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Comparative antistress effect of *Vitis vinifera* and *Withania somnifera* using unpredictable chronic mild stress model in rats

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ABSTRACT

Introduction: The human society has become complex. However, our physiological responses designed to cope with the ever-increasing adverse situations have not evolved appreciably during the past thousand years. The failure of successful adaptation during stressful situations has resulted in stress-related illnesses. Methods: The objective of the present study was to carry out a comparative assessment of anti-stress effect of Vitis vinifera and Withania somnifera using unpredictable chronic mild stress model in rats. Long-term exposure to multiple stressors can cause depression. The unpredictable chronic administration of various mild stresses, a procedure known as "unpredictable chronic mild stress", is one of the best-validated rodent models to study stress in animals, for its good etiological and predictive validity. Result: Diazepam, Withania somnifera, Vitis vinifera administration dose dependently reversed the increase in immobility period in stressed rats. In the study of locomotion activity of rats in elevated plus maze apparatus, Stress treated control group rats showed less no of entries in open arm and also less time spent in open arm. Vitis vinifera treated ($p<0.0001$), Withania somnifera treated ($p<0.0001$) and Diazepam treated group showed ($p<0.0001$) no. of entries in open arms which were more than control group and stressed groups. Stressed group produce less average time spent in open arm as compared to treatment groups as Withania somnifera ($p<0.05$), Vitis vinifera and diazepam. Withania somnifera group showed significant antistress locomotry behaviour in rats. Administration of Vitis vinifera, Withania somnifera and diazepam during stress period restored the ambulatory behaviour of the rats which can be correlated with restoration of plasma corticosterone level. Finally, the results of the present study justified that Withania somnifera, Vitis vinifera and diazepam exhibited significant antistress activity in rats.

Keywords: Diazepam, UCMS-animal model, Anti-stress, Corticosterone, Resveratrol.

INTRODUCTION

Stress is considered any condition, which results in perturbation of the body's homeostasis. If the level of stress is extreme, the homeostatic mechanisms of the organism become deficit and the survival of the organism is threatened.[1] Stress has been postulated to be involved in the etiopathogenesis of a variety of disease states, viz; hypertension, peptic ulcer, diabetes, immunosuppression, reproductive dysfunctions and behavioural disorders like anxiety due to involvement of the central nervous system (CNS), endocrine system, and metabolic system.[2] Herbal formulations have been in use for many years not only in Asian countries but also globally for human well-being. The herbal formulations claimed to enhance physical endurance; mental functions and non-specific resistance of the body have been termed as adaptogens. [3]

Vitis vinifera, known as the grape fruit, belonging to the family Vitiaceae, is native to southern Europe and Western

Asia. Grape fruit (*Vitis vinifera*) skin is a rich source of resveratrol. It is a potent antioxidant and neuroprotective drug.[4] As the antioxidant effect showing compounds also produce the anti stress effect leading to variations in different biochemical parameters.[5] *Withania somnifera* (popularly known as Ashwagandha) is a green shrub, family Solanaceae.[6,7] The plant preparation has anti-inflammatory[8], anticancer[9,10] antistress and immunomodulatory, [11] adaptogenic,[12] activities.

The aim of present investigation was to explore the potential of resveratrol as a potent antioxidant and neuroprotective drug [4]. More often than not the compounds possessing antioxidant activity also produce the anti stress effect and consequent variations in the levels of various biochemical indicators such as corticosterone. [5] Grape fruit (*Vitis vinifera*) skin is a rich source of resveratrol. *W. somnifera* is known to modulate the oxidative stress markers of the body. The root extract is reported to significantly reduce the lipid peroxidation and increase the superoxide dismutase (SOD) and catalase activity, thus possessing a free radical scavenging property.

MATERIALS AND METHODS

Plant collection and preparation of extract: For the comparative evaluation of drugs, roots of *Withania somnifera* (Ashwagandha) were collected from local market in the month of August and fruits of *Vitis vinifera* (Black Grapes) were collected from local market in Kanpur, UP. The plants were authenticated by National Vrakshayurveda Research Institute, Jhansi, India. Voucher Accession No. of *Withenia somnifera* is 21708 and of *Vitis vinifera* is 21709. For future reference specimens were preserved as herbarium. The powdered root of *Withania somnifera* was packed in soxhlet apparatus and continuously extracted with ethanol (60 - 80°C) till complete extraction. The solvent was removed by distillation and then concentrated extract was dried under reduced pressure using rotary evaporator (at temperature not exceeding 40^{o}C) and then moderate heating on water bath. A yellowish brown extract was obtained. The ethanolic extract was kept in petri dish and it was stored in dessicator at cool place. [13] The fresh fruits of black grapes were washed and dried in shade. After that, fruit skin has been separated manually and dried in the shade. Once the skin part almost dried, it was extracted with ethanol by cold maceration. The skin was dipped in ethanol in a conical flask and was shaken with the help of Orbital shaking incubator for 48 hours. After then macerated by cold maceration and separated the marc. [14]

Animals: Adult male Wistar albino rats (180-220 g) were obtained from the Central Animal House, IVRI, Izzat nagar, Bareilly. The animals were housed in polypropylene cages at an ambient temperature of 25°C and 45-55% RH, with a 12:12 h light/dark cycle. The animals were fasted overnight before experiment and given food and water *ad libitum*. Experiments were conducted as per CPCSEA guidelines. The research proposal no. (IAEC/01/13) was duly approved by IAEC through its letter no. (Ref No. - IAEC/RAP/3457) dated 17/03/2013.

Preliminary study for selection of dose and treatment of drug: The doses of *Withania somnifera* (100 mg/kg; oral) were used. [15] The dose of *Vitis vinifera* (100 mg/kg; oral) were used[16] and the dose of diazepam (5 mg/kg; oral) were used. Rats (n=5) were randomly assigned to negative-stress control, positive-stress control and treatment groups i.e. *Withania somnifera* extract, *Vitis vinifera* extract and Diazepam. Positive control group and treatment groups were unpredictable chronic mild stress model for 21 days repeatedly. Lastly, all the rats were exposed to elevated plus maze apparatus study and forced swim test.

Unpredictable Chronic Mild Stress model (UCMS): Rats were subjected to unpredictable chronic mild stress model. The study continued for 21 days. Animals were subjected to stress paradigm once a day over a period of 21 days. The animals were housed in cages at an ambient temperature of 25°C and 45-55% RH, with a 12:12 h light/dark cycle. The orders of stressors were used as follows: tail pinch (1 mm, 30 s), food and water deprivation (24 h), swimming at room temperature (25°C, 20 min), day-night reversal (24h), cage tilting (45° for 24 h), soiled bedded (150 ml water/ cage), no stress, tail pinch (1mm, 90 s).

Table: 1.Experimental protocol of Unpredictable Chronic Mild Stress model in rats

S.NO	Group	No. of Animal	Treatment	Route of Administration
1	Group I (Negative control)	05	-	-
2	Group II (Positive control)	05	Vehicle	Oral
3	Group III (*Vitis vinifera* extract)	05	*Vitis vinifera* (100mg/kg)	Oral
4	Group IV (*Withania somnifera* extract)	05	*Withania somnifera* (100mg/kg)	Oral
5	Group VDiazepam	05	Diazepam (5mg/kg)	Oral

Table: 2.Time and length of activities using UCMS Protocol

Stress	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
First Week	Tail Pinch (1 mm, 30 s) 10 am	Food and Water deprivation 10 am (24 h)	Swimming at room temperature (25°C, 20 min)11 am	Day-night reversal (24 h) 10 am to 10 pm dark, 10 pm to 10 am light	Cage Tilting (45°for 24 h) 11 am	Soiled bedded (150 ml water/cage) 11 am for 24 h	No stress 11 am for 24 h
Second Week	Tail Pinch (1 mm, 90 s) 11 am	Tail pinch (1mm, 30 s) 10 am	Food and water deprivation 10 am (24 h)	Swimming at room temperature (25°C, 20 min) 11 am	Day-night Reversal (24 h) 10 am to 10 pm dark, 10 pm to 10 am Light	Cage tilting (45° for 24 h) 11 am	Soiled Bedded (150 ml water/cage) 11 am for 24 h
Third Week	No stress 11 am for 24 h	Tail pinch (1 mm, 90 s) 11 am	Tail pinch (1 mm, 30 s) 10 am	Food and water deprivation 10 am (24 h)	Swimming at room temperature (25°C, 20 min) 11 am	Day-night reversal (24 h) 10 am to 10 pm dark, 10 pm to 10 am light	Cage tilting (45° for 24 h) 11 am

After 21 days of unpredictable chronic mild stress model, rats were used for elevated plus maze test and forced-swim test. [17]

Elevated plus maze test*:* Cognition, memory acquisition and retention was tested using an elevated plus maze test on day 22. The apparatus consisted of two crossed arms, one closed and the other open. Each mouse was placed on the open arm, facing outwards. The time taken by the mouse to enter the closed arm in the study on day 21 was noted. The cut-off time was fixed as 90 s and in the case when a mouse could not find the closed arm within this period; it was gently pushed in to one of the closed arms and allowed to explore the maze for 30 s. [18]

Forced swim test: The forced swim test (FST) was performed following the locomotor activity test at daytime, conducted as after UCMS procedure. Each rat was placed individually in a transparent cylindrical polypropylene tank (40 cm height × 30 cm diameter) containing 35 cm of water at 25 ± 1°C, without the possibility of escaping. Rats were forced to swim in water for 5 minutes. A rat was judged immobile when it floated in an upright position, and could only move slowly to keep its head above water. The duration of immobility during the final 5 minutes of the test was recorded. This immobile posture reflects a state of behavioural despair or helplessness. Rat was dried immediately and returned to its home cage after the swimming test. [19]

On day 24, animals were subjected to biochemical test. For biochemical test, blood was collected through retro orbital route puncture in heparinised tubes from over night fasted rats. [20]

Plasma Preparation: All the blood collecting tubes were placed in vacuum centrifuge at 6000 RPM for 10 min. Finally, plasma was separated in fresh heparinised tubes. Plasma was stored in cold place for further biochemical parameter studies.

HPLC method development and estimation of corticosterone in rat blood plasma: A HPLC pump system (Waters 600) with a RP-18 guard column (Applied Biosystems, 5µm, 30 mm x 4.6 mm, i.d.) under isocratic condition i.e. Acetonitrile (ACN), T.D.W. glacial acetic acid (40:60-0.1 %) at a flow rate of 1 .2 ml/min. Eluents were monitored at 245 and 254 nm with UV detector (Waters 2487 dual λ absorbance detector). Mobile phase constituting of the Solvent – A (ACN) and Solvent – B T.D.W. (0.1% v/v solution of glacial acetic acid in triple distilled water) was pumped. Both solvents were degassed by ultrasonication for 15 min before use.

Preparation of mother stock solution: 10 mg of corticosterone was weighed accurately, transferred to a 10 ml volumetric flask, and made up to the volume with ACN. The resulting solution was then vortex mixed and sonicated to get a clear solution of 1 mg/ml conc.

Preparation of working stock solution**:** Working stocks of corticosterone were prepared from the stock of 1 mg/ml. These were prepared by serial dilution technique.

Reconstitution solution: ACN (HPLC grade) was used as a reconstitution solution.

Preparation of analytical standards: Analytical standards were prepared by parallel dilution technique in the range of 2000–31.2 ng/ml in ACN for determination of recovery. Parallel dilution technique from the respective stocks of corticosterone prepared above was used for preparing analytical standards.

Preparation of calibration standards: The calibration standards for corticosterone were prepared by taking 180 µl (200 µl in blank sample) of blood and extracting with 5 ml dicholoromethane by liquid-liquid extraction. Then after extraction, 200 µl of ACN was used for reconstitution. Then 20µl was injected for analysis in HPLC.

Preparation of Quality Control (QC) Samples: Blood samples (180 µl) were spiked with the working stock solutions (spiking volume was 20 µl) and extracted with dichloromethane by liquid-liquid extraction and then 50 µl of clear supernatant was injected into HPLC.

Preparation of Blood sample: Blood samples were prepared by a simple and efficient liquid-liquid extraction technique with n-dichloromethane. To 180 µl of blood, 20 µl of drug was spiked from respective stocks of 40, 20, 10, 5, 2.5, 1.25, and 0.625 µg/ml and vortex mixed for 1 min. Then 2 ml of dichloromethane was added and vortexed again for 3 min, followed by centrifugation at 2500 rpm for 10 min. Later the organic layer was transferred (by snap freezing of aqueous layer in liquid nitrogen) into a clean tube and evaporated to dryness in speed vac-concentrator. The remaining blood from the above step was re-extracted in similar manner. The dry-residues were reconstituted in 200 µl of ACN and 50 µl of it was injected for analysis.[21]

Statistical Analysis

The results of the UCMS studies were subjected to statistical analysis by using ANOVA (One way ANOVA) followed by Tukey-compare all pairs of column test by using Graph Pad Prism Instat software. P value less than (*P<0.05, **P<0.001 and ***P<0.0001) was considered to be statistically significant when compared with control group.

RESULTS

Behavioural examination of Unpredictable Chronic Mild Stress model (UCMS): To explore whether and how depressive behaviour was induced by UCMS, rats were treated with UCMS for 3 weeks in the presence and absence of different stressors. Depressive behaviours were measured manually. After 2 weeks of the UCMS model an easily visible behavioural change was seen. The rats were looking tired and restless every time. The food and water consumption firstly decreased till 2 weeks and during 3rd week the diet excessively increased. Sleep deprivation had been observed. More fear factor was seen among rats during group caging. All the changes were dominant in the positive control grouped animals as compared to other group. Diazepam treated groups were seen almost normal and in better condition among all groups.

Effect of Drugs on immobility period in Forced Swim Test: The effects of oral administration of *Withania somnifera*, *Vitis vinifera* and diazepam on immobility time in the forced swim test are recorded (Table 3 and Figure 1). Chronically stressed rats exhibited significant increase in immobility period as compared to control animals. Diazepam, *Withania somnifera*, *Vitis vinifera* administration reversed the increase in immobility period in stressed rats in a dose dependent manner. Immobility period of stressed rats (66±1.4 sec) were less than treatment groups *(W. somnifera* 91±1.2 sec, *V. vinifera* 100±4.1 sec) indicating significant potentiating difference in the anti-immobility effect of respective drugs.

Table: 3. Effect of Drugs on immobility period in Forced Swim Test in UCMS rats

S.NO.	Groups	Immobility time period (sec)
1	Group I (Negative control)	86±2.1
2	Group II (Positive control)	66±1.4
3	Group III (*Withania somnifera*)	91±1.2
4	Group IV (*Vitis vinifera*)	100±4.1*
5	Group V (Diazepam)	98±8.7*

**P values <0.05 in compare to control; Values are mean±SEM of of 5 rats in each group.*

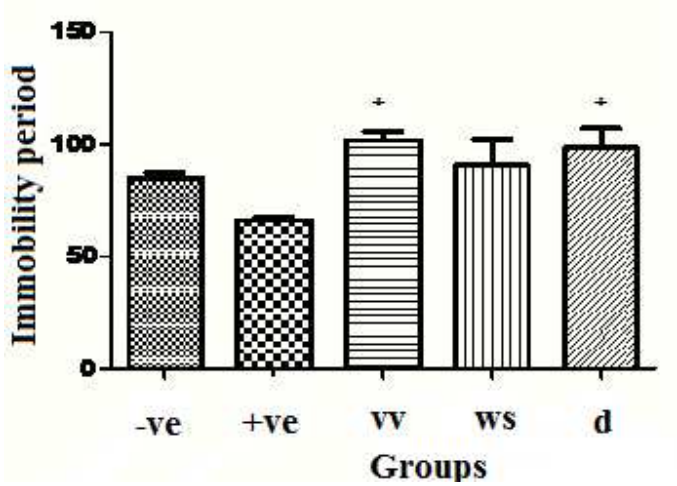


Fig. 1: Effect of control groups, *Withania somnifera* (ws), *Vitis vinifera* (vv) and Diazepam (d) on immobility time in by forced swim test

Effect of drugs on locomotion in elevated plus maze apparatus: Vehicle treated stressed rats (+ve control group) had made less number of open and closed arms entries and spent less time in open and more in closed arms respectively as compared to other groups, which indicates anxiogenic behaviour of stressed rats. Treatment of drugs during stress period significantly justified the anxiolytic behaviour as evidenced by increasing and decreasing time spent in open arm and closed arm respectively when compared to (-ve control group). Drug treated groups produced significant difference in number of entries in close and open arm as compared to positive control group.

Table: 4.Effect of drugs on locomotion in elevated plus maze apparatus in UCMS rats

S.No.	Groups	No. of entries in open arm	No. of entries in closed arm	Average Time spent in open arm (sec)	Average Time spent in closed arm (sec)
1	Group I (-ve control)	6±0.82	6.8±0.48	20±2.7	24±1.6
2	Group II (+ve control)	3±0.41	5.5±0.96	8.1±0.84	18±1.2
3	Group III (*Vitis vinifera*)	9.5±0.65***	11±0.91**	14±1.8	11±9.6
4	Group IV (*Withania somnifera*)	11±0.41***	13±0.48***	24±6.2*	16±1.3*
5	(Diazepam)	9.3±0.95***	11±0.85**	16±1.3	23±4.4*

*Values are in mean±SEM of 5 rats in each group: ***P<0.0001, **P<0.001, *P<0.05*

Stress treated control group rats exhibited less no of entries (3±0.41) in open arm and also less time spent (8.1±0.84) in open arm. *Vitis vinifera* showed (9.5±0.65***), *Withania somnifera* showed (11±0.41***) and Diazepam treated group showed (9.3±0.95***) no. of entries in open arms, which are more than control group and stressed groups. Stressed group produce less average time spent in open arm (8.1±0.84 sec) as compared to treatment groups (*Withania somnifera* (24±6.2* sec), *Vitis vinifera* (14±1.8 sec) and diazepam (16±1.3 sec). *Withania somnifera* group showed significant antistress locomotor behaviour in rats.

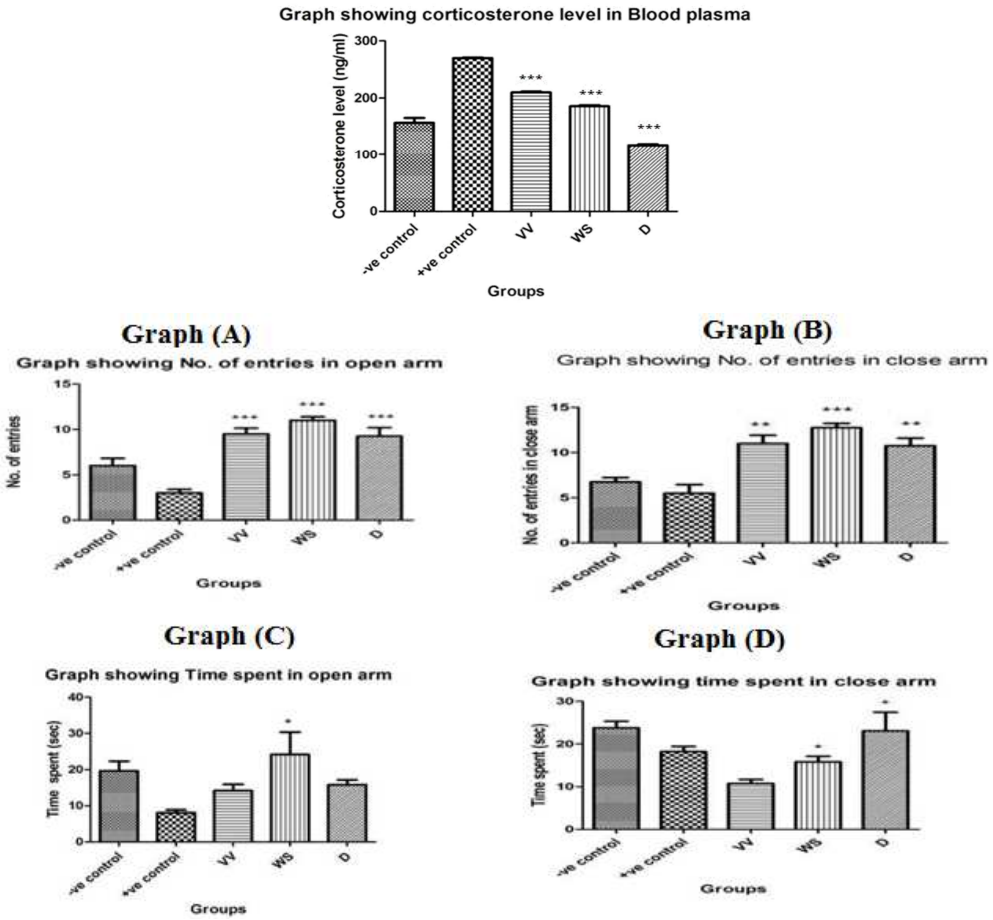
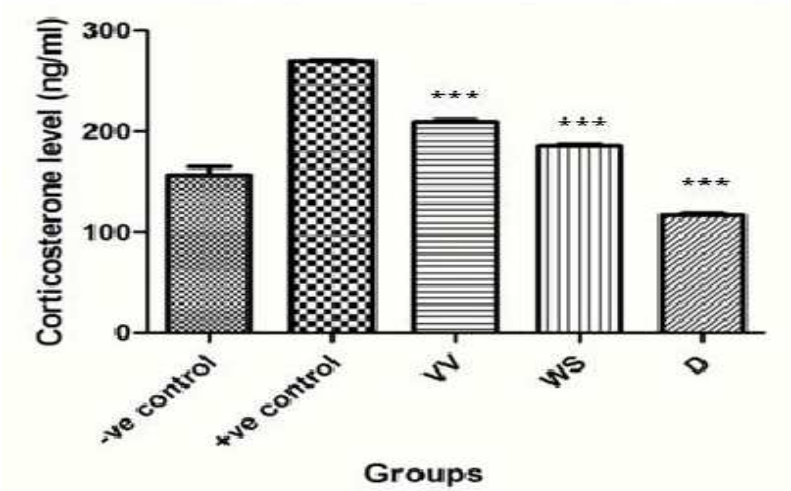


Fig. 2: Graphs (A), (B), (C), (D) showing **effect of control groups, *Withania somnifera* (WS), *Vitis vinifera* (VV) and D (Diazepam) treated groups on locomotor activity**

Fig. 3: Graph (A) Calibration curve of corticosterone
Graph (B) showing the representative chromatograms (a) analytical blank mobile phase, (b) analytical standard (2000 ng/ml), (c) blank blood, (d) calibration standard (2000 ng/ml)



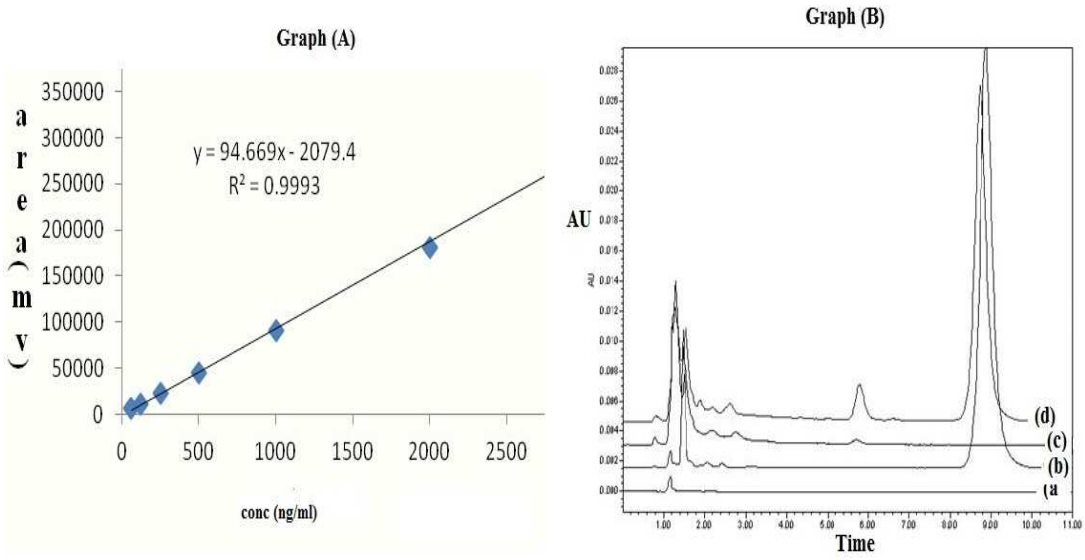


Fig. 4: Corticosterone level in blood plasma of different groups

Table: 5.Effect of drugs on plasma corticosterone level in blood plasma of UCMS rats

S. No	Groups	Result of sample (ng/ml)
1	Group I (-ve control)	140±8.5
2	Group II (+ve control)	270±0.98
3	GroupIII *(Withania somnifera)*	190±1.9***
4	Group IV (*Vitis vinifera)*	210±1.6***
5	Group V (Diazepam**)**	148±1.9***

****P values <0.0001 in compare to control; Values are in mean±SEM in UCMS rats.*

Corticosterone Estimation and HPLC Analysis

Linearity and Reproducibility: Linear least square regression analysis of the calibration graph demonstrated linearity in the range of 31.2-2000 ng/ml for corticosterone. The figure given below gives the calibration curve that was obtained by plotting the peak area of corticosterone against their corresponding concentration in spiked rat's blood. Linear least square regression analysis of the calibration data demonstrated linearity between peak area response and the corresponding concentration over the calibration range 31.2-2000 ng/ml for corticosterone, with good correlation coefficient (R^2=0.9993).

Results showed that treated groups as *Vitis vinifera* (210±1.6***), *Withania somnifera* (190±1.9***) and Diazepam (148±1.9***) had reduced corticosterone levels when compared to stressed group (270±0.98). Values for negative control group (140±8.5) considered as healthy volunteer in the study results are almost near the normal range.

DISCUSSION

The objective of the present study was a comparative anti-stress effect of *Vitis vinifera* and *Withania somnifera* using unpredictable chronic mild stress model in rats. Long term exposure to multiple stressors can cause depression. [22] UCMS depressive model is the well validated model to produce depression. Administration of *Vitis vinifera, Withania somnifera* and diazepam during stress period restored the ambulatory behaviour of the rats can be correlated with restoration of plasma corticosterone level. In the study of immobility time period of force swim test, chronically stressed rats exhibited significant increase in immobility period as compared to control animals. Diazepam, *Withania somnifera*, *Vitis vinifera* administration reversed the increase in immobility period in stressed rats in a dose dependent manner. There was significant potentiating difference observed in the anti-immobility effect of respective drugs. In the study of locomotion activity of rats in elevated plus maze apparatus, treatment of drugs during stress period significantly justified the anxiolytic behaviour as evidenced by increased number of entries in open arm and closed arm respectively when compared to control groups. *Withania somnifera* treatment produced

most anxiolytic behaviour as indicated by study in open arm as compared to close arm. Finally, the biochemical examination of stress by corticosterone estimation in blood plasma of rats showed the reduced corticosterone level in rats as compared to control group. Thus, the results of the present study justified that *Withania somnifera* is most antistress effect showing group among other groups. *Vitis vinifera* and diazepam exhibited comparative significant antistress activity in rats.

CONCLUSION

Stress is a state of threatened homeostasis provoked by psychological, physiological or environmental stressors. Stress is a stimulus either internal or external which activates the sympathetic nervous system resulting in physiological change. In the present study, stressed rats exhibited anxiogenic behaviour and depression coupled with increased corticosterone level in plasma. This indicates that the rats are in stressful condition and the alteration observed is similar to clinically related pathophysiology of depression. This study proved the best anxiolytic adaptive behaviour in stressed rats in treatment groups, thus justifying the antistress capability of respective herbal plants.

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