

Oral Administration of *Rauwolfia serpentina* Plant Extract Mitigated Immobilization Stress-Induced Behavioral and Biochemic and Deficits in Rats

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Abstract: Objectives: Now a day's global population is moving towards the herbal drugs which contain bioactive compounds, to cure the diseases. *Rauwolfia serpentina* is also a medicinally important herb with mainly effective in the treatment of hypertension and psychotic disorders. The present study was designed to investigate the effects *Rauwolfia serpentina* on acute stress. The herb extract was orally administered before immobilization for 2 h only to monitor any change in behavioral activities. We also evaluated the role of *Rauwolfia serpentina* in oxidative stress, like its effect on antioxidant enzymes activities such as catalase and superoxide dismutase and also on plasma glucose, corticosterone and leptin levels. Methods: Animals were divided into four groups that were (i) saline unstressed; (ii) *Rauwolfia serpentina* unstressed; (iii) saline stressed; (iv) *Rauwolfia serpentina* stressed, which were injected accordingly with saline (1 mL/kg) or *Rauwolfia serpentina* (30 mg/kg). Animals of the stressed group received immobilized for 2 h. Behavioral analysis was performed after the termination of 2 h immobilization period. Animals were then decapitated and plasma samples were collected for CAT, SOD, corticosterone, leptin and glucose estimation. Results: Results showed that *Rauwolfia serpentina* is an effective anxiolytic agent as it attenuate stress induced behavioral deficits and improves locomotor activity. On the other hand, it provides positive outcomes in antioxidant enzymes levels of stressed animals. Conclusion: *Rauwolfia Serpentina* was found to prevent the stress-induced increase in corticosterone and an increased in the levels of endogenous leptin attenuates the stress-induced activity of HPA axis. It is also concludes that 30 mg/kg of *Rauwolfia serpentina* was not sufficient to produce hypoglycemic effects. However, more studies are recommended to explain the particular action by which *Rauwolfia serpentina* produces its effects.

Keywords: acute stress; *Rauwolfia serpentina*; behavioral activities; oxidative enzymes; glucose; leptin

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1. Introduction

Stress exhibited an imperative role in the etiology, exacerbation and cure of affective psychopathology suggesting close interplay between the two [1]. Acute stress is a result of a traumatic event that makes a person to feel fear and helplessness [2]. A variety of diverse environmental and stressful stimuli have also been reported to alter behavioral pattern, neurotransmitter level and oxidative damage in discrete areas of brain [3,4]. However, effects of stress on the brain have long been associated with the onset and exacerbation of several neuropsychiatric disorders such as depression, anxiety, drug addiction, and epilepsy [5]. Parallel studies on experimental animals show that an uncontrollable stressor produced neurochemical changes and behavioral deficits [6,7]. Several

investigators have suggested a link between oxidative stress and certain anxiety disorders such as obsessive compulsive disorder and panic disorder indicating that oxidative metabolism can affect the regulation of anxiety.

Recognizing elements that contribute to neurodegenerative progressions in the brain is one of the chief goals of contemporary medicine. There are several hypotheses regarding the mechanisms that lead to the damage and death of brain cells in neurodegenerative diseases, [8] such as excitotoxic effects by excitatory amino acids, [9] impairment in cellular energy metabolism [10,11] and oxidative stress (OS), which is caused by free radicals or other reactive molecules [9,12]. The results of many in vitro and in vivo preclinical and clinical studies have consistently demonstrated that OS is one of the crucial players in the degeneration that occurs in the nervous system. The imbalance between OS and antioxidant defense systems seems to be a universal condition in neurodegeneration [13]. Clinical and pre clinical studies indicate that neurodegenerative diseases are characterized by higher levels of OS biomarkers and by lower levels of antioxidant defense biomarkers in the brain and peripheral tissues [14]. There is now increasing evidence that reactive oxygen species (ROS) generation is involved in the regulation of neurotransmission, in particular glutamate release, which most likely plays an important role in the “fighter flight response” [15]. Oxidative stress divulges a state of cellular imbalance, in which reactive oxygen species (ROS) production surpasses antioxidant response mechanisms which help to neutralize ROS-mediated oxidative damage to DNA, RNA and lipids leading to innumerable pathophysiological consequences [16,17]. In order to counter balance the free radical induced damage of biological molecules, antioxidant mechanisms and enzymes are activated. The role of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were identified as antioxidant enzymes that act as body’s first line of defense against ROS by catalyzing their conversion to less reactive or inert species [18]. Consequently, research has revealed that individuals with anxiety or depression show an extensive range of abnormalities in controlling fear-related responses, suggesting that deficits in emotion regulation may be linked to neurobiological differences in response to stress [1].

Now a day’s global population is moving towards the herbal drugs which contain bioactive compounds to cure the diseases [19]. *Rauwolfia serpentina* belonging to the family Apocynaceae, is an important medicinal plant in the pharmaceutical world due to the presence of its immense therapeutic properties [20,21]. It is effective in the treatment of hypertension and psychotic disorders like schizophrenia, anxiety, insomnia, insanity, and so forth [22]. Various indole alkaloids and related constituents have been isolated from the roots of this plant which have significant biological activities [23]. An in vitro study described the antimicrobial and antioxidant activities of leaf extract of this plant [24]. The principle alkaloid of *Rauwolfia serpentina* is reserpine.[24]. It is present in root, stem and leaves of the plant. It contain not less than 0.15% of Reserpine-rescinnamine group alkaloids, calculated as Reserpine [25].

Previously, numerous studies have been reported from our laboratory that establishes the capability of phytochemicals present in Rice bran Oil [26], olive oil [27] and aqueous fruit extract of Sea buckthorn [28] to attenuated/or reversed anxiety in rats. Similarly, our laboratory also explored that oral administration of Red rice bran oil averted haloperidol-induced anxiety syndrome in rats [29]. Conversely, oral administration of *Nigella sativa* (NS) and *Olea europaea* (OE) oil did not show anxiolytic effects in rats [30]. In perpetuation of our research on the plant, the present study was designed to investigate the neuroprotective effects *Rauwolfia serpentina* following acute exposure to immobilization stress in rats. The herb extract was orally administered at a non-sedative dose 30 mg/kg [31] before immobilization for 2 h to monitor any change in behavioral activities. The neuroprotective efficacy of plant extract was assessed in terms of its potency to attenuate oxidative stress induced alterations of antioxidant enzymes activities such as CAT and SOD and locomotor deficits. In order to get insight in the role of *Rauwolfia serpentina* in HPA axis, we also monitored plasma leptin, corticosterone and glucose levels. The

study would possibly help to establish that *Rauwolfia serpentina* plant extract may have potential therapeutic significance for the management of stress and related disorders.

2. Materials and Methods

2.1. Animals

Locally bred albino wistar rats, weighing 180–200 g purchased from PCSIR were housed individually on a 12 h light/dark cycle in a temperature-controlled room (24 ± 2 °C) with free access to tap water and cubes of standard rodent diet, for at least 7 days before the start of experiment (establishing familiarity with the environment). All procedures conducted were approved by the Local Institutional Animal Care and Use Committee at University of the Health Sciences and conducted in full compliance with the National Institutes of Health Guide for Care and use of Laboratory Animals.

2.2. Preparation of Plant Extract

Thirty grams of ground powder of roots of *Rauwolfia serpentina* was extracted with methanol (1 L; 95%) overnight and filtered through Whatman No.1 filter paper twice. The filtrate was then concentrated at 40°C till dryness in a rotary vacuum evaporator (Eyelane) to obtain a brown residue that was referred as methanolic root extract (MREt) [22]. This procedure yielded 3–4% (*w/w*) of the dry root. The MREt was stored in an airtight container in refrigerator below 10 °C until used.

2.3. Immobilization Procedure (Restraining Procedure)

The animals of stress groups were subjected to single exposure of immobilization stress for 2 h. Immobilization was done in separate room to prevent unstressed animals from being under stressful condition due to disturbance. The animals were immobilized by approved procedure [32,33]. Wire grids of 10" × 9" fitted with a Perspex plate of 9" × 6.5" as described earlier [33] were used. Immobilization was affected by pressing the legs of the rats through the gaps in the metal grid and tapping them together with zinc-oxide plaster. Hind limbs were also tapped and the head of animal rested on the Perspex plate. After 2 h immobilization stress, animals were released by applying acetone to the tape and returned to their home cage.

2.4. Behavioral Analysis

2.4.1. Activity in a Novel Environment (Open Field)

The locomotor activity of control and test rats was monitored in an open field apparatus. Open field is the square area of 76 × 76 cm with opaque walls of 42 cm height. The floor was divided by lines into 25 equal squares. The test was performed in a quiet room under white light to avoid any noise effect as described earlier [34,35]. Animals were placed in the center square of the open field (one at a time). Activity in open field was determined by counting number of squares crossed for 5 min [36]. Exploratory activity of control rats and test rats were monitored in a balance design to avoid order effect.

2.4.2. Light-Dark Transition Test

The light-dark transition test, a behavioral test used to monitor the anxiolytic effects of drugs in preclinical investigations, is based on the innate aversion of rodents to brightly illuminated areas. The test procedure was essentially the same as described earlier [37]. The apparatus used in the present investigation was a two-compartment light-dark box. Both the light compartment (made out transparent plastic) measured 26 × 26 × 26 cm access between two compartments was provided by a 12 × 12 cm passageway. The experiment was performed in a quiet, air-conditioned room, and the apparatus was placed under white light. An animal was introduced into the apparatus via the light compartment. Cumulative time spent in the light compartment and the numbers of entries into the light

compartment were monitored for a period of 5 min. An entry was defined as all four paws being positioned within light compartment. The degree of anxiety was assessed by a decrease in time passed in light compartment and also by a decrease in number of entries made to light compartment.

2.5. Blood Sample Collection

Blood was collected from rats in heparinized centrifuge tubes. Centrifugation was done 10 min. Plasma was collected and stored at -70 Celsius till biochemical estimation of the plasma glucose concentration in mg/dL, corticosterone concentration in $\mu\text{g}\%$, leptin concentration in ng/mL, catalase and superoxide dismutase.

2.6. Biochemical Estimation of Glucose, Catalase And Superoxide Dismutase in Plasma

2.6.1. Determination of Catalase (EC1.11.1.6)

CAT activity was estimated by method of Patterson [38]. The decomposition of H_2O_2 was measured at 240 nm taking De at 240 nm as 43.6 mMcm^{-1} . Reaction mixture (3.0 mL) consisted of 10.5 mM H_2O_2 in 0.05 M potassium phosphate buffer (pH 7.0) and the reaction was initiated after the addition of 0.1 mL enzyme extract at 25°C . The decrease in absorbance at 240 nm was used to calculate the activity. One unit of CAT activity is defined as the amount of enzyme that catalyzes the conversion of 1 mM of $\text{H}_2\text{O}_2 \text{ min}^{-1}$ at 25°C [39]

2.6.2. Determination of Superoxide Dismutase (EC.1.15.1.1)

The assay for SOD activity was performed by the method of [40]. The assay mixture consisted of 27.0 mL of 0.05 M potassium phosphate buffer (pH 7.8), 1.5 mL of L-methionine (300 mg per 2.7 mL), 1.0 mL of nitroblue tetrazolium salt (14.4 mg per 10 mL), and 0.75 mL of Triton X-100. Aliquots (1.0 mL) of this mixture were delivered into small glass tubes, followed by the addition of 20 mL enzyme extract and 10 mL of riboflavin (4.4 mg per 100 mL). The cocktail was mixed and then illuminated for 15 min in an aluminum foil-lined box, containing 25 W fluorescent tubes. In a control tube the sample was replaced by 20 mL of buffer and the absorbance was measured at 560 nm. The reaction was stopped by switching off the light and placing the tubes in the dark. Increase in absorbance due to formation of formazan was measured at 560 nm. Under the described conditions, the increase in absorbance in the control was taken as 100% and the enzyme activity in the samples were calculated by determining the percentage inhibition per minute. One unit of SOD is the amount of enzyme that causes a 50% inhibition of the rate for reduction of nitroblue tetrazolium salt under the conditions of the assay [39].

2.6.3. Estimation of Glucose in Plasma by God Pap Method

The concentration of glucose in plasma was measured by using glucose-oxidase method (GOD-PAP, Solo per USO diagnostico in vitro)

2.6.4. Estimation of Leptin and Corticosterone in Plasma by Elisa Kit

Animals were decapitated followed by the collection of blood in heparinized centrifuge tube. Centrifugation processed for 20 min at 2000 g and 4°C to obtain plasma. The samples were stored at -70 degree Celsius until the assay of plasma leptin and corticosterone using an ELIZA kit (Cat # EZRL-83K). Visit website www.milipore.com/bmia (accessed on 1 March 2022).

3. Experimental Protocol

Twenty four animals randomly divided to two equal groups of 12 each were assigned to unstressed and stressed groups. These animals were further divided into four groups of 6 rats each that were designated as (i) saline unstressed; (ii) *Rauwolfia serpentina* unstressed; (iii) saline stressed; (iv) *Rauwolfia serpentina* stressed, which were orally administered with saline (1 mL/kg) or *Rauwolfia serpentina* (30 mg/kg). Animals of the stressed

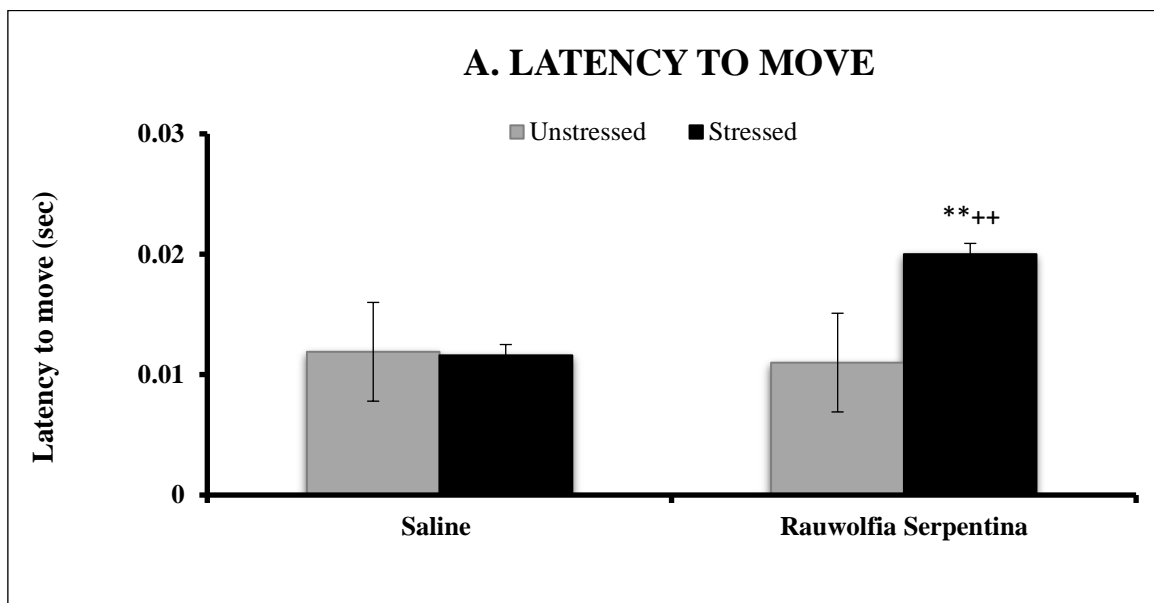
group were immobilized for 2 h commencing between 9:00–11:00 h. Animals of the unstressed group were left to their home cage during this time. Behavioral activities were monitored in open field activity and light dark transition box after the termination of 2 h immobilization period. Plasma samples were collected for CAT, SOD, corticosterone, glucose and leptin estimation. The experiment was performed in a balanced design in such a way that of control and test treated rats were measured alternately to avoid the order effect.

Statistical Analysis

Values are presented as mean ± SD. Data was analyzed by two way ANOVA. Post hoc analysis was done by Newman-keuls test. Value of $p < 0.01$ were considered as significant.

4. Results

Figure 1A Shows changes of motor activity in a novel environment in animals orally administered with *Rauwolfia serpentina* 2 h before exposing animal to acute immobilization stress for 2 h. Analysis of the data on latency to move (Figure 1A) showed significant effects of stress ($F = 7.737 p < 0.01$ df 1,20) and *Rauwolfia serpentina* ($F = 7.737 p < 0.01$ df 1,20) as well as the interaction between two factors ($F = 8.796 p < 0.01$ df 2,20).



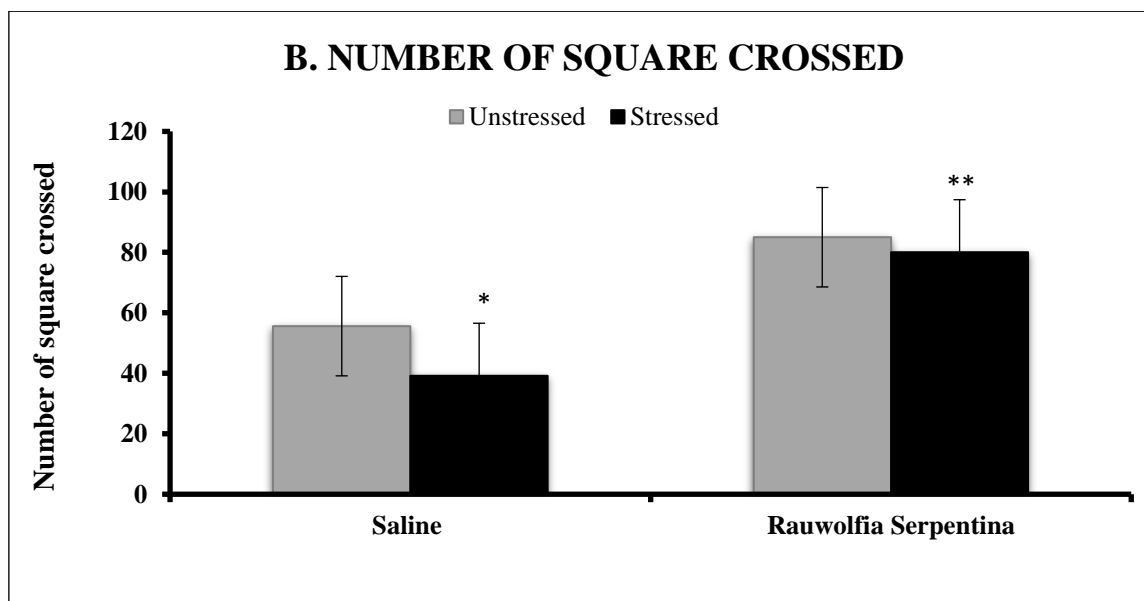


Figure 1. CHANGES OF MOTOR ACTIVITY IN A NOVEL ENVIRONMENT IN ANIMALS ORALLY ADMINISTERED WITH *RAUWOLFIA SERPENTINA* EXPOSED TO ACUTE 2 h IMMOBILIZATION STRESS. Values are means ± S.D. (n = 24). Significant differences by Newman-keuls test. ** $p < 0.01$ and * $p < 0.05$ from similarly treated unstressed control animals. ++ $p < 0.01$ from respective (unstressed or stressed) animals.

Post hoc analysis by Newman keuls revealed that administration of *Rauwolfia serpentina* at dose of (30 mg/kg) to stress rats showed increase in latency to move as compare to unstressed rats. On the other hand saline + stressed rats did *not show any significant* difference in latency to move as compare to unstressed rats. In comparison with *Rauwolfia serpentina* +stressed rats showed increase in latency to move with saline + stressed rats.

Figure 1B Shows changes of motor activity in a novel environment in animals orally administered with *Rauwolfia serpentina* 2 h before exposing animal to acute immobilization stress for 2 h. Analysis of the data on number of square crossed (Figure 1B) showed significant effects of stress ($F = 4.017 p < 0.05$ df 1,20) and *Rauwolfia serpentina* ($F = 43.136 p < 0.01$ df 1,20). The interaction between the two factors was not significant ($F = 1.143$ N.S).

Post hoc analysis by Newman keuls showed decrease number of square crossed by saline + stressed rats but not in *Rauwolfia serpentina* + stressed rats. Alone *Rauwolfia serpentina* rats showed increased locomotor activity in open field. On the other hand, stress induced decreases of locomotor activity was reversed in *Rauwolfia serpentina* administered stressed rats.

Figure 2A Shows changes of behavior in light dark transition test in animals orally administered with *Rauwolfia serpentina* 2 h before exposing animals to acute immobilization stress for 2 h. Analysis of the data on entries in light box (Figure 2A) showed significant effects of stress ($F = 16.298 p < 0.01$ df 1,20) and interaction between the two factors ($F = 5.391 p < 0.01$ df 1,20). Effects of *Rauwolfia serpentina* was not significant ($F = 1.589$ N.S).

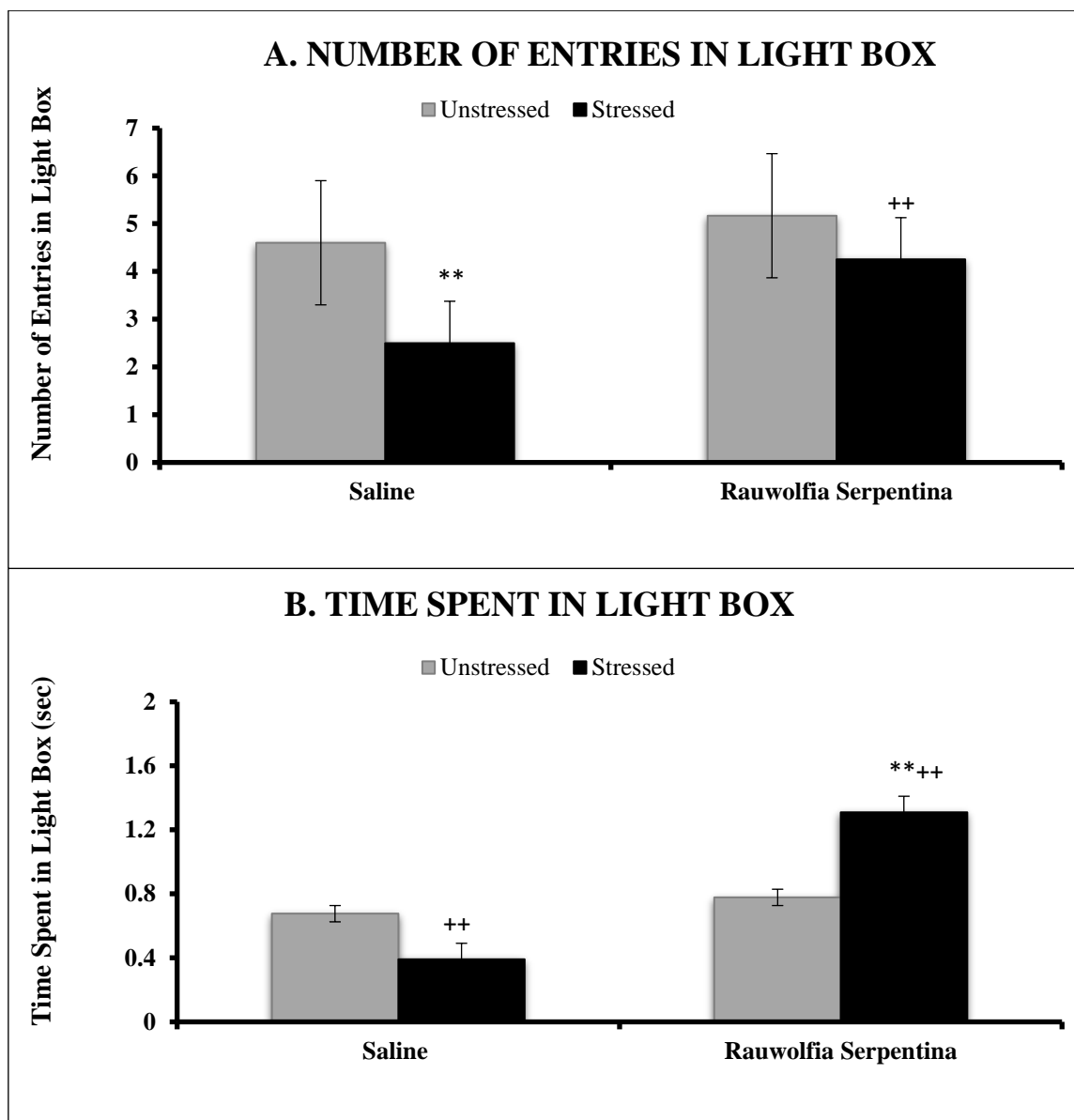


Figure 2. CHANGES OF BEHAVIOUR IN LIGHT DARK TRANSITION TEST IN ANIMALS ORALLY ADMINISTERED WITH *RAUWOLFIA SERPENTINA* EXPOSED TO ACUTE 2 h IMMOBILIZATION STRESS. Values are means \pm S.D. (n = 24). Significant differences by Newman-keuls test. ** $p < 0.01$ and * $p < 0.05$ from similarly treated unstressed control animals. ++ $p < 0.01$ and from respective (unstressed or stressed) animals.

Post hoc analysis by Newman keuls showed decrease number of entries in light dark transition box in *Rauwolfia serpentina* + stressed and saline +stressed animals as compare to their respective controls. Alone *Rauwolfia serpentina* rats showed increase number of entries in light dark transition box. On the other hand, stress induced decreases of number of entries in the light dark box was reversed in *Rauwolfia serpentina* administered rats.

Figure 2B Shows changes of behavior in light dark transition test in animals orally administered with *Rauwolfia serpentina* 2 h before exposing animals to acute immobilization stress for 2 h. Analysis of the data on time spend in light box (Figure 2B) showed non significant effects of stress ($F = 1.146$ N.S) and significant effects of *Rauwolfia serpentina* ($F = 20.861$ $p < 0.01$ df 1,20) and interaction between the two factors ($F = 7.740$ $p < 0.01$ df 2,20).

Post hoc analysis by Newman keuls showed decrease time spent in light dark transition box (sec) in saline +stressed rats but significant increased in *Rauwolfia serpentina* +stressed rats. *Rauwolfia serpentina* alone did not increased locomotor activity in a light

dark transition box. On the other hand, stress induced decrease of locomotor activity was reversed in *Rauwolfia serpentina* administered stressed rats.

Figure 3 Shows effects of stress on oral administration of *Rauwolfia serpentina* on plasma glucose level. Analysis of the data on glucose level (Figure 3) showed non significant effects of stress ($F = 0.566$ N.S), *Rauwolfia serpentina* ($F = 2.144$ N.S) as well as the interaction between the two ($F = 3.142$ $p < 0.005$ df 2,20).

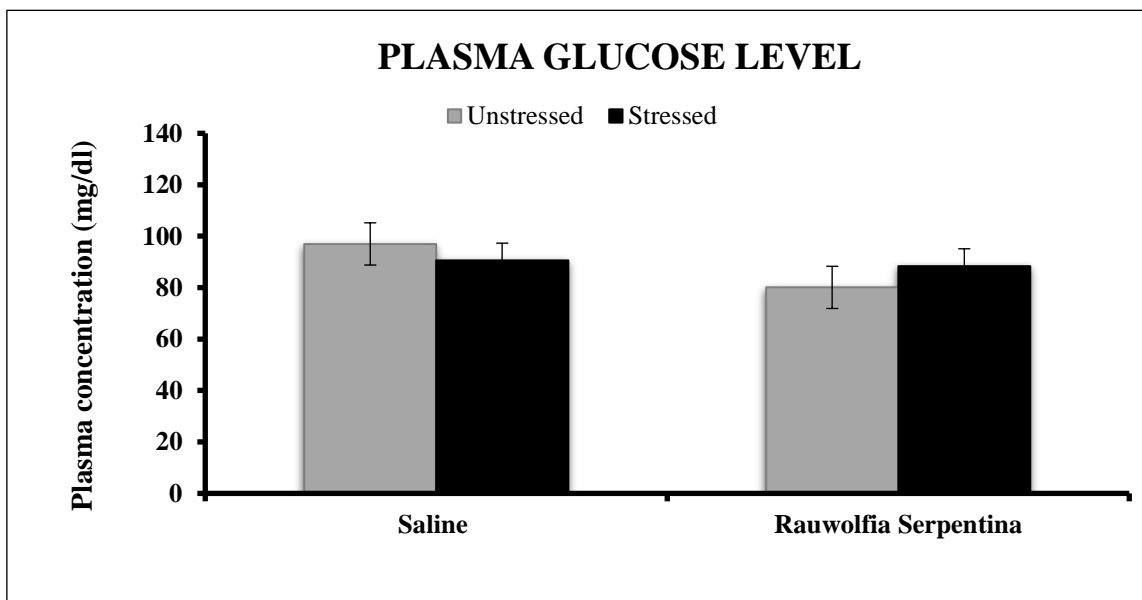


Figure 3. CHANGES IN THE LEVELS OF GLUCOSE IN ANIMALS ORALLY ADMINISTERED WITH *RAUWOLFIA SERPENTINA* EXPOSED TO ACUTE 2 h IMMOBILIZATION STRESS. Values are means \pm S.D. (n = 24).

Post hoc analysis by Newman keuls test revealed that concentration of plasma glucose was not significant in all groups.

Figure 4 Shows effects of stress on oral administration of *Rauwolfia serpentina* on plasma CAT activity. Analysis of the data on CAT activity (Figure 4) showed non significant effects of stress ($F = 0.508$ N.S) and interaction between the two factors ($F = 2.802$ N.S). Effects of *Rauwolfia serpentina* was significant ($F = 4.858$ $p < 0.05$ df 2,20).

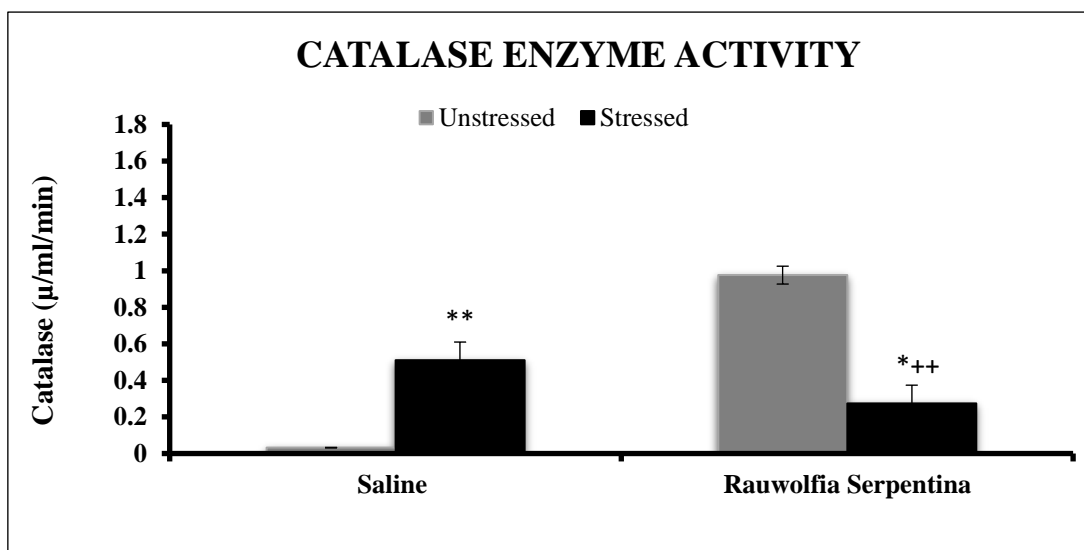


Figure 4. CHANGES IN THE LEVELS OF CATALASE ACTIVITY IN ANIMALS ORALLY ADMINISTERED WITH *RAUWOLFIA SERPENTINA* EXPOSED TO ACUTE 2 h IMMOBILIZATION

STRESS. Values are means \pm S.D. (n= 24). Significant differences by Newman-keuls test. ** $p < 0.01$ and * $p < 0.05$ from similarly treated unstressed control animals. ++ $p < 0.01$ and from respective (unstressed or stressed) animals.

Post hoc analysis by Newman keuls revealed that activity of CAT was significantly increased in saline + stressed rats but significant decreased in *Rauwolfia serpentina* + stressed rats. *Rauwolfia serpentina* alone administration increased CAT activity. On the other hand, stress induced increase of CAT activity was attenuated in *Rauwolfia serpentina* administered stressed rats.

Figure 5 Shows effects of stress on oral administration of *Rauwolfia serpentina* on plasma SOD activity. Analysis of the data on SOD activity (Figure 5) showed significant effects of stress ($F = 3.282 p < 0.05$ df 1,20). Effects of *Rauwolfia serpentina* ($F = 2.256$ N.S) and the interaction between two were not significant ($F = 1.121$ N.S).

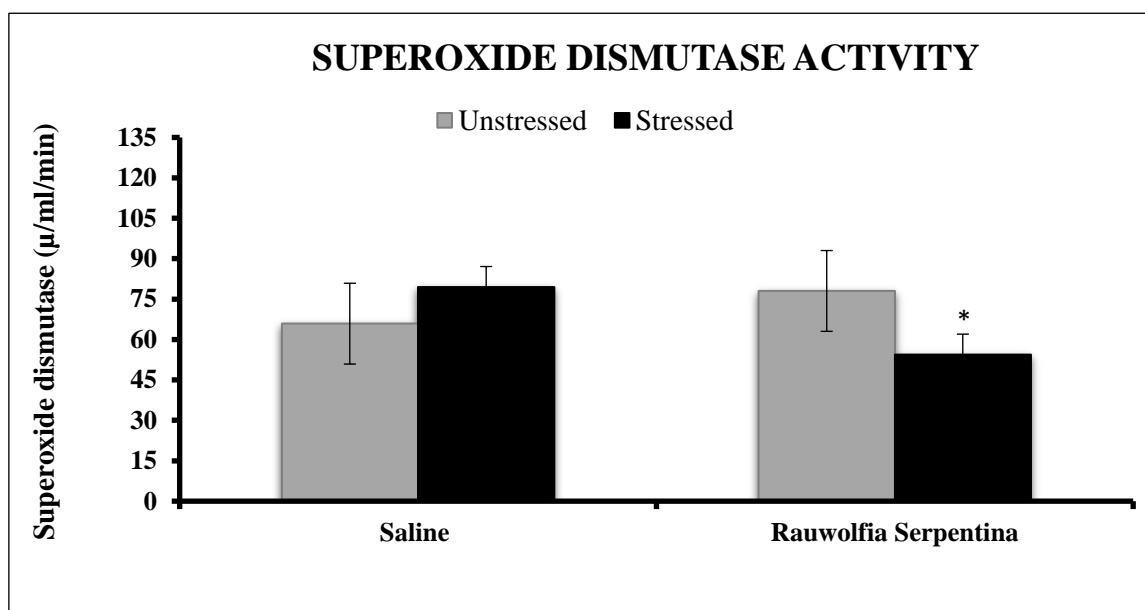


Figure 5. CHANGES IN THE LEVELS OF SUPEROXIDE DISMUTASE ACTIVITY IN ANIMALS ORALLY ADMINISTERED WITH *RAUWOLFIA SERPENTINA* EXPOSED TO ACUTE 2 h IMMOBILIZATION STRESS.

Post hoc analysis by Newman keuls showed that activity of SOD was significantly decreased in *Rauwolfia serpentina*+ stressed rats. But *Rauwolfia serpentina* alone did not alter the activity of SOD. On the other hand, the activity of SOD was not significant in other group.

Figure 6 Shows effects of stress on oral administration of *Rauwolfia serpentina* on plasma corticosterone level. Analysis of the data on corticosterone level (Figure 6) showed significant effects of stress ($F = 9.0$ df 1,20 $p < 0.01$), *Rauwolfia serpentina* ($F = 7.92$ df 2,20 $p < 0.01$) as well as the interaction between the two ($F = 26.01$ df 1,20 $p < 0.01$).

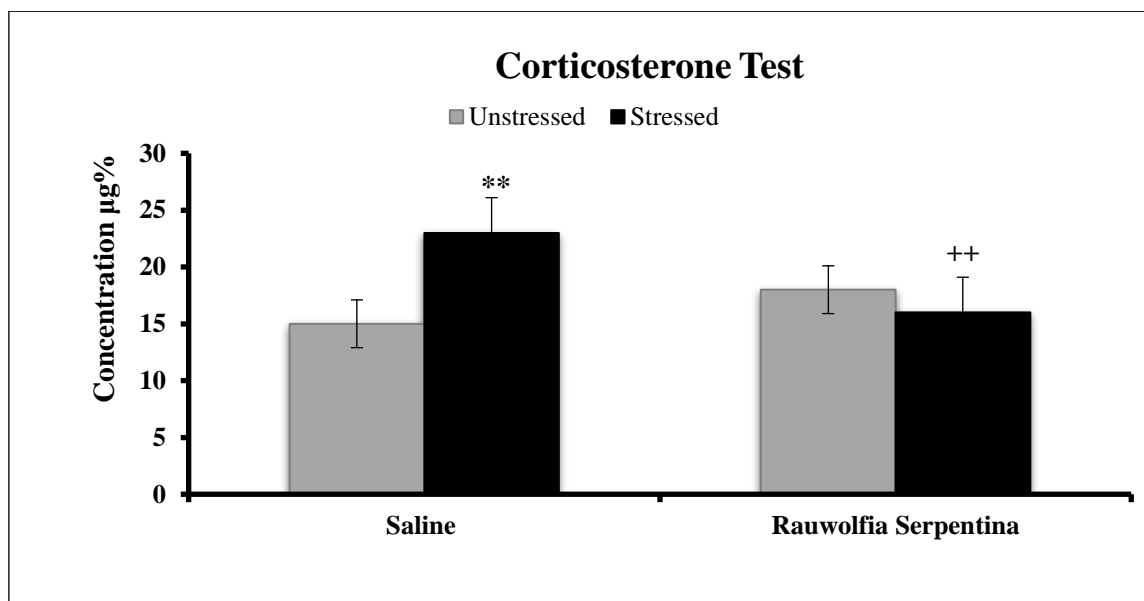


Figure 6. CHANGES IN THE LEVELS OF CORTICOSTERONE IN ANIMALS ORALLY ADMINISTERED WITH *RAUWOLFIA SERPENTINA* EXPOSED TO ACUTE 2 h IMMOBILIZATION STRESS. Values are means \pm S.D. (n = 24). Significant differences by Newman-keuls test. ** $p < 0.01$ from similarly treated unstressed control animals. ++ $p < 0.01$ and from respective (unstressed or stressed) animals.

Post hoc analysis by Newman keuls showed significant increased level of corticosterone in saline +stressed animals but decreased in *Rauwolfia serpentina* +stressed animals. On the other hand immobilization stress induced increase of corticosterone did not occur in single *Rauwolfia serpentina* administered animals.

Figure 7 Shows effects of stress on oral administration of *Rauwolfia serpentina* on plasma leptin level. Analysis of the data on leptin level (Figure 7) showed significant effects of stress ($F = 9.0$ df 1,20 $p < 0.01$) and *Rauwolfia serpentina* ($F = 7.92$ df 2,20 $p < 0.05$). A non significant interaction between the two factors ($F = 26.01$ N.S).

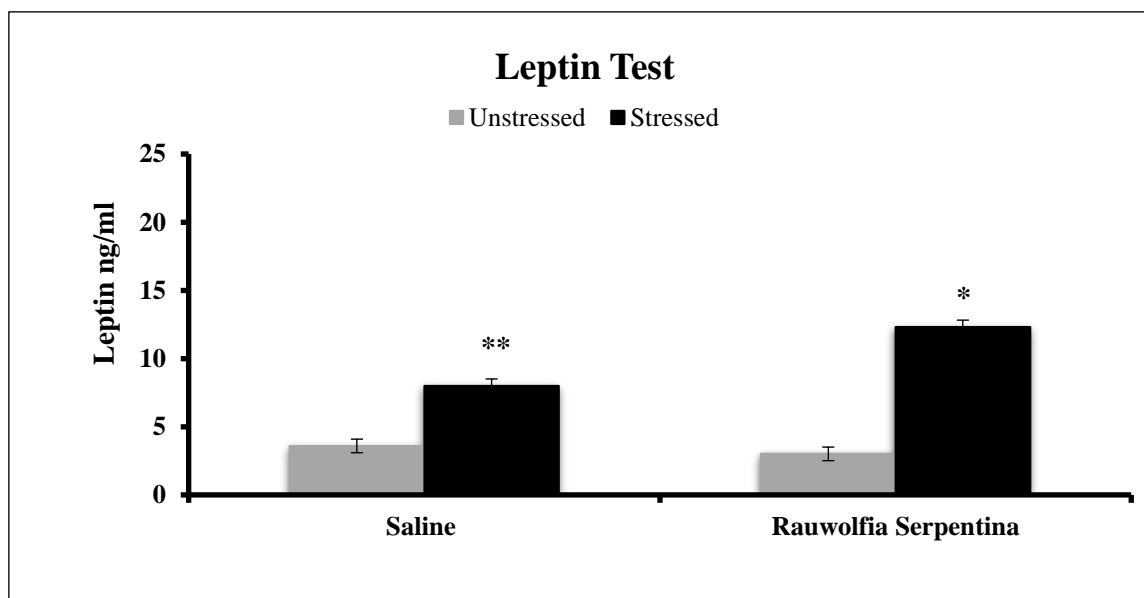


Figure 7. CHANGES IN THE LEVELS OF LEPTIN IN ANIMALS ORALLY ADMINISTERED WITH *RAUWOLFIA SERPENTINA* EXPOSED TO ACUTE 2 h IMMOBILIZATION STRESS. Values are means \pm S.D. (n = 24). Significant differences by Newman-keuls test. ** $p < 0.01$ and * $p < 0.05$ from similarly treated unstressed control animals.

Post hoc analysis by Newman keuls showed significant increased in both saline +stressed and *Rauwolfia serpentina* +stressed animals as compare to their unstressed control rats respectively. In comparison with *Rauwolfia serpentina* +stressed rats showed increase level of leptin with saline + stressed rats.

5. Discussion

Experiencing stress is an inexorable part of everyday life that serves a precarious role in shaping adaptive behavior [41]. Acute exposure to the immobilization stress has been reported to impair motor activity, cause memory dysfunction, modulate anxiety [42], pain perception [43] and depression-like behaviors [44] in the animals. The goal of the current study was to observe the neuroprotective effects of *Rauwolfia serpentina* on the behavioral activity of animals in the novel environment and light dark transition box activity following acute exposure to 2 h immobilization stress in rats. Alterations in the levels of corticosterone, glucose and leptin were also measured to establish a link between oxidative stress and HPA axis following administration of plant extract. We also probed concentration of antioxidant enzymes such as catalase and superoxide dismutase to delineate the relationship of oxidative stress with behavioral deficits in rats. A consistent finding of the present study is that an oral administration of *Rauwolfia serpentina* plant extracts attenuated immobilization stress-induced behavioral deficits and alteration in antioxidant enzymes levels in rats. Moreover, plasma leptin and corticosterone were also mitigated in these rats proposing the role of antioxidant components of plant extract which may elicit neuroprotective effects.

In the present study, we examined the effects of *Rauwolfia serpentina* on the modulation of immobilization stress-induced behavioral deficits by two extensively used behavioral models of anxiety-like behavior including open field and light dark transition test. These tests may be useful to expect anxiolytic-like or anxiogenic-like activity in mice [45]. The present results showed that 2 h immobilization exhibits a significant decrease in the number of square crossed but not latency to move in the open field as compare to the unstressed animals (Figure 1). Our findings are consistent with previous studies that showed the acute exposure to (2 h) immobilization stress produces anxiety like symptoms in rats, and it did not explore rapidly enough to find and enter the dark compartment; instead, they tended to freeze and remain immobile for a majority of the test session [46]. Therefore, immobilized stress animals avoid exploring new environment in light dark box as well as in the open field exploration test. Conversely, oral *Rauwolfia serpentina* extract alone administration increased number of square crossed in the open field in rats. On the other hand, oral administration of *Rauwolfia serpentina* extract attenuated 2 h immobilization stress-induced decreases in the locomotor activity in the open field. Similarly, a significant increase in the numbers of entries in light box and time spent in light compartment of the light dark transition box activity were also observed in these animals suggesting a reduction in novel environment-induced anxiogenic effects (Figure 2). Therefore, this anxiolytic effect of *Rauwolfia serpentina* plant extract could be explainable in terms of presence of numerous phytochemical compounds or secondary metabolites like alkaloids, carbohydrates, flavonoids, glycosides, phlobatannins, phenols, resins, saponins, sterols, tannins and terpenes in the plant extract [24,47,48]. The present results are therefore in agreement with previous findings that phenolic antioxidants present in the plant extracts could produce anxiolytic effects [49].

Oxidative stress has been implicated in the responses to stress [50] and in the pathogenesis of neurologic and psychiatric diseases [51]. An antioxidant is a substance that is present at low concentrations and significantly deferrals or prevents oxidation of the oxidizable substrate [52]. Endogenous antioxidants play a vital role in conserving optimal cellular functions. However, endogenous antioxidants may not be adequate under certain conditions that could promote oxidative stress [53,54] as observed in the current results (fig 4.4 & 4.5). Thus, elevated superoxide dismutase and catalase activities were found in rats immobilized for 2 h than control animals signifying that acute exposure to stress can

promote the formation of ROS and exhibits oxidative stress. In such cases, dietary antioxidants should be supplied to maintain optimal cellular functions. Some antioxidants can interact with other antioxidants in order to regenerate their original properties. This process is referred to as the “antioxidant network” [55]. It has been suggested that a diet rich in antioxidants can bring health benefits [56] and a lot of interest is directed towards assessing the antioxidant capacity of natural products. In recent years, many studies evidenced that majority of the antioxidant activity of plants may be from compounds such as phenolic acids, flavonoids and ascorbic acids that can provide protection against ROS [57–60]. In this perspective, plant extract containing flavonoids and ascorbic acid content of *Rauwolfia serpentina* exhibit antioxidant capacity which expand its nutraceutical values [61]. In present study, oral administration of *Rauwolfia serpentina* (Figures 4 and 5) attenuated immobilization induced increase in antioxidant enzymes CAT and SOD activities suggesting antioxidant capacity of plant extract component particularly flavonoids and ascorbic acid. Conversely, we also observed that oral administration of *Rauwolfia serpentina* alone increases CAT but not SOD antioxidant enzymes activity. It has been indicated that the balance between pro-oxidant and antioxidant compounds moderately favors pro-oxidants under physiological conditions. Consequently, it engendering a slight oxidative stress and requiring the intervention of endogenous antioxidant systems of the organism [62]. It seems possible that alkaloids and flavonoids components of *Rauwolfia serpentina* plant extract could be contributed along with endogenous antioxidant system to counteract oxidative stress under basal conditions.

It is well recognized that exposure to acute stress causes the formation of free radicals that may leads to oxidative damages [63]. The HPA axis is the neuroendocrine system that regulates responses to stress [64]. The production of high level of free radicals into the glands that comprise the HPA axis is related to the activation of a stress response system [65–67]. In terms of the activity of the HPA axis, it is now eminent that neurons in the paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing factor (CRF) to stimulate the synthesis and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then travels to the adrenal gland and induces the rapid [68] release of corticosteroids which later on activate various physiological processes to assist an organism to cope with the stressful situation and reinstate homeostasis under a potential threatening condition [34,69,70]. The present investigation demonstrates that animals subjected to immobilization stress exhibits an increased corticosterone levels (Figure 6). This is not unexpected since it has been previously reported that acute restraint stress [71] and immobilization stress [72] increases corticosterone levels and it is considered to be an important indicator of stress [73–75]. However, oral administration of *Rauwolfia serpentina* alone did not alter corticosterone levels as compare to saline plus unstressed animals. Conversely, immobilization-induced elevated levels of corticosterone were attenuated in *Rauwolfia Serpentina* treated animals (Figure 6). Previously, it was reported that chronically immobilized [63] and restraint [76] stress-induced attenuation of corticosterone levels explainable in terms of anti-stress activity. It is therefore interesting to relate the *Rauwolfia Serpentina* induced modulation of corticosterone levels in terms of suppressing HPA mobilization in responses to stress by normalizing elevated plasma corticosterone levels back to baseline. Thus, oral administration of *Rauwolfia Serpentina* reduced the adverse effects of acute exposure to (2 h) immobilization stress and thought to be beneficial for the body to prevent from stress-induced damages.

As per clinical evidences elevated level of corticosterone in response to stress also increases the plasma glucose concentration [77,78]. From the previous studies, it was reported that stress causes increase in plasma glucocorticoid levels [79–81] which stimulate liver gluconeogenesis that leads to the elevated blood glucose [82]. Regardless of the wide use of glucose as an indicator of stress, some authors [83,84] emphasized that care has to be taken when using plasma glucose as the only indicator. It has been reported that glucose measurements show many inconsistencies and should be a complement of stress tests rather than a main indicator [85]. In the present result acute (2 h) exposure to

immobilization stress unable to alter the plasma glucose concentration. Previously, preclinical studies on antidiabetic potential of methanolic root extract of *Rauwolfia serpentina* have been reported. It was found to be effective in lowering the blood glucose levels [31]. However, in our findings oral administration of *Rauwolfia serpentina* did not show any significant decrease in the levels of glucose as compare to the saline plus unstressed rats (Figure 3). Similarly, treatment with *Rauwolfia serpentina* also did not alter stress induced changes in glucose concentration in rats (Figure 3). It seems that 30 mg/kg of *Rauwolfia serpentina* was not sufficient to produce significant hypoglycemic effects in our present study paradigm. The reason for the variation between our observation and that in the mentioned study is unclear but it may be due to discrepancy in nature of stress or ambient environment.

We are here reporting for the first time about the potential therapeutic role of *Rauwolfia serpentina* on endogenous leptin and corticosterone levels. Leptin secretion is basically under the influence of neural and hormonal control [86–88]. Influence of leptin on HPA axis is one of the mechanisms by which leptin can improve stress controllability to produce antidepressant and anxiolytic-like effects. Previously, preclinical studies reported that exposure to 1 h immobilization [89], 10 min forced swimming [90], 120 dB noise [91] showed an increase in circulating levels of leptin. These studies are consistent with our present data that exposure to acute (2 h) immobilization stress resulted in significant increase in the circulating levels of leptin (Figure 7). As many components of the HPA axis contain leptin receptors, it seems promising that systemically circulating leptin can alter the stress response at every focal point of the axis [92]. On the other hand, stress-induced releases of corticosterone have an opposite influence on leptin expression in adipocytes and its secretion into the blood circulation. It has been reported that pretreatment with recombinant mouse leptin inhibited the stress-mediated stimulation of plasma ACTH and corticosterone in mice [93] and this inhibitory effects could be produced by receptors in the hypothalamus. The present results showed that oral administration of *Rauwolfia serpentina* significantly augment immobilization stress-induced increases of plasma leptin levels (Figure 7) but inhibit corticosterone levels (Figure 6). It is therefore suggested that leptin could elicit a feedback effect over the activity of the HPA axis. Thus, the role of leptin in HPA axis functioning suggest that their relationship is bidirectional [92]. However, a role of leptin in alleviating stress perception is also apparent from studies reporting anxiolytic-like effects of pharmacological doses of exogenous leptin in rodent models of anxiety and an inhibition of stress-induced anxiety in these models [93]. It has also been reported that conventional potential anxiolytic compounds inhibited the corticosterone response to an acute stressor [94,95] and reversed stress-induced behavioral deficits [96,97]. Similarly, we found that oral administration of *Rauwolfia serpentina* reversed acute (2 h) immobilization stress induced behavioral deficits (Figures 1 and 2). It is therefore suggested that oral administration of plant extract could possibly elicits an anxiolytic like effect (Figures 1 and 2) by modulating endogenous leptin levels and thus inhibiting stress induced activation of the HPA axis.

We suggest that *Rauwolfia serpentina* has potential to antagonize adverse effects of acute (2 h) immobilization stress by reducing stress perception. Despite an apparently promising role in reducing the stress perception, the molecular mechanism underlying the acute anxiolytic effects of oral administration of *Rauwolfia serpentina* plant extract remains to be determined. Future studies are also needed to determine the effects of oral administration of *Rauwolfia serpentina* plant extract on before and after exposure to unpredictable stress perception to further evaluate its potential as an anxiolytic compound and may facilitate the development of alternative treatment strategies for stress related disorder including anxiety and depression.

6. Conclusions

The present study concludes that *Rauwolfia serpentina* is an effective anxiolytic agent as it attenuate stress induced behavioral deficits and improves locomotor activity. Majority of the components present in *Rauwolfia serpentina* are beneficial and provide positive outcomes in antioxidant enzymes levels of restrained animals but in case of unstrained animals it showed increased antioxidant enzymes levels that might be due to the presence of any alkaloid. On the other hand, our results showed that *Rauwolfia serpentina* was found to prevent the stress-induced increase in corticosterone. Moreover, an increased in the levels of endogenous leptin attenuates the stress-induced activity of HPA axis and reverses the adverse effects of acute stress. It is also concludes that 30 mg/kg of *Rauwolfia serpentina* was not sufficient to produce hypoglycemic effects. However, more studies are recommended to explain the particular action by which *Rauwolfia serpentina* produces its effects.

Abbreviations

Catalase	CAT
Superoxide dismutase	SOD
Oxidative Stress	OS
Reactive oxygen species	ROS
Glutathione peroxidase	GPx
Nigella sativa	NS
Olea europaea	OE
Methanolic root extract	MREt
Paraventricular nucleus	PVN
Corticotropin-releasing factor	CRF
Adrenocorticotropin hormone	ACTH

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