 1.78[c]
Withania somnifera (100 mg/kg).	10.83± 1.34[a]	11.83± 1.11[b]	65.67± 5.93[a]	92.67± 9.03[c]	47.91± 2.5[c]	41.47± 2.52[c]
Withania somnifera (200 mg/kg).	11.83± 1.32[b]	13.67± 1.6 8[b]	72.83± 10.46[b]	108.7± 10.24[c]	47.83± 3.42[c]	40.12± 1.97[c]

Values represent ± SEM (n=2), aP<0.05; aP<0.05; aP<0.05vs. Vehicle treated control group (one-way ANOVA followed by Dunnett's "t" test); %OAE=percentage of open arm enteries;%TSOA(sec)=percentage of total time spent in open arm in seconds

Table 2: Effect of Withania Somnifera fat extract on behavior of mice in light & dark test

Treatment (mg/kg, p.o.)	Time spent in lighted box(sec)	Time spent in dark box(sec)	No of crossings	Duration of immobility (sec)
Vehicle	120.2±12.93	209±20.03	17.83±1.63	38.17±3.84
Diazepam(1)	199.2±12.74[c]	118.8±10.52[c]	27.5±3.312[a]	22±3.05[b]
Withania somnifera (100)	230±16.4c	140.2±8.67b	29.67±2.81b	23.67±1.93b
Withania somnifera (200)	254±7.44c	145.5±4.78b	28.5±1.41a	27.17±3.36a

Values represent ± SEM (n=2), aP<0.05; aP<0.05; aP<0.05vs. Vehicle treated control group (one-way ANOVA followed by Dunnett's "t"test)

Table 3: Effect of Withania Somnifera fat extract on behavior of mice in Open Field Test

Treatment (mg/kg, p.o.)	No of rearings	No of assisted rearings	No of squares crossed
Vehicle	11.17±1.87	22±3.46	119.2±6.67
Diazepam(1)	25±2.22[a]	38±4[a]	188.5±21.32[a]
Withania somnifera (100 mg/kg).	26.5±4.57[a]	41.67±4.52[b]	210.2±24.83[b]
Withania somnifera (200 mg/kg).	25.17±4.15[a]	39.33±4.38[a]	233±18.15[b]

Values represent ± SEM (n=2), aP<0.05; aP<0.05; aP<0.05vs. Vehicle treated control group (one-way ANOVA followed by Dunnett's "t"test)

Table 4 Effect of Withania Somnifera fat extract in mice on rota rod performance

Treatment (mg/kg, p. o.)	Time (sec) of animals remained without falling from revolving rod					
	15	***30***	***45***	***60***	***75***	***90 min***
Vehicle	262.2 ± 12.03	237.0 ± 9.67	218.0 ± 7.92	188.2 ± 4.96	165.5 ± 3.68	140.7 ± 6.52
Diazepam (1)	248.3 ± 11.78	203.2 ± 6.17	165.3 ± 9.50b	118.5 ± 11.98c	93.00 ± 11.07c	84.30±7.7 8c
Withania somnifera (100 mg/kg).	270.2 ± 12.97	225.7 ± 13.34	186.5 ± 10.12	141.0 ± 7.74b	116.5 ± 5.57c	91.67 ± 5.70c
Withania somnifera (200 mg/kg).	256.3 ± 14.75	189.2 ± 13.97a	145.8 ± 9.50c	86.00 ± 6.37c	67.00 ± 4.91c	56.67 ± 3.33c

Values represent mean ± SEM (n = 6);aP < 0.05;bP < 0.01;cP < 0.001 vs.vehicle-treated control group (one-way ANOVA followed by Dunnett's "t" test)

DISCUSSION

The introduction of drugs like Benzadizepines, beta blockers and others has revolutionized the treatment of anxiety. The amazing efficacy of Diazepam, Midazolam

and Alprozolam in these anxiety disorders has paved the way for the introduction and use of newer anxiolytic agents. However, the safety factor in respect of both the Diazepam and Midazolam anxiolytic drugs has been rather intriguing and hence a definite need is visualized for the introduction of safer anxiolytic drugs having no troublesome adverse effects. ***There are several studies on*** ethanolic, methanolic and aqueous root extracts of WS but studies on the fat (Grutha) root extract are sparse hence, the present study has attempted to fill the lacunae of this invaluable drug.

The major biochemical constituents of Ashwaganda root are steroidal alkaloids and steroidal lactones in a class of constituents called withanolides.[16] About 12 alkaloids, 35 withanolides, and several sitoindosides from this plant have been isolated and studied. A sitoindoside is a withanolide containing a glucose molecule at carbon 27. Much of Ashwaganda's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D.

This study examined some neuropharmacological effects of WS and established that it has anxiolytic- like activities.Anxiety, like all emotions, has cognitive, neurobiological and behavioral components. It is a negative emotion that occurs in response to perceived threats that can come from internal or external sources and can be real or imagined.[17] The present work has shown that anxiolytic activity by the *WS* as assessed by OFT, light/dark box, EPMT and motor coordination test. The experimental models of anxiety, elevated plus maze, open field and bright and dark arena, are based on the assumption that unfamiliar, non-protective and brightly lit environmental stress provokes inhibition of normal behaviour. This normal behavioural inhibition is further augmented in the presence of fear or anxiety like state.In the elevated plus maze, the open arms are more fear provoking than the closed arms. The ratio of entries, time spent and rearing behaviour in open arms to closed arms reflects the safety of closed arms with relative fearfulness of open arms.[18] The reduction in entry, time spent, rearing in open arms, ratio of open arm to total arm entries and increased defecation are the indications of high level of fear or anxiety. Anxiolytic drugs increase the proportion of entries, time spent and rearing in open arms. They also increase the ratio of open arm to total arm entries. The EPMT is used to evaluate psychomotor performance and emotional aspects of rodents. Results obtained on the elevated plus maze after treatment with *WS* (100 and 200 mg/kg) revealed anxiolytic activity, since increases in open arm entry parameters are the most representative indices of anxiolytic activity. [19] Time spent on the central platform appears to be related to decision making and/or risk assessment, and the total arm entries is a contaminated measure reflecting changes in anxiety or in general activity. [20]

In the light and dark box paradigm, the brightly lit environment is a noxious environment stressor that inhibits the exploratory behaviour of rodents. Reduction in the number of entries, time spent and rearing behaviour in the light chamber is regarded as markers of anxiety.[21] Rearing reflects an exploratory tendency of the animal that can be reduced due to a high level of fear. [22] The light-dark exploration test is an ethological-based approach-avoidance conflict test is widely used in rodents and it is sensitive to drugs that affect anxiety. The test exploits the ethological conflict between the tendencies of mice to explore a novel environment and to avoid a brightly lit, open area.[23] An increase in inter-compartment transitions, without an increase in spontaneous locomotion, is considered to reflect anxiolytic activity[24] However, the measurement that has been found to be the most consistent and useful for assessing anxiolytic-like action is the time the mice spent in the lit area, because this parameter provides the most consistent dose-effect results with drugs. [25] In this test, the number of transitions between the light and dark compartments as well as the time spent in the light side are recognized as anxiety indices, despite the transition parameter being highly dependent on locomotor activity.[24] Mice treated with *WS* (100 and 200 mg/kg) showed increase in the time spent in the light compartment and no changes in the numbers of shuttle crossings, confirming the activity upon the main anxiolytic parameter.

Exploration is a key animal behavior in response to novelty and it has been suggested that it is gradually inhibited by anxiety. [26-29] The inhibition of exploration behavior can be reversed by anxiolytic compounds.[26, 30-33]

Since rearing is an accepted indicator of exploration,28 it implies that rearing behavior can represent an indirect measurement of anxiety.26,28 The increase in frequency of rearings due to WS- and diazepam-induced behavioral disinhibition, in this study, further supports their anxiolytic-like activity.The open field paradigm is used mainly to assess the locomotor activity and emotional state of animals .The open field model examines anxiety related behavior characterized by the normal aversion of the animal to an open, brightly lit area. Thus, animals removed from their acclimatized cage and placed in environment express anxiety and fear, by showing alteration in all or some parameters. Anxiolytic treatments reduce such fearful behavior of animals in open field. [34]

Statistical analysis of the data obtained from these experiments supported anxiolytic-like activity of *WS* extract at both the doses (100 and 200 mg/kg) as its effect shows significant increase in the number of rearings, number of assisted rearings and number of squares crossed, as compared to the vehicle treated group, which indicates its anxiolytic-like effect. RRT was first introduced to screen the assay of neurotoxicity of anticonvulsants and later was reported to predict motor dysfunction produced by centrally acting drugs to determine possible alterations in the motor coordination ability of the animal, often caused by the use of sedative and antipsychotic drugs. 15 In this test, the difference in the fall of time from the rotating rod between the vehicle and extract treated groups is taken as an index of muscle relaxation. The skeletal muscle relaxation together with taming or calming effect also reduces anxiety and tension. Thus, in this study, both the doses of *WS* extract (100 and 200 mg/kg) and diazepam significantly reduced the fall of time of the mice from the rotating rod, indicating the skeletal muscle relaxant activity. About 12 alkaloids, 35 withanolides, and several sitoindosides from this plant have been isolated and studied. A sitoindoside is a withanolide containing a glucose molecule at carbon 27. The anxiolytic -like effects of WS reported here can be attributed to one or more of its chemical constituents. Indeed, WS has been shown by phytochemical analysis to contain About 12 alkaloids, 35 withanolides, and several sitoindosides and one of them may be responsible for the observed effects, Earlier reports on the chemical constituents of plants and their pharmacology suggest that plants containing withaferin A and withanolide D possess activity against many CNS disorders. further biochemical and pharmacological studies are necessary to establish the exact chemical constituents and their mechanisms of action.

CONCLUSION

The results obtained in this study suggest that the fat extract of *WS* possesses anxiolytic and muscle relaxant properties. Thus, *WS* has potential clinical applications in the management of anxiety and muscle tension disorders. This study may help in future for better understanding of anxiolytic activity of WS and thus development of newer drugs and newer modalities of treatment. Further human studies are needed to prove the safety and efficacy of long term administration of fat extract of *Withania somnifera* root. *In the light of observations made it may be envisaged that Withania somnifera can be used as a potential adjuvant in the treatment of anxiety disorders.*

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