Neuroprotective effect of Clerodendrum serratum Linn. leaves extract against acute restraint stress-induced depressive-like behavioral symptoms in adult mice

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Abstract:
Objective: The objective of this study was to study the effect of ethanol extract of Clerodendrum serratum (EECS) Linn. on acute restraint stress (ARS)-induced depressive-like behavior and biochemical alterations in mice.

Materials and Methods: Ethyl acetate and n-butanol fractions of EECS were analytically characterized for the flavonoid components, apigenin (API) and luteolin (LUT) by reverse-phase high-performance liquid chromatography. Behavioral tests, namely, forced-swim test and tail-suspension test were performed for assessing antidepressant-like effect and anxiolytic activity in mice. Oxidative stress parameters and biochemical alterations in mice brain tissue were also performed.

Statistical Analysis: Expression of data was done as mean ± standard error of mean. The normally distributed data were subjected to two-way ANOVA followed by Dunnett’s test. P < 0.05 was considered statistically significant.

Results: The study showed that flavonoids, API and LUT were present in ethyl acetate and n-butanol fractions of EECS, which significantly reversed ARS-induced depressive-like behavior without affecting locomotion. EECS also attenuated oxidative damage caused by ARS. The level of norepinephrine and 5-hydroxytryptamine was also significantly restored by pretreatment with EECS for 7 days.

Conclusion: EECS significantly alleviated ARS-induced depressive-like behavior without affecting locomotion.

Key words: 5-hydroxytryptamine, brain neurotransmitters, Clerodendrum serratum, norepinephrine, neuroprotection, restraint stress

Stress, which is a crucial determinant of health and disease, plays a significant role in the pathogenesis of neuropsychiatric disorders as these stressful events result in altered physiological, immunological, psychological, and neurobehavioral responses such as anxiety, depression, cognitive impairment, insomnia, anorexia, and activation of hypothalamic-pituitary-adrenal axis in animals and humans.11 Detrimental effects on cellular functions, as a result of stressful conditions, arise due to oxidative damage produced by the release of free radicals or reactive oxygen species (ROS) which is implicated in neuropsychiatric disorders.12 Central nervous system (CNS) is especially susceptible to free radical damage because of brain’s high oxygen demand, abundant lipid content, and relative paucity of antioxidant enzymes.13

Restraint stress exposure alters the free radical scavenging enzymes in discrete regions of brain.14 Moreover, neurotransmitters’ norepinephrine (NE) and 5-hydroxytryptamine (5-HT) are known to be involved in the expression of behavioral disorders in adult individuals of several species following stress.15 Medicinal plants rich in phytochemicals such as phenolics and flavonoids, act as free radical scavengers and metal chelators, which are useful in preventing neurodegeneration.16

Clerodendrum serratum Linn. plant has been widely used as a traditional medicine for many diseases, especially for neurological disorders.

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Materials and Methods

Plant Material and Extraction
Fresh leaves of *C. serratum* were collected during the month of June–July (2016) from Attapadi forest, Palakkad district, Kerala. It was taxonomically identified and authenticated by Dr. Arun Kumar, Assistant Professor, Department of Botany, University of Kerala and herbarium of the plant is preserved for future reference (Specimen Voucher No. 11410/3 [UCBD]). The collected leaves were washed and shade dried at room temperature for 7 days. Dried leaves were then coarsely powdered and fine powder was separated by passing through sieve no. 60. The coarse powder of the leaves (437 g) was then macerated with petroleum ether (60–80°C) for 1 day with occasional shaking and filtered. marc left after was then extracted with 70% v/v ethanol by shaking for another 6 days and filtered. Filtrate was dried under pressure to remove the solvent completely. The extract was then weighed and calculated the percentage yield in terms of air-dried crude material. The resultant EECS was kept in a refrigerator for future use. Before administration, the extract was freshly prepared with distilled water and two doses (25 mg/kg and 50 mg/kg) were selected based on previous studies.

Phytochemical Characterization of Ethanoll Extract of *Clerodendrum serratum* Using Reverse-Phase High-Performance Liquid Chromatography
Ethyl acetate and n-butanol fractions of EECS were analytically characterized for the flavonoid components, API and LUT by RP-HPLC. Analysis was carried out with Shimadzu® Japan HPLC system consisting of a solvent delivery pump, ultraviolet (UV) detector, autosampler, and system controller. Data collection and analysis were performed using LC solution. Separation was performed on enable C18 column (250 mm × 4.6 mm i.d., 5 μm particle size). The detection wavelength was set at 352 nm. The mobile phase consisted of methanol: Acetonitrile: Acetic acid: Orthophosphoric acid: Water (200 mL: 100 mL: 0.75 mL: 0.75 mL: 200 mL), with the flow rate of 0.6 mL/min.

Animals
Male Swiss albino mice (20–30 g) were obtained from Sree Venkateswara Enterprises Pvt. Ltd., Bengaluru, and maintained at constant room temperature (20–22°C) with free access to water and food, under a 12:12 h light: dark cycle. Animals were aclimatized to laboratory conditions before experiment. All the experiments were carried out between 9.00 and 17.00 h. The experimental protocols were approved by the Institutional Animal Ethics Committee of KMCH College of Pharmacy, Coimbatore, (KMC/PhD/16/2015–16) and procedures in this study were performed in accordance with guidelines of committee for the purpose of control and supervision on experiments on animals (685/Po/02/a/CPSEA). All efforts were made to minimize animal suffering and the number of animals used in the experiments.

Experimental Design
Thirty mice were randomly divided into five experimental groups. Group-I (control) mice received distilled water (1.0 mL/kg, p.o.) daily for 7 days; Group-II (stress control) mice received distilled water (1.0 mL/kg, p.o.) daily for 7 days and subjected to restraint stress on 8th day. Group-III (standard drug-treated) mice received imipramine (15 mg/kg, i.p.) on 8th day 1 h before subjecting to restraint stress. Group-IV and V mice were treated with EECS (25 mg/kg and 50 mg/kg, p.o.) daily for 7 days subjected to ARS on 8th day.

Depressive-like behavior was assessed by subjecting the mice to behavioral paradigms such as forced-swim test (FST) and tail-suspension test (TST), 40 min postrestraint stress procedure. Oxidative stress parameters such as SOD, CAT, GSH, glutathione peroxidase (GPx), and extent of LPO were analyzed in restraint stress-induced animals and control group, following behavioral tests on 8th day. Estimation of neurotransmitters such as NE and 5-HT was also performed in mice brain on the same day, following behavioral studies.

Another group of 24 animals was divided randomly into only four experimental groups for assessing locomotor activity, as these animals were devoid of ARS procedure, which may otherwise interfere with the study. Locomotor activity was performed to exclude any false positive or negative response obtained for antidepressant activity. Group-I (control) mice received distilled water (1.0 mL/kg), p.o.; Group-II (standard drug-treated) mice received diazepam (5 mg/kg, i.p.); Group-III and IV received EECS (25 mg/kg and 50 mg/kg, p.o.) on the day of the experiment.

Procedure for acute restraint stress
ARS protocol was adapted from a previously described procedure. Immobilization stress was accomplished by placing them in an individual rodent restraint device made of wire mesh for 6 h. This restrained all physical movements without subjecting the animal to pain. Animals were deprived of food and water during the entire period of exposure to stress. After 6 h, the animals were released from their enclosure and after 40 min postrelease, the animals were subjected to behavioral tests and then to biochemical estimations. In normal control group, the mice were kept in the animal cage in the experimental room.

for Shwasa (breathlessness), Kasa (cough), Vrana (wound), Shotha (swelling), and many Vataja disorders (neurological disorders). These data indicate that this plant may certainly have some therapeutic effects on CNS. Nevertheless, scientific evidence about the potential effects of this plant on neurological disorders is lacking. In this study, we sought to extend the existing literature dealing with the effect of ethanol extract of *C. serratum* (EECS) for the flavonoid components, apigenin (API) and luteolin (LUT), by reverse-phase high-performance liquid chromatography (RP-HPLC) technique and on depression-like behavioral symptoms in adult mice and to analyze brain antioxidant elements such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase; glutathione (GSH) and lipid peroxidation (LPO), and neurotransmitters such as NE and 5-HT, in relation to the behavioral responses due to acute restraint stress-induced depressive-like behavior. Hence, estimation of acute restraint stress (ARS)-induced alterations in the levels of NE and 5-HT in animal brain will help in understanding the impact of stress and the role of herbs in attenuating such aberrations, in addition to antioxidant elements.
Behavioral Tests

Tail-suspension test
Mice were suspended from the edge of a Table 50 cm above the floor[13] by the adhesive tape placed approximately 1 cm from the tip of the tail. Total duration of immobility was recorded for next 4 min during a 6 min test. Mice were considered to be immobile only when they hung passively and were completely motionless. Recording of duration of immobility of animals was done by observers blind to the treatments given to the animals under study.

Forced-swim test
FST, the widely used behavioral model for screening antidepressant agents in rodents was performed according to the method described by Porsolt et al. with some modifications.[13] Mice were forced to swim in a cylinder (diameter 15 cm, height 25 cm) containing 15 cm of fresh water maintained at 25°C ± 1°C. Water in the cylinder was changed after each animal to prevent the behavioral alteration among animals due to used water. Each animal showed vigorous movement during initial 2 min period of the test. Duration of immobility was manually recorded during the next 4 min of total 6 min testing period by the observer blind to the treatment conditions. Mice were considered to be immobile when they floated in an upright position, making only small movements to keep their head above the water level. Following swimming session, mice were dried using cotton towel and returned to home cages after experiment. A decrease in the duration of immobility is indicative of antidepressant-like effect, whereas an increase of immobility time, when compared with the control group, is associated with depressive-like effects.

Actophotometer test
To assess the effect of EECS on locomotor activity, mice were evaluated in actophotometer[14] and the number of movements within the apparatus was counted in a 5 min session. The apparatus was cleaned with a solution of 10% ethanol between tests to hide animal clues.

Biochemical Analysis

Preparation of brain tissue homogenate
All the animals were sacrificed by decapitation, after behavioral observations. The brains were quickly removed, washed in ice-cold sterile isotonic saline, and weighed. A 10% (w/v) tissue homogenates were prepared with 0.1M tris-HCl buffer (pH 7.4). The supernatant was obtained by centrifugation of the homogenate at 1000 rpm for 20 min at 5°C and used for further biochemical estimation.

Estimation of Antioxidant Elements in Mice Brain

Superoxide dismutase
SOD was assayed spectrophotometrically[15] based on the ability of SOD to inhibit auto-oxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of adrenochrome transition by 50%. The SOD enzymatic activity was expressed as units/min/mg protein.

Catalase
To measure the CAT activity,[16] the reaction was started by the addition of freshly prepared hydrogen peroxide solution and stopped by adding dichromate acetic acid reagent. The rate of hydrogen peroxide decomposition by CAT was measured spectrophotometrically at 570 nm. The CAT enzymatic activity was expressed as μM of H₂O₂ consumed/min/mg protein.

Glutathione peroxidase
GPx activity was measured using an NADPH reduction assay.[17] Tissue supernatant was added to a reaction mixture containing reduced GSH, sodium hydrogen phosphate, and hydrogen peroxide. The reaction was arrested by the addition of trichloroacetic acid and color developed on addition of DTNB reagent was measured at 412 nm spectrophotometrically. GPx activity was expressed as μg/mg protein.

Glutathione
GSH was estimated by Ellman’s procedure.[18] Trichloroacetic acid 10% was added to the reaction mixture containing brain homogenate and centrifuged. After centrifugation, free thiol groups were determined in the clear supernatant, on addition of Ellman’s reagent at 412 nm. Results were obtained by plotting a standard GSH curve and the values were expressed as μM/mg protein.

Lipid peroxidation assay
Quantitative measurement of LPO in the whole brain was assessed[19] based on the amount of malondialdehyde (MDA) formed, which was estimated by reaction with thiobarbituric acid in acidic condition to generate a pink-colored chromophore, read at 535 nm, using Shimadzu, UV-visible spectrophotometer. The results were expressed as μM of MDA per mg of protein using molar extinction coefficient of chromophore.

Estimation of Neurotransmitters (Norepinephrine and 5-Hydroxytryptamine) in Mice Brain
NE and 5-HT were estimated according to the method described by Schlumpf et al.[20] To aqueous phase of brain homogenate, iodine solution was added for oxidation, followed by addition of sodium thiosulfate solution. Reaction mixture was then heated at 100°C for 6 min and excitation-emission spectra were read at 395–485 nm for estimating NE. The fluorophore developed by heating the reaction mixture, after adding O-phthaldialdehyde reagent to 100°C for 10 min, was measured at 360–470 nm for 5-HT in spectrofluorimeter.

Statistical analysis
Expression of data was done as mean ± standard error of mean. The normally distributed data were subjected to two-way ANOVA followed by Dunnett’s test. P < 0.05 was considered statistically significant.

Results

Reverse-Phase high-Performance Liquid Chromatography Determination of Apigenin and Luteolin in Various Fractions of Ethanol Extract of Clerodendrum serratum
On application of the developed RP-HPLC method, well-separated peaks were obtained for both API and LUT in ethylacetate and n-butanol fractions of EECS. The quantitative analysis revealed that API and LUT predominated in n-butanol fraction (0.44 mg/g of API and 2.64 mg/g of LUT) compared to ethylacetate fraction (0.07 mg/g of API and 0.37 mg/g of LUT) [Figure 1].
Effects of Ethanol Extract of Clerodendrum serratum

Pretreatment on Depressive-like Behavior Induced by Acute Restraint Stress

Two-way ANOVA revealed that EECS given by oral route for 7 days, significantly \( (P < 0.001) \) decreased the duration of immobility in FST and TST, which are characteristic behavioral profiles of antidepressant-like effect. Our results showed that the immobility time increased significantly \( (P = 0.0048) \) in the mice subjected to ARS as compared to unstressed group, which is in agreement with its ability to induce depressive-like behavior. Post hoc analysis revealed that in FST, pretreatment with EECS significantly and dose-dependently attenuated ARS-induced increase in the immobility time, at both the doses, 25 mg/kg \( (P = 0.0019) \) and 50 mg/kg \( (P < 0.001) \). In TST, both the EECS-treated group showed a significant \( (P < 0.001) \) reduction in duration of immobility, compared to stressed group. Those animals, who received standard drug imipramine (15 mg/kg) also showed a significant reduction in immobility time \( (P < 0.001) \) compared to stressed group [Figure 2].

Effect of Ethanol Extract of Clerodendrum serratum on Locomotor Activity

Acute treatment with EECS, at 25 mg/kg and 50 mg/kg, was found to have no significant effect on locomotor activity in mice whereas diazepam, the standard drug exhibited a significant \( (P = 0.0086) \) reduction in locomotor activity, when compared to control group [Figure 3].

Effect of Ethanol Extract of Clerodendrum serratum on Acute Restraint Stress-induced Oxidative Stress Parameters

Results showed that ARS caused an increase \( (P = 0.0023) \) in SOD activity and this effect was attenuated to normal level by pretreatment with EECS at doses 25 mg/kg and 50 mg/kg, which was comparable with that of standard drug imipramine. Post hoc analysis indicated that EECS pretreatment, at both doses, significantly \( (P < 0.001, P = 0.0019) \) abolished increase in SOD activity caused by ARS. Furthermore, evaluation of CAT activity revealed that stressed mice presented a significant \( (P = 0.0086) \) decrease in CAT activity, which was significantly \( (P = 0.0135) \) prevented by EECS (50 mg/kg) pretreatment, when compared to unstressed group [Figure 4]. ARS significantly decreased the reduced GSH level in brain tissues \( (P = 0.0363) \) of mice as compared to that of vehicle-treated group, whereas no significant improvement was observed in EECS pretreated group, when compared to restraint stress group. Furthermore, statistical analysis revealed that ARS produced a significant \( (P = 0.0281) \) increase in GPx activity whereas EECS (25 mg/kg, 50 mg/kg) pretreatment significantly \( (P = 0.0096, 0.0049) \) abolished increase in GPx activity, compared to stressed animals. Our results showed that the treatment of ARS mice with EECS was able to prevent the increase in GPx activity caused by stress procedure [Figure 5].

The results depicted in Figure 5 illustrate that ARS significantly increased MDA \( (P = 0.0048) \) level in mice brain as compared to unstressed mice. Post hoc analysis indicated that EECS (50 mg/kg; \( P = 0.0113 \)) pretreatment and imipramine \( (P = 0.0091) \) significantly abolished increase in MDA level caused by ARS.
Statistical analysis indicated a significant decrease in NE ($P = 0.0036$) and 5-HT ($P = 0.0281$) level in ARS-induced mice brain compared to unstressed mice. Post hoc analysis revealed a significant ($P = 0.0365$) increase in the availability of both NE and 5-HT in brain tissue of mice pretreated with EECS (50 mg/kg) for 7 days, compared to stressed mice. At the same time, acute administration of imipramine also significantly ($P = 0.0238$) restored the level of NE and 5-HT in mice brain [Figure 6].

**Discussion**

Stressful events influence physiological homeostasis of the organism which might cause changes in immunological and neurobehavioral profile during adaptational processes. ARS, type of stressful event, has been reported to induce anxiety and depression-like behavior in animals which can be monitored in various rodent behavioral models effectively.\[9\] ARS which is a reliable model of anxiety and depression induced by stress, produces an inescapable physical and mental stress, in addition to impairment in the *in vivo* antioxidant defense mechanism.\[21\]

Stress-induced depression models, rodents, used for evaluating antidepressant activity include FST and TST, which assess duration of immobility as an index of depression as immobility represents a state of behavioral despair and failure to adapt to a stress. FST and TST provide rapid and reliable results for antidepressant activity and are also quite sensitive and relatively specific to all the major classes of antidepressants.\[21\]

Conventional antidepressant drugs reliably decrease duration of immobility in animals. In the present study, ARS significantly increased the duration of immobility in FST and TST, indicating depressive-like behavior as demonstrated in earlier studies.\[9,21\]

Pretreatment with EECS for 7 days, provided significant protection against ARS induced increased immobility time in FST and TST which supported the findings obtained in similar studies on evaluation of antidepressant-like effect.\[9\]

Moreover, FST has some drawbacks as of obtaining false positive or negative response because drugs that enhance motor activity may also give false positive response in FST.\[22\]

Similar to antidepressants, psychostimulants are also shown to decrease duration of immobility in FST and TST models, but at the same time, they cause a marked motor stimulation.\[23\]

Thus, to exclude motor stimulation activity of EECS, locomotor activity test was also performed. Our results are consistent with other studies\[9,23\] indicating that EECS at same doses that produce antidepressant-like activity, did not show motor stimulation. Since decreased immobility time was not accompanied by hyperlocomotion (that could produce false positive results) specific antidepressant-like activity of EECS can be confirmed from the above results.

Suppression of NE and 5-HT levels in different regions of mouse and rat brain subjected to restraint stress was clearly differentiated in several studies on depression.\[5\] Previous studies also demonstrated that currently used antidepressants act by increasing the concentration of the monoamine
neurotransmitters in the brain.\textsuperscript{[24]} From the results, it can be concluded that antidepressant-like activity of EECS, might be due to its modulatory effect on central monoamines mainly NE and 5-HT, the findings of this study were similar to those obtained with ethanol extract of \textit{Uncaria lanosa}, where antidepressant-like mechanism was well elucidated with the help of neurotransmitter and monoamine oxidase (MAO) enzyme estimation.\textsuperscript{[23]}

**Figure 5:** Effect of EECS pretreatment on ARS induced changes on GPx, reduced GSH activity and lipid peroxidation. NC: Normal control; ARS: Acute restraint stress; EECS: Ethanol extract of \textit{C. serratum}. Values are expressed as mean ± standard error of mean (\(N=6\)). ***\(P<0.01\), **\(P<0.05\). a versus NC group and b versus ARS group

**Figure 6:** Effect of EECS pretreatment on ARS induced changes on noradrenaline and serotonin level. NC: Normal control; ARS: Acute restraint stress; EECS: Ethanol extract of \textit{C. serratum}. Values are expressed as mean ± standard error of mean (\(N=6\)). ***\(P<0.001\), **\(P<0.01\), *\(P<0.05\). a versus NC group and b versus ARS group
Several studies have reported that flavonoids act as inhibitors of MAO-A and MAO-B enzymes. MAO-A preferentially oxidizes 5-HT and NE, whereas DA appears to be substrate for both isoenzymes. Thus, selective inhibitors of MAO-A can elevate the levels of monoamines mainly NE and 5-HT in postsynaptic sites. Another study demonstrated that four flavonoids isolated from standardized Ginkgo biloba extract by HPLC method, namely, kaempferol, quercetin, API, and chrysin were identified as MAO-A inhibitors, along with their K<sub>i</sub> values (kaempferol [0.7 μM], API [1 μM], chrysin [2 μM], and quercetin [5 μM]). Significant increase in monoamine levels of NE and 5-HT, exhibited by EECS, might be due to the presence of plant constituents flavonoids mainly API and LUT, as revealed by RP-HPLC quantification, which is strongly supported by the study of Nair et al. Since EECS contains flavonoid API, antidepressant-like activity may be related to selective MAO-A inhibitory activity, which might be responsible for elevation of monoamines in the brain.

Various studies have shown that stressful events are associated with oxidative damage in the brain as a result of increase in the production of ROS which also plays an important role in the pathogenesis of depression. Huge increase in SOD activity and decrease in CAT activity induced as a result of ARS, is an index of pro-oxidative conditions. In the present study, 6 h restraint stress induced a significant oxidative damage, as indicated by increased SOD and decreased CAT, which was significantly reversed by EECS pretreatment.

Lipid peroxidation is considered as a critical mechanism in causing cell injury during oxidative stress. Several studies have demonstrated that restraint stress significantly elevated LPO level in the hippocampus of rats. Our results are in line with these findings, showing significant LPO, evidenced by increased amount of MDA, which was attenuated by EECS treatment. Thus, the antidepressant-like effect of EECS could be associated with its capacity to prevent the lipid peroxidative damage, induced by ARS.

Glutathione system is an important indicator which mediates protection against pro-oxidant molecules in the brain, alteration of which is involved in several neuropathological conditions. A recent study clearly demonstrated antidepressant-like response of GSH in FST in mice. Various other studies reported that ARS caused depletion of reduced GSH content, and results of our study are consistent with these findings. In this study, EECS pretreatment significantly restored GSH content, suggesting its antioxidant-like effect. The neuroprotection offered by EECS pretreatment might be attributed to the antioxidant constituents such as flavonoids and phenolics in the leaves of C. serratum.

This study has few limitations too. MAO activity in mice brain tissue could not be determined to substantiate our findings. Moreover, mechanism of antidepressant action of EECS could be better explained with the help of in vitro cell line models. However, the previous findings on flavonoids may very well be sufficient to elucidate and explain the mechanism of antidepressant effect of C. serratum.

Conclusion

C. serratum, is a rich source of flavonoids, mainly API and LUT, responsible for its significant antioxidant potential, and may be helpful in eliciting neuroprotective effects, thereby preventing or slowing the progression of various oxidative stress-induced disorders. Based on these findings, it may be concluded that EECS demonstrated significant antidepressant and neuroprotective effect with the speculation that antidepressant effect of EECS is not due to inhibition of locomotor activity. This study thus provides justification for the traditional usage of C. serratum plant for the treatment of neurological disorders and may be recommended as supplement with the synthetic antidepressant drugs.

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Conflicts of Interest
There are no conflicts of interest.

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