Effect of Beta vulgaris Linn. Leaves Extract on Anxiety- and Depressive-like Behavior and Oxidative Stress in Mice after Acute Restraint Stress

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ABSTRACT
Background: Stress plays a significant role in the pathogenesis of neuropsychiatric disorders such as anxiety and depression. Beta vulgaris is commonly known as “beet root” possessing antioxidant, anticancer, hepatoprotective, nephroprotective, wound healing, and anti-inflammatory properties. Objective: To study the protective effect of Beta vulgaris Linn. ethanolic extract (BVEE) of leaves against acute restraint stress (ARS)-induced anxiety- and depressive-like behavior and oxidative stress in mice. Materials and Methods: Mice (n = 6) were pretreated with BVEE (100 and 200 mg/kg, p. o.) for 7 days and subjected to ARS for 6 h to induce behavioral and biochemical changes. Anxiety- and depressive-like behavior were measured by using different behavioral paradigms such as open field test (OFT), elevated plus maze (EPM), forced swim test (FST), and tail suspension test (TST) 40 min postARS. Brain homogenate was used to analyze oxidative stress parameters, that is, malondialdehyde (MDA) and reduced glutathione (GSH) level. Results: BVEE pretreatment significantly (P < 0.05) reversed the ARS-induced reduction in EPM parameters, that is, percentage entries and time spent in open arms and in OFT parameters, that is, line crossings, and rearings in mice. ARS-induced increase in the immobility time in FST and TST was attenuated significantly (P < 0.05) by BVEE pretreatment at both the dosage. An increase in MDA and depletion of GSH level postARS was prevented significantly (P < 0.05) with BVEE pretreatment at both the dosage (100 and 200 mg/kg). Conclusion: BVEE exhibits anxiolytic and antidepressant activity in stressed mice along with good antioxidant property suggesting its therapeutic potential in the treatment of stress-related psychiatric disorders.

Key words: Acute restraint stress, antioxidant, anxiety, Beta vulgaris, depression, oxidative stress

SUMMARY
Stress plays major role in the pathogenesis of anxiety and depression
ARS-induced anxiety- and depressive-like behavior through oxidative damage in mice
BVEE pretreatment reversed ARS-induced behavioral changes, that is, anxiety and depression
ARS-induced oxidative stress was prevented by BVEE pretreatment in mice.

INTRODUCTION
World Health Organization report says that approximately 450 million people are suffering from psychiatric disorders contributing 12.3% of the global burden of disease, and this figure will rise to 15% by 2020.[1] Worldwide, there are 10–20 million suicide attempts every year which are associated with mental disorders.[2] Unfortunately, these disorders are under-diagnosed and undertreated.[2] Moreover, most of the clinically available anxiolytics and antidepressants are not effective for all patients and inundated by adverse effects, slow onset of actions, and poor patient compliance.[3] The knowledge of basic neuroscience helps us in improving our understanding about disease pathophysiology, identifying
novel mechanisms which can be targeted for effective treatments, and screening of drugs from herbal sources. These considerations implicate the search for novel anxiolytic and antidepressant agents which have a fast onset of action with maximum efficacy and minimum toxicity. Several plant species have shown their pharmacological effectiveness in the variety of animal models of psychiatric disorders, and are being used as complementary and alternative medicines for the management of these disorders.

There are ample evidences which demonstrate that stress play a significant role in the pathogenesis of neuropsychiatric problems including anxiety and depression. Stress may be defined as a state of body to reestablish homeostasis by producing adaptive physiological and neurobehavioral changes, and depends on severity, type, and duration of stressful events. A consequence of stressful events is the altered physiological and psychological responses that affect different organs and systems, including the central nervous system (CNS) and increased susceptibility to different psychiatric diseases, including depression.

Moreover, stress induces immunological and neurobehavioral responses such as anxiety, depression, cognitive impairment, insomnia, anorexia, and activate the hypothalamic-pituitary-adrenal axis resulting in elevated corticosterone levels in animals and humans. Stress exerts detrimental effects on cellular functions through oxidative damage due to release of free radicals. Acute restraint stress (ARS) stimulates several cellular events resulting in enhanced reactive oxygen species (ROS) production. These free radicals damage the body parts, especially CNS because of brain's high oxygen consumption, abundant lipid content, and relative paucity of antioxidant enzymes.

Similarly, restraint stress in rodents precipitates many neurochemical, hormonal, and behavioral abnormalities that are often associated with an imbalance in the brain's intracellular redox state. Numerous studies have reported that restraint stress enhances lipid peroxidation and decreases antioxidant enzyme activities in rodents.

Various diseases are now being treated with the help of indigenous drugs, and rebirth of herbal treatment options are happening all over the globe. At present, the herbal products are considered to be safe while synthetics are regarded as unsafe to human and the environment due to their side effects. Nowadays, peoples are showing faith on products of natural origin due to safety and security and decreasing their dependence on products of synthetic origin. Epidemiological studies have suggested positive association between the consumption of phenolic-rich foods or beverages and the prevention of diseases. These effects have been attributed to antioxidant components such as plant phenolics, flavonoids, and phenyl propanoids among others.

_Beta vulgaris_ (BV) Linn. is a native to Mediterranean region and extensively cultivated in America, Europe, and throughout India. The parts of this plant are harbored with numerous medicinal values and used in traditional Indian medicine. Leaves possess diuretic, purgative, and anti-inflammatory activity and useful in alleviating paralysis, spleen, and liver diseases. Seeds are expectorant and having carminative effect. Roots possess expectorant, diuretic, sedative, emmenagogue effects, and employed in the treatment of mental problems and liver diseases. It is also used as a natural food colorant for dairy and meat products.

In addition, leaves contain various phytoconstituents such as betalains, that is, betacyanins (red-violet pigments) and betaxanthines (yellow pigments), flavonoids, polyphenols, vitamins, and minerals. BV possesses antioxidant, anticancer, hepatoprotective, nephroprotective, wound healing, and anti-inflammatory activities. Leaves are a good source of natural antioxidant and widely consumed as vegetables due to its high nutritional value. On the basis of literature review, it can be concluded that little work has been done to explore its biological activities. Moreover, no scientific report is available regarding its anti-anxiety and antidepressant activity, to the best of our knowledge. Therefore, to validate the traditional use, the present study was undertaken to evaluate the effect of BV leaves extract against restraint stress-induced neurobehavioral and biochemical alterations in mice.

**MATERIALS AND METHODS**

**Chemicals**

Fluoxetine, thiobarbituric acid, 5,5’-dithiobis (2-nitrobenzoic acid), and L-reduced glutathione (GSH) were purchased from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals were of analytical grade purchased from Sigma-Aldrich chemicals unless otherwise mentioned.

**Animals**

The protocol of this study was approved by the Institutional Animal Ethics Committee of Sapience Bioanalytical Research Lab., Bhopal, India (Approval no. 1413/PO/a/11/Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA]) in accordance with the CPCSEA guidelines for the safe use and care of the experimental animals. The adult male Swiss albino mice (22–30 g) were procured from Sapience Bioanalytical Research Lab., Bhopal, (Madhya Pradesh). The animals were kept under the experimental housing and normal feeding conditions for 7 days prior to the test. All the experiments were carried out between 9:00 and 17:00 h. The animal room was maintained at 22°C ± 2°C temperature, 60% ± 5% relative humidity, with 12:12 h light–dark cycle. Food and water were available ad libitum. Considering the animal ethical issues, all animals were kept under best hygienic conditions and animals were inspected daily for any signs of pain, discomfort, or distress.

**Plant material and extraction**

The fresh leaves of BV were procured from the market of Bhopal, India, and authenticated by Dr. Zia Ul Hasan, Professor, Department of Botany, Saifia College, Bhopal (Madhya Pradesh), India (Specimen voucher no. 483/Bot/Saifia/14). The fresh leaves were washed with tap water, dried under shade, and powdered. The powdered leaf material (160 g) was successively extracted with petroleum ether (50–60°C) and 95% ethanol (60–70°C) using Soxhlet extractor. The resultant ethanol extract was air dried for 5 days to remove solvents and kept in a refrigerator until use and in this study without any further purification. The suspension of plant extract in 0.5% carboxymethylcellulose (CMC) was prepared, and the dose level 100 and 200 mg/kg body weight was selected based on the previous studies.

**Experimental design**

Thirty-six mice were randomly divided into six experimental groups, each consisting of six mice. Group I (normal control) mice received 0.5% CMC (1 mL/kg, p.o.) daily for 7 days. Group II (stress control) mice received 0.5% CMC (1 mL/kg, p.o.) daily for 7 days and subjected to restraint stress on 8th day [Figure 1]. Group III-IV mice were treated with _Beta vulgaris_ Linn. ethanolic extract (BVEE) (100 and 200 mg/kg, p.o.) daily for 7 days and subjected to ARS on 8th day. Groups V-VI mice were treated with BVEE (100 and 200 mg/kg, p.o.) daily for 7 days and no stress was given on 8th day. Anxiety- and depressive-like behavior were measured by submitting the mice to different behavioral paradigms such as open field test (OFT), elevated plus maze (EPM) test, forced swim test (FST), and tail suspension test (TST) 40 min poststress procedure. Oxidative stress parameters such as lipid peroxidation and reduced GSH level were also analyzed following behavioral tests.
Acute restraint stress procedure

Restraint stress protocol was adapted from a previously described procedure.[8,17,25] The animals were divided into six groups as mentioned above. Stressed groups were administered with vehicle and BVEE, and 1 h after the treatment, they were submitted to the stress protocol. The immobilization stress was accomplished by placing them in an individual rodent restraint device made of Plexiglas fenestrate for a period of 6 h. This restrained all physical movements without submitting the animal to pain. The 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 the behavioral tests or biochemical estimations. In unstressed group, the mice were kept in the animal cage with soft bedding in the experimental room.

Behavioral tests

Elevated plus maze test

EPM test has been proposed for selective screening of anxiolytic and anxiogenic drugs. It was made of two open arms (35 cm × 5 cm) perpendicular to two closed arms (35 cm × 5 cm × 20 cm) size with a small central square (5 × 5) between arms. The maze was elevated 50 cm from the floor in a dim room. Each mouse was placed at the center of EPM with head facing toward the open arm. During 5 min test period, the parameters measured were: Number of open arm entries and closed arm entries. Subsequently, the percentages of open arm entries and time spent on open arms were calculated from open arm entries and time spent on open arms was divided by the total number of entries in both open and closed arms and time spent on open arm exploration was divided by total time spent in both open and closed arms, respectively. The procedure was conducted in sound attenuated room.[13,26]

Open field test

OFT is a useful tool to assess the effect of restraint stress on motor and behavioral changes in the mice. Wooden box (60 cm × 60 cm × 30 cm) with its floor divided into 16 equal sized squares (15 cm × 15 cm) was used. Four squares were considered as the center and the 12 squares along the walls were considered the periphery. Each mouse was placed in the very center of the open field and (a) number of central crossing, (b) number of peripheral crossing, and (c) rearing were observed during a 5 min exposure period for both the control and treated animals.[13,26]

Forced swim test

The FST, the most commonly employed behavioral model for screening antidepressant agents in rodents, was performed as per the method described by Porsolt et al.[27] with some modifications. Briefly, 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. At this height of water, mice were not able to support themselves by touching the bottom or the side walls of the cylinder with their paws or tail. 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. The duration of immobility was manually recorded during the next 4 min of the total 6 min testing period by the observers 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. Following swimming session, mice were dried using room heater or towel and returned to their home cages.[1] A decrease in the duration of immobility is indicative of an antidepressant-like effect,[27] whereas an increase of immobility time, when compared with the control group, is associated with depressive-like effect.[25]

Tail suspension test

TST is a frequently used behavioral model to study the antidepressant-like activity in mice. The TST was performed as previously described by Steru et al.[28] with some modifications. Briefly, each mouse was individually suspended to the edge of a table, 50 cm above the floor, by adhesive tape, placed approximately 1 cm from the tip of the tail in dim lighted room that was acoustically and visually isolated. The immobility time of each mouse was manually recorded for 6 min but immobility time during the last 4 min was analyzed and presented. Animal was considered to be immobile when it did not show any body movement, hung passively, and completely motionless. Recording of the immobility of animals was done by the observers blind to the treatments given to the animals under study.[1,4]

Biochemical analysis

Preparation of brain homogenate

All the animals were sacrificed by decapitation on the same day immediately after behavioral assessments. The brains were quickly removed, washed in ice cold sterile isotonic saline, and weighed. A 10% (w/v) tissue homogenates were prepared with 0.1 M phosphate buffer (pH 7.4). The supernatant was obtained by centrifugation of the homogenate at 12000 ×g for 20 min at 4°C and used for further biochemical analysis.

Glutathione levels

Reduced GSH levels in the brain homogenates were determined according to the Ellman's method.[29] Briefly, homogenates were mixed with 50% trichloroacetic acid solution. After centrifugation (3000 rpm/15 min), the supernatant of the homogenate was collected and mixed with 0.4 M tris HCl buffer (pH 8.9) and 0.01 M 5,5-dithiobis (2-nitrobenzoic acid). The resultant yellow color was immediately read at 412 nm using a ultraviolet (UV)-visible spectrophotometer. Results were calculated based on a standard GSH curve and expressed as µM of GSH/mg of protein.

Lipid peroxidation assay

The quantitative measurement of lipid peroxidation in the whole brain was assessed according to the method of Jangra et al.[30] The amount of malondialdehyde (MDA) formed was measured by the reaction with thiobarbituric acid at 532 nm using Shimadzu UV-visible spectrophotometer. The results were expressed as µM of MDA per mg of protein using the molar extinction coefficient of chromophore (1.56 × 105/M/cm).
**RESULTS**

**Effect of Beta vulgaris Linn. ethanolic extract pretreatment on restraint stress-induced anxiety - like behavior tested in elevated plus maze**

Analysis of EPM data revealed that ARS (6 h) induced an anxiety - like behavior as observed by significant reduction in the percentage open arm entries ($P < 0.01$) and percentage time spent in open arms ($P < 0.01$) as compared to control group. BVEE pretreatment, at both the dosage (100 and 200 mg/kg p.o. for 7 days), reversed the restraint stress-induced changes in EPM parameters. Chronic pretreatment of BVEE increased the percentage of open arm entries [Figure 2a] and percentage time spent in open arms [Figure 2b] significantly at both the dosage 100 and 200 mg/kg, respectively ($P < 0.05$ and $P < 0.01$), as compared to the stress group.

**Effect of Beta vulgaris Linn. ethanolic extract pretreatment on restraint stress-induced anxiety - like behavior tested in open field test**

Restraint stress-induced a significant decrease in the number of central crossing ($P < 0.01$), peripheral crossing ($P < 0.01$), and rearing ($P < 0.001$) as compared to the control group. Pretreatment of BVEE attenuated the restraint stress-induced changes in the open field parameters. BVEE pretreatment at low dose (100 mg/kg) produced significant increase in the frequency of central ($P < 0.05$) and peripheral crossings ($P < 0.05$) and rearings ($P < 0.05$) whereas higher dose of BVEE (200 mg/kg) showed more significant effect on the number of central ($P < 0.01$) and peripheral crossings ($P < 0.01$) and rearings ($P < 0.01$) [Figure 3a-c].

**Effect of Beta vulgaris Linn. ethanolic extract pretreatment on restraint stress-induced changes in immobility time in forced swim test**

To evaluate the influence of treatment of mice with BVEE on the depressive-like behavior elicited by ARS procedure, the immobility time of mice in the FST was measured [Figure 4a]. ARS caused a significant increase in the immobility time, which is in agreement with its ability to induce depressive-like behavior. In the FST, our results showed that the immobility time was increased significantly ($P < 0.001$) in the mice subjected to ARS as compared to control group. Pretreatment with BVEE attenuated restraint stress-induced increase in the immobility time at both the dosage 100 mg/kg ($P < 0.05$) and 200 mg/kg ($P < 0.01$) significantly.

**Effect of Beta vulgaris Linn. ethanolic extract pretreatment on restraint stress-induced changes in immobility time in tail suspension test**

Mice subjected to ARS exhibited a significant increase in the immobility time ($P < 0.001$) as compared with that of control group, whereas pretreatment with BVEE significantly reversed ARS-induced increase in the immobility time in TST at both the dosage 100 mg/kg ($P < 0.05$) and 200 mg/kg ($P < 0.01$) [Figure 4b]. BVEE pretreatment exhibited significant protective effect against ARS-induced increase in the immobility time in TST.

**Effect of Beta vulgaris Linn. ethanolic extract pretreatment on restraint stress-induced oxidative stress parameters**

ARS (6 h) significantly decreased the reduced GSH level in brain tissues ($P < 0.001$) of mice as compared to that of vehicle treated group. Pretreatment of BVEE resulted in significant improvement in GSH level at both the dosages, that is, 100 mg/kg ($P < 0.05$) and 200 mg/kg ($P < 0.01$) when compared to the restraint stress group [Figure 5a].

The exposure of 6-h ARS significantly increased MDA level ($P < 0.001$) in mice brain as compared to control (unstressed) mice. Chronic (7 days) pretreatment with BVEE significantly attenuated the rise in MDA level at both the dosage, that is, 100 ($P < 0.05$) and 200 mg/kg ($P < 0.01$) as compared to the restraint stress group [Figure 5b].

**DISCUSSION**

Stressful events are known to influence the physiological homeostasis of the organism and complex mechanisms, lead to the changes in immunological and neurobehavioral profile in the course of adaptational processes. Stress plays the significant role in the pathophysiology of psychiatric disorders including anxiety and depression. It alters various neurological functions at both central and peripheral level through activation of hypothalamus-pituitary-adrenal axis. Any type of stress influences brain functions by causing long-term changes in the multiple neural systems. ARS has been reported to precipitate anxiety- and depression-like behaviors in the animals. Stress-induced behavioral alterations can be monitored in the rodents effectively. EPM and OFT are the most commonly employed test to study the effect of anxiolytics on behavioral parameters of animals. In the...
present study, ARS (6 h) caused anxiety-like behavior as evidenced by significant reduction in the EPM parameters such as percentage number of entries and percentage time spent in open arms. Similarly, restraint stress caused significant decrease in central and peripheral ambulation and rearing in the OFT, indicating fear or anxiety. The results are in an agreement with previous studies. Increase in the percentage number of entries and percentage time spent in open arms in EPM was reversed by the chronic pretreatment of BVEE at both the dosage level, might be due to the chemical constituents such as betaine and betalains present therein. Similarly, pretreatment of BVEE increased number of central and peripheral crossings and rearings. The observed results indicate decreased fear or anxiety. Thus, BVEE pretreatment provided anxiolytic effects against ARS.

Figure 3: Effect of Beta vulgaris ethanolic extract pretreatment on ARS-induced anxiety-like behavior tested in open field. (a) Number of Central and (b) Peripheral crossings, and (c) Rearings. NC: Normal control; ARS: Acute restraint stress; BVEE: Beta vulgaris ethanolic extract. Values are expressed as mean ± standard error of mean (N = 6 mice). ***P < 0.001, **P < 0.01, *P < 0.05. a versus NC group and b versus ARS group

Figure 4: Effect of Beta vulgaris ethanolic extract pretreatment in ARS-induced changes in immobility time in (a) forced swim test (FST) and (b) tail suspension test. NC: Normal control; ARS: Acute restraint stress; BVEE: Beta vulgaris ethanolic extract. 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

Figure 5: Effect of BVEE pre-treatment on ARS-induced changes in oxidative stress parameters (a) GSH (b) MDA. MDA: Malondialdehyde; GSH: Glutathione; NC: Normal control; ARS: Acute restraint stress; BVEE: Beta vulgaris ethanolic extract. Values are expressed as Mean±S.E.M (N = 6). ***P < 0.001, **P < 0.01, *P < 0.05. a Vs NC group and b Vs ARS group

There are ample of evidences which showed association of depression with stressful events. Therefore, stress-induced depression models in rodents are used to evaluate antidepressant drugs. FST and TST are the two most commonly employed tests in rodents for screening of antidepressants, are quite sensitive and relatively specific to all the major classes of antidepressants. In the current study, ARS significantly increased the immobility time in the FST and TST indicating depressive-like behavior. These results are in accordance with earlier studies which demonstrated that restraint stress-induced depressive-like behavior as evidenced by increased immobility time in FST and TST. Pretreatment of BVEE provided significant protection against ARS-induced increased in the immobility time in FST and TST. This protective activity of BVEE might be due the presence of betalains and betaine, an active constituent of BV Linn., which are useful in the treatment of depression.
Oxidative stress generates ROS that exerts detrimental effects on the cellular components such as lipids, proteins, and DNA resulting in cellular damage and neurodegeneration. [2,3,36] Restraint stress is a well-known method to produce oxidative damage by causing derangement in the antioxidant defense mechanism. [17] In the present study, 6-h restraint stress caused significantly oxidative damage as indicated by increased lipid peroxidation and depleted reduced GSH level. The results are in line with the earlier findings that exposure of restraint stress for 6 h cause imbalance in antioxidant defense mechanism resulting in oxidative stress. [3,17] Study, reported an increased oxidative damage and weak antioxidant defense mechanisms, are implicated in anxiety and depression. [18] In the present study, BVEE pretreatment significantly attenuated lipid peroxidation and restored GSH activity suggesting its antioxidant-like effect. The protection offered by BVEE pretreatment might be attributed due to the presence of antioxidant constituents such as polyphenols (e.g., betalains and betaine), flavonoids, and Vitamin C in the leaves of BV Linn. [14,19] Our results also indicate that BVEE could be a beneficial dietary supplement to combat various neurodegenerative diseases.

CONCLUSION

The results of the current study demonstrate the anxiolytic and antidepressant-like activities of BVEE against ARS-induced anxiety- and depressive-like behavior and oxidative damage in mice. These observed effects may be mediated by the central serotonergic neuro-transmission (5-HT) and antioxidant profile of BVEE. Further research is needed to be performed to characterize the specific phytoconstituents involved as well as to ascertain their individual contributions and exact mechanism of action pertaining to CNS activity. However, this study provides indication that BVEE can be used in the treatment and management of stress-induced disorders.

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Conflicts of interest

There are no conflicts of interest.

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