EVALUATION OF ANTI-SRESS ACTIVITY OF ETHANOLIC EXTRACT OF ALTERNANTHERA SESSILIS (LINN.) AND ITS SILVER NANOPARTICLES IN CHRONIC VARIABLE STRESS MODEL.

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ABSTRACT
Biosynthesis of nanoparticles incorporating the herbal extracts can potentially eliminate the toxicity problem. These particles can be prepared easily by different chemical, physical, and biological approaches. But the biological approach is the most emerging approach of preparation, because, this method is easier than the other methods, eco-friendly and less time consuming. The Green synthesis of silver nanoparticles was done by using the ethanolic extract of Alternanthera sessilis (Linn.) and Silver nitrate. The anti stress activity of ethanolic extract of Alternanthera sessilis (Linn.) (EEAS) and its silver nanoparticles (ASAgNPs) were evaluated using forced swim (FST) and open field test following chronic variable stress (CVS). The effect of EEAS (200 mg/kg) and ASAgNPs (20mg/kg) were assessed by the ex vivo biochemical assays by measuring the plasma corticosterone and glucose levels. The behavioural parameters such as rearing, self-grooming, ambulation activity and Immobility were analysed using open field apparatus and Forced swim test respectively. EEAS and ASAgNPs have shown protective effect against stress in chronic variable stress model. Significant neuroprotection was produced in chronic variable stress model by EEAS. It resulted in a significant (p < 0.001) decrease in immobility time along with a significant increase in locomotion as compared to the stress only group. The reduction in the plasma corticosterone level and
glucose levels indicate the reduction in stress in the animals. It is concluded that *Alternanthera sessilis* (Linn.) may be used as a phytomedicine for Anti-stress. The ethanolic extract exhibited better activity compared to its nanoparticles.

**KEYWORDS:** Chronic variable stress, *Alternanthera sessilis* (Linn.), *Alternanthera sessilis* (Linn.) silver nanoparticles.

**INTRODUCTION**

Chronic stress affects structure, physiology, and behaviour. Numerous studies have also shown an increased risk for memory deficit and changes in learning by chronic stress.[1] Normal structure and function of the brain are altered by chronic and/or severe stress. A variety of changes, including neuronal excitability, neurochemistry, and morphological as well as functional plasticity of some brain areas, such as medial prefrontal cortex and hippocampus are adversely affected during stress.[2] Learning and memory impairing was reported after chronic stress in both animal models and human beings. A variety of chemical compounds including synthetic and natural substances have been used to prevent or ameliorate the effect of stress on the behavioural changes in animals and humans. Animal health including human has been shown to be affected by the stressful events of life inducing situation which alters cognition, learning memory and emotional responses, causing mental disorders like stress, depression and anxiety. The present study aims to evaluate the effects of behavioural changes in chronic variable stress model in rats.

Exposure to stress and hyperactivity of the Hypothalamic-Pituitary-Adrenal axis have long been recognized as risk factors for the development of psychiatric and neurodegenerative disorders including Alzheimer's disease.[2] Although underlying mechanisms remain poorly understood, chronic Hypothalamic-Pituitary-Adrenal axis activation has been associated with decreased neurogenesis, neuronal loss, and altered connectivity.[3]

Recently, considerable attention has been paid to utilize herbal medicines for the treatment or prevention of neurodegenerative disease. The current study envisages in evaluating the anti stress effect of ethanolic extract of *Alternanthera sessilis* (Linn.) (EEAS) and its silver nanoparticles (ASAgNPs). *Alternanthera sessilis* (Linn.) is commonly known as sessile joy weed, found in humid and warm regions of the world.[4] In the present investigation the preliminary phytochemical tests on EEAS and ASAgNPs gave positive results for flavonoids, steroids, glycosides, carbohydrates and sterols, and that may be responsible for its biological
activity. The use of nanoparticles in drug delivery therapy holds much promise in targeting remote tissue. Silver nanoparticles used as alternative strategies for drug delivery to Alzheimer brain are able to cross the Blood brain barrier and penetrate into the cell cytoplasm and induce underlying cellular change.\cite{5} The extracts of *Alternanthera sessilis* (Linn.) have shown to exert significant antioxidant activity as in FARP and DPPH radical scavenging assay and has shown Improved Superoxide dismutase and catalase activities in the livers of ovariectomized mice.\cite{6-7} Hence in the present study an attempt was made to explore the possible anti stress activity of *Alternanthera sessilis* (Linn.), keeping in mind its anti oxidant potential.

**MATERIALS AND METHODS**

**Experimental animal**

Healthy male Wistar albino rats (150 - 200 gm) were obtained from the animal house of Department of Pharmaceutical sciences, M.G University, Cheruvandoor, Kottayam. They were housed in well ventilated, large spacious hygienic cages under standard animal husbandry conditions (22-28°C) with relative humidity of 55±5 % and alternate 12 hour light-dark cycle. The animals were fed with standard food and water ad libitum. All animals were acclimatized to the experimental environment prior to study.

**Plant**

*Alternanthera sessilis* (Linn.) whole plant were collected from Kanjiramattom village of Ernakulum district, Kerala, India and were authenticated at CMS College, Kottayam, Kerala. A voucher specimen is preserved at the Herbarium with collection number. 782.

**Drugs and chemicals**

95 % Ethanol, Silver nitrate (3Mm), Fluoxetine.

**Preparation of A. sessilis (Linn.) Silver Nanoparticles**

20 ml of the plant extract was mixed with 80 ml of 3mM of silver nitrate solution.\cite{8} The colour changed from yellow to reddish brown colour indicates the formation of silver nanoparticles. The ASAgNPs thus obtained was purified by repeated centrifugation at 7000 rpm for 10 min. The pellet was collected and dried. The chemical tests were carried out in ASAgNPs for Proteins and Vitamin C.
Characterization of biosynthesized silver nanoparticles of *A. sessilis* (Linn.)

UV spectra analysis: The reduction of pure silver ions was confirmed by measuring the UV spectrum of the reaction mixture against distilled water as a blank. The Spectral analysis was done using double beam Shimadzu 1800, Japan spectrophotometer at a resolution of 1 nm from 250 to 450 nm.

SEM analysis: Morphological characterization of the samples was done using FE-SEM (JEOL JSM 3600). A pinch of dried sample was coated on a carbon tape. It was again coated with platinum in an auto fine coater and then the material was subjected to analysis.

Particle size measurement: Particle sizing experiments were carried out by means of laser diffractometry, using Nano- ZS, Malveren Instrument (Zetasizer Ver. 7.03) serial No.MAL1008884. Measurements were taken in the range between 0.1 and 10,000 nm.

**Acute oral toxicity study**

The oral acute toxicity study was carried out in adult female albino rats by the “fixed dose” method of OECD Guideline No.420, the fixed dose method as in Annex 2d (OECD, 2000).

**Chronic variable stress model**

Male wistar rats were taken for the study and divided into five groups of six rats each. Group-I: Vehicle control Group-II: Stress Group , Group-III: Stress treated with Fluoxetine (10mg/kg) p.o daily. Group-IV: Received Stress + ASE (200mg/kg). Group-V: Stress + ASAgNPs (20mg/kg).

Rats in stressed groups were exposed to the following stressors once daily for 5 consecutive weeks: 2-h immobilization; 24-h food deprivation; 24-h water deprivation; 5-min cold swim at 4°C; wet bedding; 1-min tail pinch with a clothes-pin placed 1cm distal from the base of tail. The same stressor will not be applied successively so that rats cannot anticipate the occurring of stress.

The performance testing was done using Forced swimming test and Open field test. Further, the blood samples were collected by Retro-orbital (non-surgical/terminal) method into separate tubes containing heparin as the anticoagulant for the estimation of corticosterone in plasma.
Forced swimming test
The test will be carried out on 2 successive days after the last stress period. Briefly, rats were forced to swim in a vertical plastic cylinder (diameter 21 cm, height 50 cm) containing 25 cm of water maintained at 25±1°C. On the 1st day of experiment, rats will be forced to swim for 15 min. On the following day, rats will be re-exposed to the forced swimming for 5 min. During the second test session, the immobility time will be evaluated. A rat is judged to be immobile whenever it remains floating passively in the water in a slightly hunched but upright position with its head just above the surface. At the conclusion of the swim test, the animal will be removed from the cylinder, dried by a towel, and returned to its home cage.

Open field apparatus
Open field apparatus consist of squares (61 × 61) were used for the study. Blue lines were drawn on the floor with a marker. The lines divided floor into sixteen squares. A central square was drawn on the middle of open field. The rats were centrally placed in the open field apparatus and were allowed to walk without restraint inside the area for 5 minutes and following behavioral aspects were noted.

• Ambulation
this was measured in terms of the number of squares crossed by the animal.

• Rearing frequency
partial or total elevation on to hind limbs.

• Self grooming
number of times animal groomed facial region, and licked /washed/ scratched various part of the body. Two consecutive days animals were exposed to the apparatus for habituation. The open field was cleaned with a 5% water-alcohol solution before behavioral testing to eradicate possible bias due to smells left by previous rats.

Biochemical estimations
Estimation of plasma corticosterone
The fluorimetric method was used to estimate the plasma corticosterone as an index of hypothalamic-pituitary-adrenocortical function. A reaction mixture consisting of 1 ml of plasma and 7.5 ml of dichloromethane was shaken for 2 minutes, centrifuged (in order to separate the phases), and then the plasma layer was removed. At zero time, 2.5 ml of
fluorescent reagent (7:3 v/v concentrated sulfuric acid and ethanol) was added and shaken for 2 s. The supernatant was discarded and exactly after 12 minutes, the acid extracts were read at 530 nm emission with 470 nm excitation.[10]

Standard: Corticosterone standard (20 µg./ml.), 20 mg. of corticosterone dissolved in 5 ml. absolute ethanol and then diluted quantitatively to 1 liter with distilled water, diluted before use to 0.1 or 0.2 µg/ml. with distilled water.[11]

Statistical analysis
The results of studies were expressed as mean ± SEM. The difference between control and treated means were analyzed using one way analysis of variance (ANOVA). P-values < 0.05 were taken to be statistically significant. Tukey’s multiple comparisons test was used for multiple comparisons. The statistical analysis was done by using graph pad prism version 6.05.

RESULTS
Characterization of biosynthesized Silver nanoparticles of *Alternanthera sessilis* (Linn.)

UV–vis spectra analysis
Extracts from whole plants under study (*A. sessilis* Linn) showed rapid conversion of silver nitrate into silver nanoparticles indicated by color changes from pale yellow to red-brown within few min of extract addition in 3mM AgNO₃ solution. A representative scheme of biosynthesis and UV-Vis spectrum is given in Figure 1. Synthesized silver nanoparticles primarily characterized by UV-visible spectroscopy. ASAgNPs give typical spectrum having maximum absorption in range of 420-450 nm. This absorption is unique property of metal nanoparticles called SPR (Surface Plasmon Resonance) arises due to conduction of electrons on surface of AgNPs.[12]

Scanning electron microscopy
Characterization of ASAgNPs under the study by Scanning electron microscopy revealed that nanoparticles formed by *A. sessilis* Linn. are spherical in shape. (Figure 2).

Particle Size Analysis
Characterization of plant nanoparticles under the study by Particle size analysis revealed that nanoparticles formed by *A. sessilis* (Linn.) had an average size of 122 - 396 nm. A well dispersed ASAgNPs were found with respect to intensity in this range. (Figure 3).
Forced swim Test
The EEAS and ASAgNPs were administered to male Wistar albino rats and at the end of the study period, duration of immobility was measured in forced swim test. The results obtained from the forced swim test were expressed as Mean±SEM. The immobility time was significantly decreased in animals treated with Fluoxetine (P<0.001) and EEAS (P<0.001) in comparison with the stress only group. (Figure 4).

Open field Test
The open field test was done in order to determine the effect of EEAS and ASAgNPs in CVS. The response was recorded before induction of stress after two weeks of stress and after five weeks of stress. EEAS treated group showed significant antistress activity as evidenced from increase in the grooming (p<0.05), rearing (p<0.05) and ambulation (p<0.001). Increases in activity were also seen in ASAgNPs treated group but non significant. (Figure 5a, 5b, 5c)

Biochemical estimation
Plasma corticosterone
CVS significantly increased the plasma corticosterone levels. Treatment with EEAS significantly (P<0.05) decreased the plasma corticosterone level when compared to vehicle groups. (Figure 6).
Figure 3: DLS (Size distribution by intensity).

Figure 4. Effect of EEAS and ASAgNPs on Immobility time in FST.

Figure 5a. Effect of EEAS and ASAgNPs in ambulation frequency on Open field test in CVS model.
Figure 5b. Effect of EEAS and ASAgNPs in rearing frequency on Open field test in CVS model.

Figure 5c: Effect of EEAS and ASAgNPs in self grooming on Open field test in CVC model.

Figure 6: Effect of EEAS and ASAgNPs on Plasma corticosterone.

Values are expressed as Mean ±SEM (n=6), analyzed by one-way ANOVA followed by Tukey’s multiple comparisons test, * Represents P < 0.05, **P <0.01, ***P<.0.001 when compared to stress only group. “a” Represents P<.0001, “b” P <0.01 and “c” P < 0.05 when compared to vehicle only group.
DISCUSSION AND CONCLUSION

In the present investigation the preliminary phytochemical tests on EEAS and ASAgNPs gave positive results for flavonoids, steroids, glycosides, carbohydrates, terpenoids and sterols, and that may be responsible for its biological activity. *A. sessilis* (Linn.) is a commonly known as sessile joy weed, found in humid and warm regions of the world.[13] The use of nanoparticles in drug delivery therapy holds much promise in targeting remote tissue. Silver nanoparticles used as alternative strategies for drug delivery to Alzheimer brain are able to cross the blood brain barrier and penetrate into the cell cytoplasm and induce underlying cellular change.[14] Increased generation of oxidative free radicals (OFR) or impaired antioxidant defence mechanism have been implicated in neurodegenerative diseases and in chronic stress induced perturbed homeostasis including immunosuppression, inflammation, diabetes mellitus, peptic ulceration, depression and other stress related diseases.[15] The extracts of *A. sessilis* (Linn.) have shown to exert significant antioxidant activity as in ferric reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay.[16] and it has shown to improve the levels of superoxide dismutase and catalase in the livers of ovariectomized mice.[17] Hence in the present study an attempt was made to explore the possible neuroprotective activity of *A. sessilis* (Linn.), keeping in mind its anti oxidant potential.

The anti-stress activity of EEAS and ASAgNPs was estimated using chronic variable stress model. The treatment with EEAS had shown significant anti stress activity in open field test which was evidenced by the increase in grooming, rearing and ambulation parameters (Figure: 5a,5b,5c). The EEAS showed a significant increase in the grooming (p<0.05), rearing (p<0.05) and ambulation (p<0.001) activities in comparison to the stress group. The open field behavioural model studies the exploratory and locomotor activity in animals. The positive results in the open field test shows that the EEAS has the ability to reverse or normalize the locomotor suppressant behavior in experimental animals and hence may help to cope with immobility associated with stress. The decrease of immobility time in the forced swim test in treated groups also showed the significant anti stress activity by EEAS (Figure:4). The EEAS decreased the duration of immobility time (sec) to 292.20 ± 8.98 (P<0.001) while the animals which received stress alone throughout the study period showed a immobility time (sec) of 328.60 ± 3.96. In this study significant reduction in immobility time (by increase in swimming time and climbing) was observed following oral administration of EEAS (Figure: 4). Drugs with predominant nor adrenaline or dopaminergic
elevating effects reduce the immobility by increasing climbing behaviour, whereas, drugs with predominant serotonin elevating effect reduce immobility time by increasing the swimming behaviour.\[18\] Hence here the possible mechanism of anti stress activity may be due to the increase in monoamine levels in the synaptic cleft. Previous phytochemical studies have reported the isolation of flavonols, triterpenoids, steroids and tannins; βsitosterol, stigmasterol, campesterol, lupeol being few of its important constituents.\[19\] *A.sessilis* posses high levels of flavonoids (70.42 mg/100 g) and phenolics (103.75 mg/100 g).\[20\] The increased plasma corticosterone level in stress group is in accordance with previous studies.\[21-24\] which shows that plasma corticosterone is an important indicator of stress. It is also reported that exposure to glucocorticoids or to stress may lead to oxidative injury in various tissues.\[25\] Plasma corticosterone levels in all stress groups were found to be significantly elevated with respect to the vehicle group. In the present study, results are in accordance with the previous studies that the increased level of plasma corticosterone has been one of the most important indicators of stress.\[26-27\] There are several studies which showed an increased level of plasma corticosterone depending on stress.\[28\] In accordance with the previous researchers, in this study also there was an increased corticosterone level in stress animals. After EEAS treatment, it decreased the plasma corticosterone levels. Upon treatment with EEAS, the decreased level of corticosterone in the stressed group indicates that the EEAS may have the anti-stress activity. (Fig: 6).

From the study we found that that EEAS and ASAgNPs have shown protective effect against CVS model. The neuroprotective and *ex-vivo* antioxidant activity of plants may be due to the presence of flavonoids and phenolic compounds. Significant neuroprotection was produced by EEAS. Further studies are required for screening and evaluation of the particular phytoconstituents present in plant, which might exhibit a protective effect in this study. In addition, further studies are needed to explore other possible mechanisms involved in the neuroprotective effect of *A.sessilis*.

REFERENCES


