Proceeding Paper

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

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Abstract: Objectives: Nowadays, the global population is moving towards herbal drugs, which contain bioactive compounds, to cure diseases. *Rauwolfia serpentina* is a medicinally important herb that is mainly effective in the treatment of hypertension and psychotic disorders. The present study was designed to investigate the effects of *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, including 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, which were (i) saline unstressed, (ii) *Rauwolfia serpentina* unstressed, (iii) saline stressed and (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 immobilization for 2 h. Behavioral analysis was performed after the termination of the 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 attenuates stress-induced behavioral deficits and improves locomotor activity. On the other hand, it provides positive outcomes regarding the antioxidant enzymes levels of stressed animals. Conclusion: *Rauwolfia Serpentina* was found to prevent the stress-induced increase in corticosterone, and an increase in the levels of endogenous leptin attenuates the stress-induced activity of the HPA axis. It is also concluded that 30 mg/kg of *Rauwolfia serpentina* is 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

1. Introduction

Stress has 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 causes a person to feel fear and helplessness [2]. A variety of diverse environmental and stressful stimuli have also been reported to alter behavioral patterns, neurotransmitter levels and oxidative damage in discrete areas of the brain [3,4]. However, the 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 showed 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 the oxidative metabolism can affect the regulation of anxiety.

Recognizing elements that contribute to neurodegenerative progression 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 preclinical 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, particularly glutamate release, which most likely plays an important role in the “fight or flight response” [15]. Oxidative stress creates a state of cellular imbalance, in which reactive oxygen species (ROS) production surpasses the antioxidant response mechanisms that help to neutralize ROS-mediated oxidative damage to DNA, RNA and lipids, leading to innumerable pathophysiological consequences [16,17]. In order to counterbalance the free-radical-induced damage of biological molecules, antioxidant mechanisms and enzymes are activated. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were identified as antioxidant enzymes that act as the 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].

Nowadays, the global population is moving towards herbal drugs, which contain bioactive compounds, to cure diseases [19]. Rauwolfia serpentina, belonging to the family Apocynaceae, is an important medicinal plant in the pharmaceutical world due to its immense therapeutic properties [20,21]. It is effective in the treatment of hypertension and psychotic disorders such as 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 the leaf extract of this plant [24]. The principle alkaloid of Rauwolfia serpentina is reserpine [24]. It is present in the root, stem and leaves of the plant. It contains less than 0.15% of reserpine and rescinnamine group alkaloids [25].

Previously, numerous studies have been reported from our laboratory that establish the capability of phytochemicals present in rice bran oil [26], olive oil [27] and the aqueous fruit extract of sea buckthorn [28] to attenuate/or reverse anxiety in rats. Similarly, our laboratory also observed that the 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 continuation of our research on the plant, the present study was designed to investigate the neuroprotective effects of Rauwolfia serpentina following acute exposure to immobilization stress in rats. The herb extract was orally administered at a non-sedative dose of 30 mg/kg [31] before immobilization for 2 h to monitor any change in behavioral activities. The neuroprotective efficacy of the 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 obtain an insight into the role of Rauwolfia serpentina in the HPA axis, we also monitored plasma leptin, corticosterone and glucose levels. The study establishes 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 and 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 the experiment (establishing familiarity with the environment). All procedures conducted were approved by the Local Institutional Animal Care and Use Committee at the University of the Health Sciences and conducted in full compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Preparation of Plant Extract

Thirty grams of ground powder of the 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 (Eyela-NE) to obtain a brown residue that was referred to 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 a refrigerator, below 10 °C, until use.

2.3. Immobilization Procedure

The animals of the stress groups were subjected to a single exposure of immobilization stress for 2 h. Immobilization was done in a separate room to prevent unstressed animals from being placed under stressful conditions due to disturbance. The animals were immobilized by an approved procedure [32,33]. Wire grids fitted with a Perspex plate, as described earlier [33], were used. Immobilization was affected by pressing the legs of the rats through the gaps in the metal grid and taping them together with zincoxide plaster. Hind limbs were also taped and the head of the animal rested on the Perspex plate. After 2 h of 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. The open field was a 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 the open field was determined by counting the number of squares crossed for 5 min [36]. Exploratory activity of control rats and test rats was monitored in a balance design to avoid order effects.

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 compartments (composed of transparent plastic) measured 26 × 26 × 26 cm and access between the 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 the light compartment. The degree of anxiety was assessed by a decrease in time passed in the light compartment and also by a decrease in the number of entries made to the light compartment.
2.5. Blood Sample Collection

Blood was collected from rats in heparinized centrifuge tubes. Centrifugation was done for 10 min. Plasma was collected and stored at −70 Celsius till biochemical estimation of the plasma glucose concentration in mg/dL, corticosterone concentration in µg%, leptin concentration in ng/mL and 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 the method of Patterson [38]. The decomposition of H₂O₂ was measured at 240 nm, taking De at 240 nm as 43.6 mM cm⁻¹. Reaction mixture (3.0 mL) consisted of 10.5 mM H₂O₂ 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 H₂O₂ 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. The increase in absorbance due to the 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 was 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 the 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 tubes. Centrifugation proceeded for 20 min at 2000 × g and 4 °C to obtain plasma. The samples were stored at −70 degrees Celsius until the assay of plasma leptin and corticosterone using an ELISA kit (Cat # EZRL-83K).

3. Experimental Protocol

Twenty-four animals randomly divided into two equal groups of 12 each were assigned to the 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 and (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 and 11:00 h. Animals of the unstressed group were left in their home cage during this time. Behavioral activities were monitored in open field activity and light–dark transition box after the termination of the 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 control and test rats were measured alternately to avoid an order effect.
Statistical Analysis

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

4. Results

Figure 1A shows changes in motor activity in a novel environment in animals orally administered with *Rauwolfia serpentina* for 2 h before exposing the animal to acute immobilization stress for 2 h. Analysis of the data on latency to move (Figure 1A) showed significant effects of stress ($F = 7.737$ $p < 0.01$ df1,20) and *Rauwolfia serpentina* ($F = 7.737$ $p < 0.01$ df1,20), as well as the interaction between two factors ($F = 8.796$ $p < 0.01$ df2,20).

Figure 1. Changes in motor activity in a novel environment in animals orally administered with *Rauwolfia serpentina* exposed to 2 h acute immobilization stress. Values are means ± S.D. ($n = 24$). Significant differences evaluated 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 the administration of *Rauwolfia serpentina* at a dose of (30 mg/kg) to stressed rats resulted in an increase in latency to move...
as compared to unstressed rats. On the other hand, saline + stressed rats did not show any significant difference in latency to move as compared to unstressed rats. *Rauwolfia serpentina* + stressed rats showed an increase in latency to move in comparison with saline + stressed rats.

Figure 1B shows changes in motor activity in a novel environment in animals orally administered with *Rauwolfia serpentina* for 2 h before exposing the animal to acute immobilization stress for 2 h. Analysis of the data on the number of squares crossed (Figure 1B) showed significant effects of stress (F = 4.017 p < 0.05 df1,20) and *Rauwolfia serpentina* (F = 43.136 p < 0.01 df1,20). The interaction between the two factors was not significant (F = 1.143 N.S.).

Post hoc analysis by Newman–Keuls showed a decreased number of squares crossed by saline + stressed rats but not *Rauwolfia serpentina* + stressed rats. Rats treated with *Rauwolfia serpentina* alone showed increased locomotor activity in the open field. On the other hand, stress-induced decreases in locomotor activity were reversed in *Rauwolfia serpentina*-administered stressed rats.

Figure 2A shows changes in behavior in the light–dark transition test in animals orally administered with *Rauwolfia serpentina* for 2 h before exposing animals to acute immobilization stress for 2 h. Analysis of the data on entries in the light box (Figure 2A) showed significant effects of stress (F = 16.298 p < 0.01 df1,20) and an interaction between the two factors (F = 5.391 p < 0.01 df1,20). Effects of *Rauwolfia serpentina* were not significant (F = 1.589 N.S.).

Post hoc analysis by Newman–Keuls showed a decreased number of entries in the light–dark transition box in *Rauwolfia serpentina* + stressed and saline + stressed animals as compared to their respective controls. Rats treated with *Rauwolfia serpentina* alone showed an increased number of entries in the light–dark transition box. On the other hand, stress-induced decreases in the number of entries in the light–dark box were reversed in *Rauwolfia serpentina*-administered rats.

Figure 2B shows changes in behavior in the light–dark transition test in animals orally administered with *Rauwolfia serpentina* for 2 h before exposing animals to acute immobilization stress for 2 h. Analysis of the data on time spent in the 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 df1,20), as well as an interaction between the two factors (F = 7.740 p < 0.01 df2,20).

![A. NUMBER OF ENTRIES IN LIGHT BOX](image)

**Figure 2. Cont.**
Figure 2. Changes in behavior in light–dark transition test in animals orally administered with *Rauwolfia serpentina* exposed to 2 h acute immobilization stress. Values are means ± S.D. (n = 24). Significant differences evaluated 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 decreased time spent in the light–dark transition box (sec) in saline +stressed rats but significantly increased in *Rauwolfia serpentina* +stressed rats. *Rauwolfia serpentina* alone did not increase locomotor activity in the light–dark transition box. On the other hand, the stress-induced decrease in locomotor activity was reversed in *Rauwolfia serpentina*-administered stressed rats.

Figure 3 shows the effects of stress with the oral administration of *Rauwolfia serpentina* on the plasma glucose level. Analysis of the data on glucose level (Figure 3) showed non-significant effects of stress (F = 0.566 N.S.) and *Rauwolfia serpentina* (F = 2.144 N.S.), as well as the interaction between the two (F = 3.142 p < 0.005 df2,20).

Figure 3. Changes in the levels of glucose in animals orally administered with *Rauwolfia serpentina* exposed to 2 h acute immobilization stress. Values are means ± S.D. (n = 24).
Post hoc analysis by Newman–Keuls test revealed that the concentration of plasma glucose was not significant in all groups.

Figure 4 shows the effects of stress with the 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 an interaction between the two factors ($F = 2.802$ N.S.). Effects of *Rauwolfia serpentina* were significant ($F = 4.858$ $p < 0.05$ df2,20).

![Figure 4](image-url)

**Figure 4.** Changes in the levels of catalase activity in animals orally administered with *Rauwolfia serpentina* exposed to 2 h acute immobilization stress. Values are means ± S.D. (n = 24). Significant differences evaluated 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 the activity of CAT was significantly increased in saline + stressed rats but significantly decreased in *Rauwolfia serpentina* + stressed rats. *Rauwolfia serpentina* administration alone increased CAT activity. On the other hand, the stress-induced increase in CAT activity was attenuated in *Rauwolfia serpentina*-administered stressed rats.

Figure 5 shows the effects of stress with the oral administration of *Rauwolfia serpentina* on plasma SOD activity. Analysis of the data on SOD activity (Figure 5) showed significant effects of stress ($F = 3.282$ $p < 0.05$ df1,20). Effects of *Rauwolfia serpentina* ($F = 2.256$ N.S) and the interaction between the two were not significant ($F = 1.121$ N.S.).

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

Figure 6 shows the effects of stress with the oral administration of *Rauwolfia serpentina* on the plasma corticosterone level. Analysis of the data on corticosterone levels (Figure 6) showed significant effects of stress ($F = 9.0$ df1,20 $p < 0.01$), *Rauwolfia serpentina* ($F = 7.92$ df2,20 $p < 0.01$), as well as the interaction between the two ($F = 26.01$ df1,20 $p < 0.01$).

Post hoc analysis by Newman–Keuls showed significantly increased levels of corticosterone in saline +stressed animals but decreased in *Rauwolfia serpentina* +stressed animals. On the other hand, an immobilization-stress-induced increase in corticosterone did not occur in single *Rauwolfia serpentina* administered animals.
Figure 5. Changes in the levels of superoxide dismutase activity in animals orally administered with *Rauwolfia serpentina* exposed to 2 h acute immobilization stress. *p* < 0.05.

Figure 6. Changes in the levels of corticosterone in animals administered with *Rauwolfia serpentina* exposed to 2 h acute immobilization stress. Values are means ± S.D. (n = 24). Significant differences evaluated by Newman–Keuls test. **p** < 0.01 from similarly treated unstressed control animals. ++p < 0.01 and from respective (unstressed or stressed) animals.

Figure 7 shows the effects of stress with the oral administration of *Rauwolfia serpentina* on plasma leptin levels. Analysis of the data on leptin levels (Figure 7) showed significant effects of stress (*F* = 9.0 df1,20 *p* < 0.01) and *Rauwolfia serpentina* (*F* = 7.92 df2,20 *p* < 0.05). A non-significant effect of the interaction between the two factors was noted (*F* = 26.01 N.S.).

Post hoc analysis by Newman–Keuls showed significant increases in both saline +stressed and *Rauwolfia serpentina* +stressed animals as compared to their unstressed control rats, respectively. *Rauwolfia serpentina* +stressed rats showed increase levels of leptin in comparison with saline + stressed rats.
Figure 7. Changes in the levels of leptin in animals orally administered with Rauwolfia serpentina exposed to 2 h acute immobilization stress. Values are means ± S.D. (n = 24). Significant differences evaluated by Newman–Keuls test. ** $p < 0.01$ and * $p < 0.05$ from similarly treated unstressed control animals.

5. Discussion

Experiencing stress is an inexorable part of everyday life and plays a significant role in shaping adaptive behavior [41]. Acute exposure to immobilization stress has been reported to impair motor activity, cause memory dysfunction, modulate anxiety [42] and pain perception [43] and elicit depression-like behaviors [44] in animals. The goal of the current study was to observe the neuroprotective effects of Rauwolfia serpentina on the behavioral activity of animals in a 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 the HPA axis following administration of the plant extract. We also probed the concentrations 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 the oral administration of Rauwolfia serpentina plant extracts attenuated immobilization-stress-induced behavioral deficits and alterations in antioxidant enzymes levels in the rats. Moreover, plasma leptin and corticosterone were also mitigated in these rats, suggesting a role of the antioxidant components of the 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 with two extensively used behavioral models of anxiety-like behavior, including the open field and light–dark transition test. These tests may be useful to examine anxiolytic-like or anxiogenic-like activity in mice [45]. The present results showed that 2 h immobilization exhibited a significant decrease in the number of squares crossed but not latency to move in the open field as compared to the unstressed animals (Figure 1). Our findings are consistent with previous studies that showed that acute exposure to (2 h) immobilization stress produces anxiety-like symptoms in rats, and the animals did not explore rapidly enough to find and enter the dark compartment; instead, they tended to freeze and remain immobile for the majority of the test session [46]. Therefore, immobilized stressed animals avoided exploring the new environment in the light–dark box as well as in the open field exploration test. Conversely, administration of the oral Rauwolfia serpentina extract alone increased the number of squares 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 locomotor activity in the open field. Similarly, a significant increase in the numbers of entries in the light box and time spent in the light compartment of the light–dark transition box 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 the presence of numerous phytochemical compounds or secondary metabolites, such as 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 the phenolic antioxidants present in plant extracts could produce anxiolytic effects [49].

Oxidative stress has been implicated in the response 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 inhibits or prevents the 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 (Figures 4 and 5). Thus, elevated superoxide dismutase and catalase activities were found in rats immobilized for 2 h compared to control animals, signifying that acute exposure to stress can promote the formation of ROS and lead to 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 the majority of the antioxidant activity of plants may be from compounds such as phenolic acids, flavonoids and ascorbic acids, which can provide protection against ROS [57–60]. From this perspective, the plant extract containing flavonoids and the ascorbic acid content of *Rauwolfia serpentina* exhibit antioxidant capacity, which expands its nutraceutical value [61]. In the present study, oral administration of *Rauwolfia serpentina* (Figures 4 and 5) attenuated the immobilization-induced increase in the antioxidant enzymes CAT and SOD’s activities, suggesting the antioxidant capacity of the plant extract components, particularly flavonoids and ascorbic acid. Conversely, we also observed that oral administration of *Rauwolfia serpentina* alone increases CAT but not SOD activity. It has been indicated that the balance between pro-oxidant and antioxidant compounds moderately favors pro-oxidants under physiological conditions. Consequently, it leads to slight oxidative stress and requires the intervention of the endogenous antioxidant systems of the organism [62]. It seems possible that the alkaloid and flavonoid components of *Rauwolfia serpentina* plant extract could contribute, along with the endogenous antioxidant system, to counteracting oxidative stress under basal conditions.

It is well recognized that exposure to acute stress causes the formation of free radicals, which may lead to oxidative damages [63]. The HPA axis is the neuroendocrine system that regulates responses to stress [64]. The production of high levels of free radicals in the glands that comprise the HPA axis is related to the activation of the 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 activate various physiological processes to assist an organism in coping with a stressful situation and reinstate homeostasis under a potentially threatening condition [34,69,70]. The present investigation demonstrates that animals subjected to immobilization stress exhibit increased corticosterone levels (Figure 6). This is not unexpected, since it has been previously reported that acute restraint stress [71] and immobilization stress [72] increase corticosterone levels, and this is considered to be an
important indicator of stress [73–75]. However, oral administration of *Rauwolfia serpentina* alone did not alter corticosterone levels as compared 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 the chronically immobilized [63] and restraint [76] stress-induced attenuation of corticosterone levels is 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 response 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 is thought to be beneficial for the body to prevent stress-induced damages.

As per clinical evidence, elevated levels of corticosterone in response to stress also increase the plasma glucose concentration [77,78]. From previous studies, it was reported that stress causes an increase in plasma glucocorticoid levels [79–81], which stimulates liver gluconeogenesis, which then leads to elevated blood glucose [82]. Regardless of the wide use of glucose as an indicator of stress, some authors [83,84] have 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 complemented to stress tests rather than a main indicator [85]. In the present results, acute (2 h) exposure to immobilization stress was unable to alter the plasma glucose concentration. Previously, preclinical studies on the antidiabetic potential of the methanolic root extract of *Rauwolfia serpentina* have been reported. It was found to be effective in lowering 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 compared 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 the discrepancy in the nature of the stressful or ambient environments.

We report, for the first time, the potential therapeutic role of *Rauwolfia serpentina* in endogenous leptin and corticosterone levels. Leptin secretion is basically under the influence of neural and hormonal control [86–88]. The influence of leptin on the 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] and 120 dB noise [91] showed an increase in circulating levels of leptin. These studies are consistent with our present data where in exposure to acute (2 h) immobilization stress resulted in a 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 the 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 effect could be produced by receptors in the hypothalamus. The present results showed that oral administration of *Rauwolfia serpentina* significantly augments immobilization-stress-induced increases in plasma leptin levels (Figure 7) but inhibits 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 suggests 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 the oral administration of the plant extract could possibly elicits an anxiolytic-like effect (Figures 1 and 2) by modulating endogenous leptin levels and thus inhibiting the stress-induced activation of the HPA axis.

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

6. Conclusions

The present study concludes that *Rauwolfia serpentina* is an effective anxiolytic agent as it attenuates stress-induced behavioral deficits and improves locomotor activity. The majority of the components present in *Rauwolfia serpentina* are beneficial and provide positive outcomes regarding the antioxidant enzyme levels of restrained animals, but in the case of unstrained animals, it showed increased antioxidant enzyme 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 increase in the levels of endogenous leptin attenuates the stress-induced activity of the HPA axis and reverses the adverse effects of acute stress. It is also concluded 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.

**Author Contributions:** W.B.A. and E.S. designed the study. W.B.A., S.K., N.J., M.M. and N.M. conducted the laboratory experiments. W.B.A. and N.J. contributed to laboratory analyses. E.S. and W.B.A. analyzed the data. W.B.A. wrote the manuscript and E.S. and D.J.H. commented on the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was designed and conducted in accordance with the National Institute of Health (NIH) guidelines for the Care and Use of Laboratory Animals and a protocol (ASP No.: 2018-0001) approved by the Institutional Committee for Animal Care and Use.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Catalase</td>
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<tr>
<td>Superoxide dismutase</td>
<td>SOD</td>
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<td>Oxidative stress</td>
<td>OS</td>
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<td>Reactive oxygen species</td>
<td>ROS</td>
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<td>Glutathione peroxidase</td>
<td>GPx</td>
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<td>Nigella sativa</td>
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<td>Olea europaea</td>
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<td>Methanolic root extract</td>
<td>MREt</td>
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<td>Paraventricular nucleus</td>
<td>PVN</td>
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<td>Corticotropin-releasing factor</td>
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<td>Adrenocorticotropic hormone</td>
<td>ACTH</td>
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