Evaluation of anti-stress activity of *Withania Somnifera* in experimental animals

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Abstract:

This study aimed to evaluate the anti-stress activity of the ethanolic extract derived from the roots of *Withania somnifera* (WS) in a rat model using the Elevated Plus Maze (EPM) apparatus. Stress-related disorders are a growing concern, and WS, a well-known adaptogen in traditional medicine, holds promise as a natural remedy. The EPM apparatus is a validated tool for assessing anxiety-like behavior in rodents.

Rats were subjected to stress induction through various paradigms and subsequently, they were treated with the ethanolic extract of WS. Behavioral responses were then observed in the EPM apparatus, focusing on parameters such as open-arm entries, closed-arm entries, and time spent in each arm. The results demonstrated that the ethanolic extract of WS had a significant impact on stress-induced anxiety-like behavior in the rats. Open-arm entries and time spent in the open arms increased, while closed-arm entries decreased, indicating reduced anxiety levels. These observations suggest that WS extract possesses anxiolytic

properties. In conclusion, this study provides evidence for the anti-stress and anxiolytic potential of the ethanolic extract of WS roots in a rat model, as demonstrated by behavioral changes in the EPM apparatus. Further mechanistic studies and clinical trials are warranted to elucidate the underlying mechanisms and validate the therapeutic efficacy of WS in managing stress-related disorders.

Keywords: *Withania somnifera,* Elevated Plus Maze, stress, anxiolytic, ethanolic

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1. Introduction

Defining stress comprehensively poses a challenge due to its subjective interpretation across various disciplines. It represents a reaction to adverse stimuli and can be characterized as the amalgamation of bodily responses that disrupt the typical physiological equilibrium, leading to a perturbed state of homeostasis. Stress is a notion familiar to most individuals, universally acknowledged and amplified by the progression of industrialization in our demanding society (1-4).

Stress is a ubiquitous experience encountered by each person. Excessive stress, when left unaddressed, can prove detrimental to the body, warranting appropriate intervention. Furthermore, stress is intricately linked to the development of a spectrum of illnesses encompassing psychological conditions like depression and anxiety, compromised immune response, endocrine irregularities such as diabetes mellitus, male reproductive issues, cognitive impairments, peptic ulcers, elevated blood pressure, and ulcerative colitis (5)

From this perspective, stress pertains to an individual's state that emerges when the perceived environmental requirements surpass their evaluated capacities. In simpler terms, stress materializes when a person acknowledges their inadequate ability to effectively manage the situation they find themselves in. While the concept of stress holds a degree of ambiguity, within the research framework, it primarily alludes to the physiological and psychological responses that activate an organism's protective mechanisms against internal or external threats to its well-being, also known as stressors (5). Physical illness is frequently cited as the prevailing consequence of unaddressed stress, with some scholars positing that stress could potentially underlie a majority of illnesses and disorders. Specifically, within the realm of anxiety, complexities might encompass elevated rates of coexisting mental and

physical conditions, heightened hospitalization rates, and suboptimal occupational performance (Milrod & Busch, 1996). Meanwhile, depression frequently accompanies an overarching diminishment in life quality and compromised social functioning (6-9).

Numerous endeavors have been undertaken to mitigate the adverse impacts of stress, encompassing practices such as yoga, meditation, and pharmaceutical interventions like anxiolytic benzodiazepines (BDZ). Yet, despite assertions of their efficacy, the applicability of these nonpharmacological and pharmacological strategies seems constrained. Anxiolytic or hypno-sedative medications are often employed to address stress-related concerns, albeit they come with inherent drawbacks, prompting consideration for the substitution of these agents with safe natural alternatives. Abundant medicinal plants boast potential antistress properties, thereby offering a promising avenue for exploration (10-11). *Withania somnifera* (WS) Dunal, a member of the Solanaceae family, is renowned as ashwagandha in Ayurveda, an ancient Hindu medicinal system with a history of over 2500 years. Its roots are classified as Rasayana, acknowledged for their potential to enhance health and longevity by bolstering immunity, curbing the aging process, rejuvenating the body during periods of debility, reinforcing the individual's resistance against adverse environmental factors, and nurturing mental well-being. Notably, the roots of W. somnifera have been a subject of interest due to their constituents, Sitoindoside VII and sitoindoside VIII, two glycosides extracted from W. somnifera roots. These compounds exhibited substantial antistress effects and exhibited the potential to enhance learning and memory retention in both young and elderly rats (12-13). Based on literature review and presence of phytoconstituents, an attempt was made to evaluate the anti-stress activity of ethanolic extract of *Withania somnifera* roots in experimental animals.

2. Materials and Methods

2.1 Collection of Plant Material

Withania somnifera (WS) roots were collected from the local market and subjected to authentication by a renowned botanist. A voucher specimen was prepared and preserved for future reference, ensuring the accuracy and reliability of the plant material used in the study.

2.2 Preparation of ethanolic Extract:

The roots were cleaned by washing with running water and shade dried and the milled to pass through 100-mesh sieve. The powder was extracted by maceration for three days with

80% ethanol at room temperature. The extracts were concentrated at 45^0 C using Rotary vacuum evaporator to yield 80% fraction. The concentrated extracts were kept in refrigerator at 4°C until further use.

2.3 Phytochemical Screening

The ethanolic root extract of *WS* underwent phytochemical analysis to determine the presence of various compounds. The analysis aimed to identify the existence of volatile oils, carbohydrates, alkaloids, glycosides, polyphenols, flavonoids, tannins, propanoids, sterols, terpenoids, ketones, and alcohols in the extract.

2.4 Experimental Animals

Albino Wistar rats weighing between 150-200g were chosen as subjects for the experimental study. The animals were housed in an animal facility approved by the Institutional Animal Ethics Committee (IAEC-CPCSEA) and maintained under standard laboratory conditions. The laboratory environment was set at a temperature of 22 ± 2℃, relative humidity of 50 ± 15%, and a 12-hour light/dark cycle. Throughout the study, the rats had unrestricted access to standard pellets as their food and water was provided *ad libitum.*

2.5 **Acute Toxicity Study**

An acute toxicity study was conducted on the ethanolic root extract of *WS* following OECD guidelines no. 423. Healthy male Albino Wistar rats were randomly divided into four groups, with three animals in each group. The rats were fasted overnight, only provided with water. The ethanolic extract was orally administered to the rats using increasing doses (5, 50, 300 and 2000mg/kg) via an intra-gastric tube, employing the up and down method to determine safe doses. The animals were continuously observed for 1 hour and then frequently monitored for 4 hours up to the end of 24 hours (14)

2.6 Methodology

2.6.1 Chronic restraint stress (CRS)

Restraint was performed by daily placing male rats (160-180g at start of experiment) in 20 × 7 cm plastic tubes for 6 h (10-16h) for 21 days. There were several 3-5 mm holes for breathing. They allowed plenty of air but animals were unable to move. No feed and water was supplied during restraint. Drugs were administered 30 min prior to stress procedure. Unstressed rats were placed in separate room from stressed rats. The treatment schedule for 21 days was as follows-

Group 1: Control- No stress (n=6)

Group 2: Stressed Control

Group 3: Diazepam (Standard) (1mg/kg, i.p.)

Group 4: Low Dose (200 mg/kg/p.o.)

Group 5: High Dose (400 mg/kg/p.o.)

2.6.2 Assessment of Anxiety: Elevated Plus Maze (EPM)

On day 21, after last restraint rats were placed individually in the centre of the elevated 'plus' maze facing one of the open arms. During 5 min test period the following measures were taken: the number of entries and time spent into the open and enclosed arms. An entry was defined as entering into one arm with all four feet. An increase in open arms entries and increase in time spent in open arms were interpreted as an index of potential anxiolytic activity.

2.6.3 Chronic unpredictable stress (CUS)

For CMS, male rats (160-180g at start of experiment) were exposed to a variable sequence of mild, unpredictable stressors. These stressors were applied on daily basis for 21 consecutive days in the following manner (table)

Sr. No	Stressor/day Duration of stressor	Days
1	Immobilization for 30 min	D1, D11
2	Forced swimming for 5 min	D2, D12
3	Tail pinch for 5min	D3, D13
4	Tilt cage- 45° for 12 hrs	D4, D14
5	Damp cage for 24 hrs	D5, D15
6	Food deprivation for 24 hrs	D6, D16
7	Water deprivation for 24 hrs	D7, D17
8	Crowding for 24 hrs	D8, D18
9	Immobilization for 60 min	D9, D19
10	Forced swimming 10 min	D10, D20
11	Individual housing	D 21

Drugs were administered 30 min prior to stress procedure. Unstressed rats were placed in separate room from stressed rats. The treatment schedule for 21 days was as per 2.6.1. All the testing's carried out on Day 21 after last stressor.

3.0 Results

3.1 WS and behavior of rats in EPM

As shown in table analysis of behavior, the elevated plus-maze revealed that there was significant increase in the time spent in the open arms as compared with unstressed and stressed rats. Diazepam, the standard used here in the test also increases significantly the stay in open arms than other drugs used in the study.

Table 1: Effect of WS on exploratory behavior in elevated plus maze test in rats

Treatment (mg/kg)	Time spent (Sec)		Entries	
	Open arm	Enclosed arm	Open arm	Enclosed arm
Unstressed Control	41.5 ± 2.44	154.0 ± 5.57	4.0 ± 0.25	5.70 ± 0.40
Stressed Control	21.24±1.80	188.22±6.22	2.12±0.22	7.88±0.33
Diazepam (1mg/kg, i.p)	79.02 ± 2.39 *	144.2 ± 5.53	8.20 ± 2.18*	6.30 ± 1.12
WS (200)	62.4 ± 2.43 *	144.4 ± 4.26	4.9 ± 0.29*	5.90 ± 0.35
WS (400)	75.3 ± 4.32 *	118.4 ± 6.04	6.34 ± 0.53*	4.30 ± 0.39

All the data were subjected to One Way ANOVA followed by Dunnett's test

3.2 WS, CUS and behavior of rats in EPM

As shown in table 2, analysis of behavior of rats subjected to CUS for 21 days in the elevated plus-maze revealed that there was a significant decrease in the time spent in open arms following CUS.

Table 2: Effect of WS treatment and CUS (21 days) on exploratory behavior in elevated plus maze test in rats

Treatment (mg/kg) + CUS	Time spent (Sec)		Entries	
	Open arm	Enclosed arm	Open arm	Enclosed arm
Unstressed Control	32.4 ± 1.87	134.2 ± 6.53	2.0 ± 0.35	5.50 ± 0.41
Stressed control	7.10 ± 3.22	232.3 ± 5.39	1.32 ± 0.15	3.21 ± 0.43
Diazepam (1mg/kg, i.p)	59.3 ± 2.41#	189.2 ± 3.31#	3.90 ± 0.35#	3.20 ± 0.48
WS (200)	49.0 ± 5.05#	199.2 ± 11.20 #	3.10 ± 0.20#	4.32 ± 1.01
WS (400)	51.2 ± 4.33#	193.6 ± 7.26 #	3.60 ± 0.39#	5.00 ± 0.61#
Imipramine (10)	53.8 ± 9.57#	200.4 ± 8.35 #	2.60 ± 0.25	2.53 ± 0.41
WS (200)+Diazepam (1mg/kg, i.p)	94.4 ± 12.49#	149.6 ± 14.6#	5.40 ± 0.51#	6.33 ± 0.51#
WS (400)+Diazepam (1mg/kg, i.p)	51.5 ± 2.57#	195.6 ± 9.06#	4.60 ± 0.51#	5.40 ± 1.50
WS (200)+ Imipramine (10 mg/kg, i.p.)	48.0 ± 4.72 #	209.8 ± 8.22#	2.18 ± 0.37	5.00 ± 0.55
WS (400)+ Imipramine (10 mg/kg, i.p.)	42.6 ± 4.25 #	207.4 ± 8.99	2.20 ± 0.37	4.20 ± 0.34

All the data were subjected to One Way ANOVA followed by Dunnett's test.

4.0 Discussion

The modern society has grown increasingly intricate and presents heightened demands on individuals. Nevertheless, our physiological reactions, which evolved to handle mounting adversities, have seen limited progression over the last millennium. The lack of effective adaptation to stressors has led to the emergence of stress-related disorders, often stemming from the disruption of the stress response regulation (16-17). Certain plant-derived medicines known as adaptogens seem to initiate a condition of generalized resilience, empowering the organism to effectively respond and acclimate to diverse stressors that might otherwise harm the physiological equilibrium (18). Numerous botanicals exhibit adaptogenic effects and Ayurvedic literature highlights various plants, including *Withania somnifera* (WS), classified as rasayanas. The attributes attributed to rasayanas in Ayurveda bear striking resemblance to adaptogenic traits. WS has undergone empirical investigations involving immediate stress models, unveiling substantial stress-mitigating qualities and mood-modulating behavioral impacts in animal experiments (19-20). *Withania somnifera* (WS) has undergone comprehensive chemical and biological assessments, establishing its potential as a highly promising therapeutic botanical. The root extracts of WS demonstrate characteristics that align with the adaptogenic classification. The key bioactive components of ashwagandha, primarily responsible for its medicinal attributes, revolve around the influence of specific steroidal alkaloids and steroidal lactones belonging to the category of compounds known as withanolides (21-22).

The correlation between stress and anxiety has garnered significant attention. Prolonged stress triggers mood-related disturbances in mammals, encompassing humans, and could potentially play a pivotal role in the genesis of anxiety. In an endeavor to elucidate this association, the current investigation assessed the impacts of chronic restraint stress (CRS) and chronic unpredictable stress (CUS) on rodent behavior within the Elevated plus Maze (EPM), a well-recognized model for studying anxiety-like behaviors and screening anxiolytic compounds (23-24)

The focus turned towards comprehending the diverse impacts of stress and investigating the potential efficacy of *Withania somnifera* in alleviating anxiety, as assessed through the performance of rats in the Elevated plus Maze (EPM) paradigm. Prior studies have highlighted that the behavior of animals within the EPM is subject to modulation by various

stressors, including electric shocks, forced swimming, surgical stress, social defeat, and exposure to aversive scents such as cat odor (25-67)

5.0 Conclusion

This study delved into the intricate relationship between stress and anxiety, with a specific focus on assessing the potential therapeutic effectiveness of *Withania somnifera* (WS) in mitigating anxiety-related behaviors in a rodent model. The investigation utilized the Elevated plus Maze (EPM), a well-established tool for evaluating anxiety-like responses. Further research is needed to explore bioactive moieties mitigating the stress.

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