Anti-stress Activity Of Methanolic Extract Of *Withania Coagulans* Fruit On Chronic Unpredictable Stress Model In Mice

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Abstract-

Background: Chronic stress can have a wide range of health impacts, including an increased risk of neuropsychiatric diseases and immune response dysregulation. The chronic unpredictable stress (CUS) protocol is frequently used to research the impact of stress exposure in a variety of animal models and comprises of random, intermittent. CUS has regularly been proven to cause behavioural and immunological changes characteristic of the chronic stress response. The development of an alternate CUS procedure that allows mice to be used in chronic stress investigations is necessary. Here, we show that prolonging the CUS regimen to two weeks can elicit a chronic stress response in mice. We also saw stress-related behavioural changes, such as increased anxiety and depression, as well as decreased exploratory behaviour. Many anti-stress medications used today cause hazardous pharmacological responses.

Aim and objectives: To evaluate the effect of Anti-stress activity of *withania coagulans* fruit on chronic unpredictable stress model in mice.

Materials and methods: CUS paradigms are use to induce the stress and EPM and Marble burying test was used for check the behaviour response of mice where the EPM shows the positive effect and also marble burying shows the positive effect.

Result: There was statistically substantial (P<0.001) association observed between ethanolic extract of *W. coagulans* fruits with stress action in SAM by EPM and marble burying.

Conclusion: Recent psychiatry research has shown growing interest in herbal medication due to its improved compliance and fewer adverse effects. Flavonoids in herbal formulations have demonstrated promising effects on psychiatric illnesses, primarily through serotonergic and GABAergic activation. Further research is needed to fully understand the molecular mechanisms and neuropathological changes underlying their effectiveness in treating anti-stress conditions like Chronic Unpredictable Stress (CUS).

Keywords: CUS, neuroprotective, *withania coagulans*, anxiety, depressive-like behavior, compulsive or repetitive behaviors.

1 INTRODUCTION

Stress is defined as the body's response to physical, mental, and emotional stress. Stress can cause Chemical changes in the body, increased blood pressure, heart rate, and blood sugar levels. It may also cause depression, anxiety, anger, or moodiness. This can be caused by normal life conditions or situations, such as stress, injury, or illness. Chronic stress or high levels of stress can cause mental and physical problems. stress is the body's reaction to pressure that can be mental, physical, or emotional. Stress can increase blood pressure, heart rate, and blood sugar levels because it alters the body's chemistry. Anger, frustration, anxiety, or depression may also result from it. Events like disease or trauma can also lead to stress, as can everyday activities. Issues with mental and physical health might result from prolonged or excessive stress.[1] A stressor is sometimes defined as a particular condition that causes an individual to react profoundly in physical, mental, and emotional ways. body responds to demands of any type by producing stress.[2] Numerous physiological stress responses are triggered by psychosocial stress, and these responses are necessary for the short term to maintain homeostasis. The hypothalamic-pituitary-adrenal (HPA) axis is the primary endocrine component

of the stress response and governs a wide range of adaptive physiological and psychological responses. Stress arises from an organism's perception of a disturbance or potential threat to its homeostasis, which prompts a compensating response. Early indicators of stress include anxiety, diversionary anxiety, excessive worry, altered sleep patterns, impatience, anger, melancholy, intolerance, thoughts of hurting oneself or others, palpitations, tension headaches, and internal pressure.[3]Chronic unpredictable stress (CUS), one of the most clinically relevant stress paradigms in rodents, mimics a number of behavioural characteristics observed in patients with anxiety, depression and related mood disorders.[4-5] Chronic stress can cause anxiety and sadness, which can increase the risk of physical illnesses including inflammatory bowel disease.[6-7-8]

2. MATERIALS AND METHODS

2.1 Experimental animal

Swiss Albino mice of either sex weighting 25-35 gm or 8-12 weeks age was obtained from the animal house of Department of Pharmacology, Vidyabharti College of pharmacy Reg.NO:1504/PO/RE/11/CPCSEA, Amravati. All the animals are acclimatized to the animal house prior to use. The animals were housed on a 12-h light/dark cycle under controlled temperature (22°C ± 2°C) and humidity (50± 10%). Experiments are performed in accordance with the committee for the purpose of controland supervision of experimental animals (CPCSEA) guidelines after approval of the experimental protocols by the institutional animal ethics committee (IAEC). Animal are fed on pellets and tap water ad libitum. The care and handling of animals in accordance with the internationally accepted standard guideline of use of animals (CPCSEA).

2.2 PREOARATION OF EXTRACT

Extraction Procedure

Withania Coagulans fruits were processed by washing with clean water, drying in air, crushing and sieving through a 0.3 mm sieve. The instrument consists of several parts including a heated round bottom flask, Soxhlet extractor and condenser. Hard, coarsely ground fruit (500 g) was placed in a thimble and placed in the extractor. The bottom of the extractor was connected to a round bottom flask containing a solvent (1500 ml of methanol was selected as the solvent) and connected to a reflux condenser. The bottom flask was heated until the solvent (methanol) boiled and the vapor rose through the extractor nozzle, condensed and dropped into the sleeve, which was then extracted by bringing the solvent (methanol) into contact with the solid. When the surface of the solvent (methanol) exceeded the highest point of the siphon, the solvent containing the extract was returned to the round bottom flask. This cycle was repeated until all substances were extracted from the solid fruit powder. Soxhlet extractors can run indefinitely without any additional work, making them an excellent choice for extracting substances over a period of hours or days. No filtering required. This saves a lot of time, energy and financial costs. After calculating the percent yield of the extract, various phytochemical tests were performed on the extract.

2.3 Preparation of dose

Diazepam was diluted to 2mg/10 ml with distilled water. two different concentrations (200 mg/kg, and 400 mg/kg) of the methanolic extract were prepared by dissolving the extracts in distilled water. All solutions were freshly prepared at the time of administration to the animals. Extract solution and vehicle (0.9% NaCl) were given orally and standard drug (diazepam) intraperitoneally.

2.4 TREATMENT PROTOCOL

Table 1. Groping and Dosing of animals

Sr.no	Treatment	No. of Animals	Treatment/Dose	Route of Administration
1	I (Control -Without CUS)	6	Normal saline 1ml/kg	IP
2	II Stress control (CUS)	6	Normal saline 1ml/kg	IP
3	III (DZP + CUS)	6	DZP (2 mg/kg)	IP

4	IV (MEWC + CUS)	6	MEWC (200 mg/kg)	Oral
5	V (MEWC + CUS)	6	MEWC (400 mg/kg)	Oral

2.5 Chronic Unpredictable Stress Procedure

A single severe or recurring unmanageable stressor can either cause event promotes the emergence of psychopathologies. Among these disorders, major depressive disorder is one that is linked to a combination of genetic/developmental predis positions and environmental stressors. [9] Therefore the CUS model has been adopted for this study. The stress procedure was performed as described with significant alterations. In this stress model for animals, long-term consequences are not induced just by any of the diverse, unpredictable stressors that the animals are chronically exposed. [10] To avoid habituation, the stressors were presented weekly in a different order and with different variants every animal was subjected to one stress each day. The CUS technique was then employed for 14 consecutive days. The stressors in this paradigm were applied at different times in every day and in an apparently random order in order to maximise unpredictability. 30 min before the stress procedure the drug were given to the mice. **Restrain stress**: Day 1 For 1 hr restraint, mice were placed in a restraining device made of plexiglass and flexible nylon, thus restricting movement but allowing free respiration andair circulation.[11] **Rotation stress:** Day 2 Rotation procedure was carried out by placing rats in rotating spinner (50 rpm)for 1hr. **Inversions of light and dark cycle**: Day 3 Inversions of light and dark cycle were performed by placing the mice in dark in day time and light in night time.[12] **Tail pinch stress:** Day 4 Tail pinch involved placing the mice in the previously described restraining device, and applying a clothespin 2 cm from the base of the tail for 20 min. **Hot air stream:** Day 5 mice were exposed to a hot air stream from a hairdryer for 10 min **Tilted cage:** Day 6 home cages were tilted in a 45° angle during 1 h. Stressors were administered in a random and unpredictable order.

Shaking Day 7 mice were placed in a plastic box container and placed in an orbital shaker for 1 hat 150 rpm. [13] Animals were held in a recovery area for an hour after each stressor, after which they were transferred to the housing facility in clean cages with brand-new bedding. Mice were housed separately for the same amount of time, and the CUS paradigm involved daily exposure to one of the unpleasant stimuli listed below table 2.

Table 2. The CUS paradigm involved daily exposure to mice

Weeks	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	RS	RTS	ILCS	TPS	HAS	TCS	SS
Week 2	ILCS	HAS	TCS	RTS	TPS	SS	RS

RS- Restrain stress; RTS- Rotation stress; ILCS- Inverted light cycle; TPS- Tail pinch stress; HAS- Hot air steam; TCS- Tilted cage; SS- Shaking Stress.

3. BEHAVIORAL TEST

The behavioral testing was done at least 24 h after the last dose and last stressor in order to avoid the acute effects of drug treatment. One hour before the behavioral assessment, the mice were acclimatized to the testing room which was free from stray light and noise. The temperature and humidity of the testing room was identical to that of the animal housing unit. The apparatus/exploratory area was sprayed with alcohol and scrupulously wiped between trials in order to eliminate residual odor left by the previous animal. Following Behavioral model Elevated plus maze, Marble burying test would assess the effect of medicinal plant extract for antistress activity.

3.1 Elevated plus maze:

The EPM has been widely used as a tool in the investigation of the psychological and neurochemical basis of anxiety, for screening anxiety-modulating drugs. The elevated plus maze apparatus consisting of two open arms (35 x 5 cm) and two closed arms (30 x 5 x 15 cm) with an open roof which was 50 cm elevated from the floor used to observe anxiolytic behavior in animals. On day 1 Animal placed on the elevated plus-maze apparatus 30 min after the stress procedure. Each animals were placed in the center of the elevated plus-maze with its head facing towards the open arms. The behavioral effects of the mice observed for 5 min with a

different kind of parameter such as total time spent in open arms and the number of entries in the open arms were recorded. The animals were allowed to socialize during the entire experiment. These different parameters observed using MASTER MAZE software in our video tracking room.[15]

3.2 Marble burying test

The marble burying test is commonly used preclinical behavioural assay, primarily used to evaluate anxiety-like behaviour and compulsive or repetitive behaviours in mice. the experimental room is quiet and free from disturbances. Ensure controlled lighting conditions and temperature of experimental room. the apparatus consisted of an opaque plastic chamber (40 cm × 25 cm × 12 cm) similar to the home cage. the floor of the chamber was evenly covered with 5 cm of bedding material. 15 polished glass marbles (2 cm diameter) were arranged on the bedding, On day 2The mice was singly placed in the cage. the number of marbles buried (at least 2/3rd) by the mice in the 30-min observation period was recorded.[16]

3.2 STATISTICAL ANALYSIS

All experimental results are given as mean ± standard error of mean (SEM) for each group. The statistical analysis was performed by using one- way ANOVA followed by Dunnett's test.

4. RESULTS

4.1. Pharmacognostical examination

% yield = (weight of the extract / weight of powder taken) × 100

Table 3. Percentage yield of withania coagulans fruit

Drug	*Withania coagulans* fruit
Percentage yield	12.4% w/w

Table 4. Physical examination of extract

Extract	Colour	Odour	Solubility
MEWC	Brown	Characteristic	In water

4.2 Phytochemical analysis of methanolic extract of withania coagulans

Table 5. Phytochemical investigation of *Withania Coagulans*

Sr.no	**Phytochemical Analysis**	**Test perform**	**Results**
1	Alkaloids	Wagner's test	(+)
2	Flavonoids	Alkaline Reagent test	(+)
3	Steroid	Liberman's Test	(+)
4	Tannins	Ferric chloride Test, Leas acetate Test	(+)
5	Glycoside/Sugar	Killer-Killani Test, Legals Test	(+)
6	Saponins	Forth Test	(+)
7	Terpenoids	Liberman's Test	(+)
8	Protein and carbohydrate	Biuret Test	(+)

Where, (–) indicates Absent, (+) indicates Present.

Phytochemical testing carried out find out the secondary metabolite because secondary metabolic posses biological activity. Phytochemical studies of Withania Coagulans performed for the presence of Alkaloids, Flavonoids, Steroid, Tannins, Glycoside/Sugar, Saponins, Terpenoids, Protein and carbohydrate.

4.3 PHARMACOLOGICAL STUDIES

4.3.1 Elevated Plus Maze Test:

Table 6. Antistress effect of MEWC on Elevated Plus Maze test in mice.

Group	Treatment	No. of entries in open arms	Time spent into open arms (300 seconds)
I	Control	6.667±0.4944	23.83±0.9098
II	Stress control	1.000±0.3651	9.333±0.4944
III	DZPM 2mg/kg	7.000±0.3651*	45.33±3.756*
IV	MEWC 200mg/kg	4.333±0.333*	26.00±1.528*
V	MEWC 400mg/kg	5.000±0.5774*	33.00±1.095*

Values expressed as mean ± SEM, (n=6), One way (ANOVA) followed by Dunnett's test,(* p<0.0001) compared to Stress control.

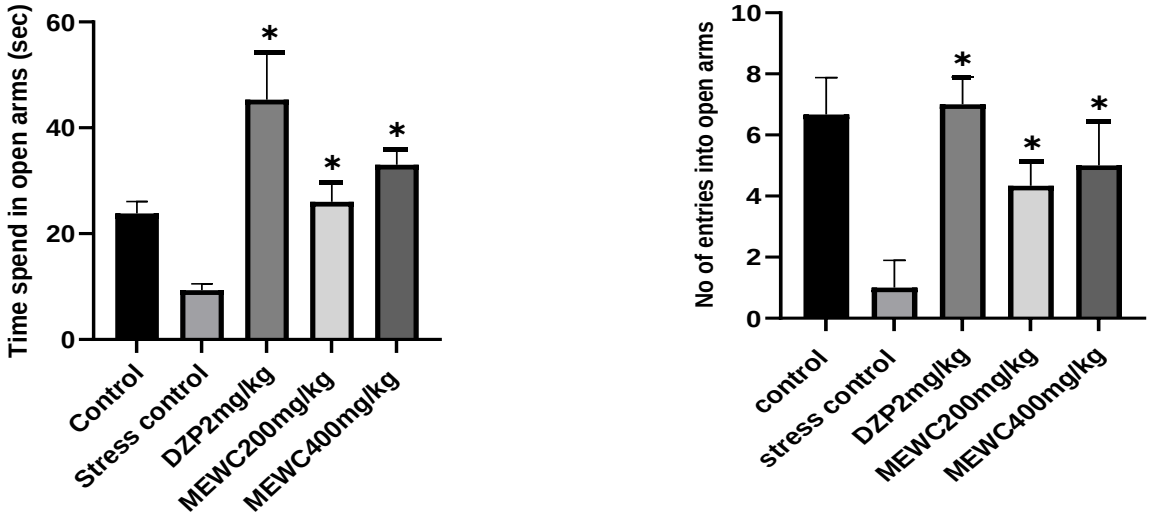


Figure 1. Antistress Effect of MEWC on the number of entries into open arms and Time spend in open arms in EPM test.

As shown in the table.6 and figure 1, the groups (II, IV, V) receiving the MEWC showed a significant difference in terms of anxiety behaviour using elevated plus-maze in comparison with those in the Stress control group (Grp II). Diazepam (Grp III) showed significantly increased in the number of entries in open arms and Time spend in open arms when compared with (Stress Control) group. In the subgroups mice were showed the significant (P<0.0001) increased entries in the open arms at doses 200 mg/kg and 400 mg/kg.

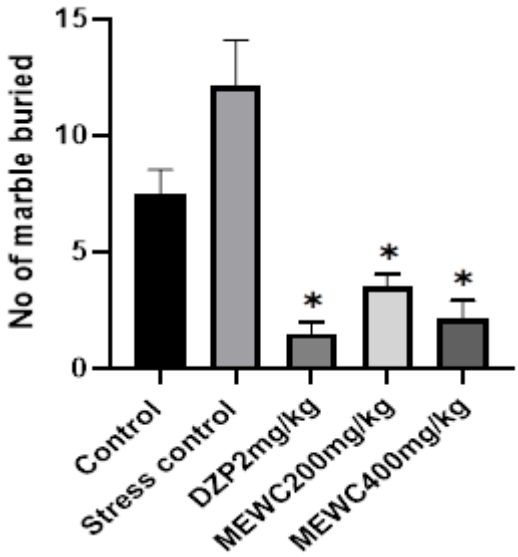
4.3.2 Marble burying Test:

Table 7. Antistress effect of MEWC on Marble burying Test in mice.

Group	Treatment	NO. of marble burying
I	Control	7.500±0.4282
II	Stress control	12.17±0.7923
III	DZP 2mg/kg	1.500±0.2236*
IV	MEWC 200mg/kg	3.500±0.2236*
V	MEWC 400mg/kg	2.167±0.3073*

Values expressed as mean ± SEM, (n=6), One way (ANOVA) followed by Dunnett's test,
(* p<0.0001) compared to Stress control.

As shown in the table 7 and figure 21, the groups (II, IV, V) receiving the *W. coagulans* fruit extract showed a significant difference in terms of antidepressant behaviours using Marble burying test in comparison with those in the Stress control group (Grp II). Diazepam (Grp III) showed significantly decreased the no of marble buried when compared with (Stress Control) group. In the subgroups mice were showed the significant (P<0.0001) decreased the number of marble buried at doses 200 mg/kg and 400 mg/kg.



DISCUSSION

Withania Coagulans is well known for its essential constituents, compounds such as flavonoids, alkaloids, Tannins, saponins, terpenoid, protein and carbohydrate. A wide range of biological functions, such as Antioxidant activity, antidiabetic activity, hepatoprotective activity, decreasing blood pressure and heart rate. In medical or biological terms, stress is defined as a physical, mental, or emotional factor that causes physiological or mental tension. Herbal psychopharmacology research has revealed a number of interesting drugs that may be useful in the treatment of stress induce anxiety, depression, social isolation, hypervigilance and memory impairment. Natural anxiolytic drugs have been studied in the hunt for a more targeted, potentially cost-free therapy. Various pharmacological models have been employed over time to analyse medicinal plants for neuropharmacological activity in order to uncover botanicals and pharmaceuticals with beneficial effects in the treatment of various CNS disorders.

The present investigation involve various CUS stress models to induce consistent stress-response in mice. CUS exposure is used as model of neuropsychiatric disorders, renders an effective CUS protocol in mice. Seven different chronic unpredictable stress (CUS) models were applied, with one stressor randomly chosen each day over a span of 14 days. In this protocol involving mice, inconsistencies in their behavioral outcomes were observed. Following a 24-hour resting period, behavioral tests were conducted to assess the mice responses and reactions.

On day 15, behavioral parameters were assessed using the Elevated Plus Maze (EPM) model, revealing an enhanced anxious-like behavior characterized by increased time spent in the closed arms and decreased time in the open arms of the EPM. Subsequently, groups (II, IV, V) receiving the MEWC showed a significant difference in terms of anxiety behaviors using elevated plus-maze in comparison with those in the Stress control group (Grp II). Diazepam (Grp III) showed significantly increased in the number of entries in open arms and Time spend in open arms when compared with (Stress Control) group. In the subgroups mice were showed the significant (P<0.0001) increased entries in the open arms at dose 200 mg/kg and 400 mg/kg. On day 16, the Marble Burying Test was performed, revealing repetitive or compulsive-like behaviors as well as anxiety behavior. the groups (II, IV, V) receiving the MEWC showed a significant difference in terms of antidepressant behaviors using Marble burying test in comparison with those in the Stress control group (Grp II). Diazepam (Grp III) showed significantly decreased the no of marble buried when compared with (Stress

Control) group. In the subgroups mice were showed the significant (P<0.0001) decreased the number of marble buried at dose 200 mg/kg and 400 mg/kg.

Accumulating evidence demonstrates that the dysfunction of serotonin (5-HT) and GABA plays an important role in the etiology of CUS. Given the complicated etiology of CUS, the important role of 5-HT and GABA in anti-Stress therapy, and the crosstalk between these neurotransmitters, it has been suggested that drugs that normalize the levels of 5-HT and GABA may exert increased therapeutic efficacy Consequently, it is reasonable to speculate that the anti- Stress -like effects of MEWC may be associated with the levels of GABA in the brain. More study supports this speculation. MEWC produced significant antianxiety like effects closely related to the normalization of GABA in the hippocampus, which were similar to the effects of the classic benzodiazepines. Therefore, the significant role of GABA neurotransmitters in the anti-Stress effects of MEWC warrants further study.

Many studies about chemical constituents of plants extract suggest that plants containing flavonoids, alkaloids, phenolic acids, saponins and tannins have beneficial effects against a wide range of CNS disorders. The efficacy of most herbal remedies is attributed to various active principles, in combination. Results of phytochemical screening of MEWC showed the presence of flavonoids, alkaloids, Tannins, saponins, terpenoid in this plant. The exact mechanism of MEWC is still unclear. It is possible that phytoconstituents especially tannins and flavonoids may be influencing CUS induced anxiety through GABA-A. There is possibility that MEWC might be increasing the level of GABA in the brain regions such as hippocampus, amygdala and prefrontal cortex.

In any given case, a sole mechanism may be in effect, or there may be complex interactions among active constituents and multiple neurotransmitter system in brain. Since the effects of MEWC which we observed in this study are obtained by using methanolic extract and not in isolate compound, it is important to understand the effects of active constituents in combination and in isolation and their interactions with other neurochemicals.

CONCLUSION

Herbal medication has piqued the interest of the psychiatry research community in recent years due to its improved compliance and decreased adverse effects. This is largely due to the fact that several herbal formulations have been shown in experimental animal and human research to have helpful effects on a variety of psychiatric illnesses. Although the behavioural and transmitter changes observed in the current model suggest that flavonoids may be an effective herbal candidate for treating Anti-stress, primarily through serotonergic and GABAergic activation, more research is needed to determine the exact molecular mechanisms underlying its effects and to better understand the neuropathological changes in Antistress.

In conclusion, The present study constitutes an extensive behavioural data characterizing the effect of CUS and action of *W. Coagulans*, on behavioural features resembling the sequelae of Stress following CUS. The present study suggested that, the CUS can be useful model of, Anti- stress, as was evidenced in behavioural assays, rather than only a model of major Stress and hypothesis was strengthens with the potential role of *W. Coagulans* reversing the symptoms of Anti-stress. Future studies will be aimed at the molecular and neurochemical substrate mediating the effects of CUS as well as the effect of CUS on neurogenesis relevant for stress.

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