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In vivo anxiolytic activity of *Gynura procumbens* (Asteraceae) leaves extract by gamma-aminobutyric acid (Gaba) mediated hyperpolarization in mice

Sharmin Sultana[1*], Md Lokman Hossain[2]
[1] Pharmacy Discipline, Life Science School, Khulna University, Khulna, Bangladesh
[2] Department of Pharmacy, Stamford University Bangladesh, Dhaka, Bangladesh

Abstract
Gynura procumbens of Asteraceae family is a commonly found medicinal plant in Bangladesh, India, China, Thailand, Indonesia, Malaysia, and Vietnam. Traditionally, it is extensively used in different countries for the management of a wide range of disorders such as kidney discomfort, rheumatism, diabetes mellitus, constipation, and hypertension. As part of our current research of traditional medicinal plant, this study assessed the anxiolytic activity of Ethanol Extract of *Gynura procumbens* (EEGP) leaves. Anxiolytic activity was screened by elevated-plus maze, light-dark box, hole-board and marble-burying test method in mice at the doses of 200 and 400 mg/kg body weight where Diazepam was used as the positive control. The phytochemical analysis of EEGP also carried out by standard methods. Elevated plus maze (EPM) and light-dark box test results explicated that mice preferred open arms and light part instead of close arms and dark part significantly ($p<0.001$). In hole-board and marble-burying test EEGP (200 & 400mg/kg body weight) reduced ($p<0.001$) the number of head dipping and number of marble burying, respectively. Phytochemical screening of EEGP revealed the presence of tannin, flavonoids, steroids, glycoside, alkaloids and terpenoids. The experimental result indicates that *G. procumbens* contains phytoconstituents that possess anxiolytic activity which traditionally used as depression and anxiety management. So, the plant may be further subjected to chemical investigation to isolate the bioactive compound(s) responsible for its pharmacological activity.

Keywords: *Gynura procumbens*, anxiolytic, elevated-plus maze, light-dark box, hole-board and marble-burying

1. Introduction
Nature has been a supply of medicinal agents since times old. A huge variety of scientific reports proved of exploitation the medicinal plants as natural remedies. Nowadays, the medicinal plant exploitation as another to artificial medication [1]. The importance of herbs in the management of human ailments cannot be overstated. It is clear that the plant kingdom harbours an everlasting source of active ingredients precious in the management of many intractable diseases [2]. Edible herbs provide the minerals like sodium, potassium, magnesium, iron, calcium, phosphorus etc. to maintain healthy life.

An herbal-based traditional medical practice that uses various plant materials in modalities considered both preventive and therapeutic. Phytomedicine or the use of herbal medicine with therapeutic properties has played a major role throughout history [3, 4]. Studies on the natural product are aimed to determine medicinal values of plants by investigation of current scientific knowledge, traditional uses and finding of potential therapeutic agents.

Phytochemicals are used as templates for lead optimization programs, which are anticipated to make safe and effective drugs [5, 6]. A number of recent drugs like aspirin, atropine, ephedrine, digoxin, morphine, quinine, reserpine, tubocurarine, and artemisinin are examples, which were originally discovered through observations of traditional cure methods of native people [7].

Plants are the idea of the many ancient drugs systems throughout the globe for thousands of years and still give man with new remedies [8]. In developing countries like Bangladesh, about 75% of the populations rely on different forms of traditional medicine for their primary health care [9]. The high cost of imported conventional drugs or inaccessibility to western health care facility, imply that traditional model of health care is the main form of health care that is reasonable and available to our rural people [6]. However, the potential benefits of herbal medicines could lie in their high acceptance by patients, efficacy, relative safety and low costs [10].

Gynura procumbens is a well-known traditional herb in South East Asia that belongs to the Asteraceae Family. This plant is about 10-25 cm high and it is presented with succulent, elliptic and glossy purplish leaves [11]. The leaves of G. procumbens have been served as food for decades in Malaysia, where it is generally consumed raw as salad. Besides, the plant is extensively used to treat inflammation, kidney discomfort, high cholesterol level, diabetic, cancer and high blood pressure. Indeed, *G. procumbens* is used traditionally in South East Asia for its valuable medicinal property. The small molecular weight compounds extracted from *G. procumbens* have been reported to display anti-cancer, anti-oxidant, anti-inflammatory, antihyperglycemic and anti-hyperlipidemic activities [12, 13]. Moreover, we have detected the presence of valuable plant defense secondary metabolites from the leaves of G. procumbens. We hope that the data found will be useful for the future intervention of secondary metabolites-based drug for discovery [13]. As a part of our long-term study about medicinal activity of natural resources [14, 15, 16, 17, 18, 19, 20], here we evaluated the anxiolytic activity of ethanol extract of *Gynura procumbens* leaves and saw whether it has CNS action. Regarding the previous reports and our phytochemical analysis, it has been

revealed that *G. procumbens* contains several bioactive compounds which have anxiolytic activities.

2. Materials and methods

2.1 Collection of Plants and identification

The leaves of *G. procumbens* was collected from Gopalganj, Bangladesh in January, 2019. The plant was identified and verified by the senior scientific officer of Bangladesh National herbarium, Mirpur, Dhaka and the given accession code was DACB: 48247.

2.2 Preparation of plant extract

About 200 gm of the powdered material was taken in a clean and flat-bottomed glass beaker and soaked in 5000 mL methanol (95%) (Merck, Germany) at 25±2 ºC for 15 days associated regular shaking and stirring. The solvent mixture was filtrated by a piece of sterile and white cotton material and finally using Whatman No. 1 filter paper. The solvent was removed by air drying and obtained 5 gm extract. The prepared extract was used for the phytochemical screening as well as pharmacological studies.

2.3 Collection and maintenance of animals

Swiss-Albino mice of either sex having aged 4-5 weeks, obtained from the animal breeding house of Jahangirnagar University, Savar, Dhaka, Bangladesh were used for the experiment. They were kept in standard environmental condition and fed International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) formulated food and water. As these animals are very sensitive to environmental changes, they are kept before the test for at least 4-5 days in the laboratory. Animals were maintained under normal conditions (temperature: (24.0±1.0 ˚C), relative humidity: 55-65% and 12 hrs light/12 hrs dark cycle) with proper cleaning of husk and excreta.

2.4 Drugs and Chemicals

The standard drugs Diazepam was collected from Aristoparma Ltd. Dhaka, Bangladesh. Saline water which was used for dilution purpose was prepared was obtained from Opsonin pharma.

2.5 Phytochemical Screening

The crude ethanol extract of *G. procumbens* was qualitatively tested for the detection of different phytochemical groups like alkaloids, glycosides, flavonoids, tannins, reducing sugar, carbohydrates, steroids and saponins following standard procedures [21].

2.6 Determination Anxiolytic activity

Drugs acting on the central nervous system (CNS) were first discovered by primitive humans and are still the most widely used group of pharmacologic agents CNS Action [22]. The effects of drugs on the central nervous system CNS with reference to the neurotransmitters for specific circuits, attenuation should be developed to general organizational principles of neurons. The view that synapses represent drug-modifiable control points within neuronal networks. It requires explicit delineation of the sites at which given neurotransmitters may operate and the degree of specificity with which such site that may be affected [23].

2.6.1 Elevated plus-maze test

The elevated plus-maze (EPM) test consisted of two open arms (30 × 5 × 0.25 cm) and two closed arms (30×5×15 cm) emanating from a common central platform (5×5 cm). Two pairs of identical arms were opposite to each other. The entire apparatus was elevated to a height of 40 cm above floor level. At the beginning of the session, a mouse was placed at the centre of the maze, its head facing an open arm and allowed to explore the maze for 5 minutes, and the following parameters were scored: the time spent and number of entries in each type of arms. The plus maze was carefully cleaned with a wet towel after each animal test. The mice were divided into four groups (5 mice/group). The control group received vehicle (saline water 0.1mL/mice). Diazepam (1 mg/kg b.w., IP) was used as the positive control or standard group and EEGP extract at doses of 200 and 400 mg/kg body weight, orally, in the two remaining groups. After each trial, the EPM apparatus was wiped clean with alcohol (70%) Solution [24].

2.6.2 Light-Dark Box Test

The apparatus (45×21×21cm) consisted of two compartments with one third painted white and two thirds painted black, and these compartments were separated by a divider with a 3.5×3.5 cm opening at floor level. The small compartment was painted black and illuminated by a dim red light (60 W; 4 lx), whereas the large compartment was painted white and brightly illuminated with a 60-W (400 lx) light source. The compartments were equipped with infrared beam sensors (four in the white area, three in the black one). Each mouse was tested by placing it in the center of the white area, facing away from the dark one, and was allowed to explore the novel environment for 5 min and thereby enabling the detection of locomotion in each zone, time spent in each zone, latency of the first crossing from one compartment to the other, and shuttle crossings between both compartments. The data for these four parameters were directly collected by observer. This test exploited the conflict between the animal's tendency to explore a new environment and its fear of bright light [25, 26]. The mice were divided into four groups (5 mice/group). The control group received vehicle (saline water 0.1 mL/mice). Diazepam was used as the positive control or standard group and EEGP extract at doses of 200 and 400 mg/kg body weight, orally, in the two remaining groups.

2.6.3 Hole-Board Test

The hole-board apparatus consists of a gray wooden box (40×40 cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor. Animals were kept in a quiet laboratory before this experiment, at least, one hour prior to testing. Each animal was placed singly in the centre of the board opposite the observer and the number of head dipping into the hole was recorded over a 5-minute exploration period on the board. Head dipping was recorded only when both eyes disappeared into the hole [27]. The mice were divided into four groups (5 mice/group). The control group received vehicle and Diazepam was used as the positive control or standard group and EEGP extract at doses of 200 and 400 mg/kg body weight, orally, in the two remaining groups.

3. Statistical analysis

Results were expressed as mean ± S.E.M. Variance was analyzed using One-way Analysis Of Variance (ANOVA), followed by Newman – Keul's multiple comparisons test. $P < 0.05$ was considered to be statistically significant.

4. Results & Discussion

In the preliminary phytochemical screening the extract showed the presence of reducing sugar, tannin, glycoside, gum, and protein.

4.1 Elevated plus maze test

In EPM, the ethanol extracts of leaves of *Gynurea procumbens* was tested at the doses of 200 and 400 mg/kg body weight. If the EEGP had anxiolytic effect the time spent in open arm would rise gradually and time spent in closed arm would decrease gradually. The results presented in table 1 indicate that treating mice with EEGP at the doses of 200 and 400 mg/kg body weight increased the percentage of time spent in open arm compared with the control group. In addition, diazepam (1 mg/kg) as a widely used anxiolytic drug in clinical practice significantly increased the percentage of time spent in the open arms in the EPM. Conversely, the number of entries and the time spent in the closed arms were reduced (Table 1).

4.2 Light-Dark Box test

In LDB, the ethanol extracts of leaves of *Gynurea procumbens* was tested at the doses of 200 and 400 mg/kg body weight. If the EEGP had anxiolytic effect, the time spent in light area would rise gradually and time spent in dark area would decrease gradually. The results presented in table 2 indicate that treating mice with EEGP at the doses of 200 and 400 mg/kg body weight increased the percentage of time spent in light area compared with the control group. In addition, diazepam (1 mg/kg body weight) as a widely used anxiolytic drug in clinical practice significantly increased the percentage of time spent in the light area in the LDB (Table 2). This result shows statistically significant.

4.3 Hole-Board Test

In the hole board test, pre-treatment with diazepam significantly decreased ($p<0.05$) number of head dipping when compared to the control. Also, pre-treatment with EEGP at the doses of 200 and 400 mg/kg body weight also significantly decreased the number of head dipping, when compared to the control group and showed the result significant statistically (Table 3).

Table 1: Effect of EEGP on mice in spent time in open and closed arm

Treatment	Dose (mg/kg b. w.)	Time in open arm (sec)	Time in closed arm (sec)
Control	0.1 mL/mice	13 ±0.89	287 ±0.89
Standard	1 mg/kg	49.7 ±1.019*	251.2 ±1.019*
EEGP	200 mg/kg	24.6 ±0.50*	274.4 ±0.50*
EEGP	400 mg/kg	76.4 ±0.87*	232.6 ±0.88*

Values are expressed as Mean ±SEM (n=5); *P< 0.05 compared with the control group (Dunnett's Test)

Table 2: Effect of EEGP on mice in spent time in light and dark area

Treatment	Dose (mg/kg b. w.)	Time in light area (sec)	Time in dark area (sec)
Control	0.1 mL/mice	63.4 ±0.67	261.4 ±0.68
Standard	1 mg/kg	251.4 ±2.22*	45.6 ±2.22*
EEGP	200 mg/kg	95.0 ±1.58*	201.0 ±1.57*
EEGP	400 mg/kg	124.2 ±3.56*	169.8 ±3.57*

Values are expressed as Mean ±SEM (n=5); *P< 0.05 compared with the control group (Dunnett's Test)

Table 3: Effect of EEGP on mice in hole-board test

Treatment	Dose and Route	No. of Head Dipping into hole
Control	0.1 mL, Oral	55.2 ±1.46
Standard	1 mg/kg, i.p.	23.0 ±1.58*
EEGP	200 mg/kg, Oral	57.6 ±1.02*
EEGP	400 mg/kg, Oral	43.4 ±1.36*

Values are expressed as Mean ±SEM (n=5); *P< 0.05 compared with the control group (Dunnett's Test)

The cause of most anxiety disorders are not fully understood, but various studies has shown the involvement of GABAergic, serotonergic neurotransmission in etiology, expression and treatment of anxiety. Benzodiazepines (BZDs), barbiturates, Tricyclic antidepressants (TCA's) have been used for long time to treat anxiety disorders. The serious side effects associated with these drugs, namely rebound insomnia, sedation, muscle relaxation, withdrawal and tolerance (BZD's, barbiturates and alcohol), sexual dysfunction, anticholinergic, antihistaminic effects (TCA's) have limited their use in patients. Buspirone, the non-sedative anxiolytic agent is not effective in a high percentage of patients. It is also associated with tachycardia, palpitation, gastric discomfort etc [28].

The results of the study indicate that the neuropharmacological effects of EEGP at different doses in mice models. In EPM test, if the EEGP had anxiolytic effect the time spent in open arm would rise gradually and time spent in closed arm would decrease gradually and our experimental data showed that having of anxiolytic properties of EEGP. In LDB, The delayed latency of mice in light area indicates anxiolytic properties of EEGP like the benzodiazepine group drug involves the binding with GABA receptor. If the EEGP had anxiolytic effect the time spent in light area would rise gradually and time spent in dark area would decrease gradually.

Phytochemical screening of EEGP revealed the presence of glycosides, reducing sugar, protien, gum and tannins. It has been reported that the presence of glycosides, and flavonoids in plant extract possess anxiolytic effect through the interaction with GABA receptors [29]. Considering our results and previously published reports, it is possible that the aforesaid chemical components in the extract might contribute at least in part to the observed pharmacological activities. We may, therefore, conceive that the ethanol extract of *G. procumbens* contains psychoactive principles that are anxiolytic in nature.

The elevated plus maze is a widely used behavioral model in rodents and has been validated to investigate the anxiolytic potential of different pharmacological agents [30]. The open arm activities of the animals in EPM reflect a conflict between the mice innate behavior to keep itself in a protected area (e.g., closed arms) and motivation to explore in a novel environment, where the anxiolytic agents induce the exploratory activities of the rodents in the open arm [31]. If the EEGP had anxiolytic effect the time spent in open arm would rise gradually and time spent in closed arm would decrease gradually and the experimental data showed same scenario. So, the effects treatment of mice with EEGP on time spent in open and time spent in close arm were significant, which indicates of having possibility anxiolytic properties of EEGP.

In light dark box is also widely used for rodents as a model for screening anxiolytic or anxiogenic drugs, based on the

innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, that is, a novel environment and light [32]. It has been reported that simply the measurement of the time spent in the light area, but not the number of transfers, is the most consistent and useful parameter for assessing an anxiolytic action [33]. However, the effects of treatment of mice with EEGP on Latency time, Time spent in light, no of transition. If the EEGP had anxiolytic effect the time spent in light area would rise gradually and time spent in dark area would decrease gradually and the experimental data showed this exact scenario. So, the effects of treatment of mice with EEGP on time spent in light and time spent in dark area were statistically significant, which indicates of having possibility of anxiolytic properties of EEGP. Anxiolytics (e.g. diazepam) are known to exert their pharmacological action by increasing the gamma aminobutyric acid (GABA) content of mice cerebral hemisphere. Further neuropharmacological studies will be required to ascertain if *Gynurea procumbens* actually mediates its action via similar mechanism [27].

5. Conclusions

The present investigation revealed that the ethanol leaves of *Gynurea procumbens* produced significant anxiolytic activity. The results suggest that the mechanisms of this anxiolytic effect may be by increasing the gamma aminobutyric acid (GABA) content of mice cerebral hemisphere. The investigation provided an empirical evidence for the use of *G. procumbens* extract in folkloric treatment of anxiety disorders. These findings suggest that *Gynurea procumbens* could serve as a potential new source of natural drug with anti-inflammatory activities.

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7. Ethics Approval and Consent to Participate

All the experimental mice were treated following the Ethical Principles and Guidelines for Scientific Experiments on Animals (1995) postulated by the Swiss Academy of Medical Sciences and the Swiss Academy of Sciences. The Institutional Animal Ethical Committee (SUB/TAEC/11.01) of Stamford University Bangladesh permitted all experimental rules.

8. Competing Interests

The authors declare that they have no conflict of interests.

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