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ANXIOLYTIC ACTIVITY OF THE AQUEOUS EXTRACT OF *BRIDELIA MICRANTHA* (HOCHST.) BAILL. (EUPHORBIACEAE) IN MICE: POSSIBLE INVOLVEMENT OF GABA-A RECEPTOR COMPLEX

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ABSTRACT

Anxiety has become the most common mental health problem associated with immense health care costs and a high burden of disease. Current drugs used for the treatment of anxiety are associated with a wide variety of prominent side effects. Medicinal plant extracts have become popular due to their efficacy, fewer undesirable effects, and can serve as sources of new pharmaceutical drugs. The aim of this study was to assess the anxiolytic properties of the bark aqueous extract of *Bridelia micrantha* (Euphorbiaceae) and its possible mechanisms of action. Hyperthermia-induced stress (HIS), Open field (OF), elevated plus maze (EPM) and hole-board tests were used in this study. Groups mice were treated with distilled water, diazepam, phenobarbital or *B. micrantha* extract (30, 76, 152 and 305 mg/kg). Evaluations of behavioural profile were done 1 h post-treatment and the duration of observation was 5 minutes. The involvement of the $GABA_A$ receptor complex in the activity of *B. micrantha* extract was inferred from experiments with known $GABA_A$-targeting agents. Extract administration caused significant dose-dependent decrease of HIS compared to negative control. Like diazepam, the extract significantly induced anxiolytic effects in mice in the open arms of the EPM, and during the open field and the hole-board tests. The plant extract significantly inhibited the anxiogenic activities of Bicuculline, Flumazenil and N-methyl-β-carboline-3-carboxamide. Thus, bark aqueous extract of *B. micrantha* possesses anxiolytic activity that might be due to interactions with the benzodiazepine-binding site of the $GABA_A$ receptor complex.

KEYWORDS: Mice, *B. micrantha*, anxiety, anxiolytic, GABA-A receptor complex.

INTRODUCTION

Anxiety is an unpleasant state of inner turmoil, often accompanied by nervous behaviour, somatic complaints and rumination.[1] When anxiety becomes excessive, it may be considered as an anxiety disorder, and can critically decrease the quality of life inducing several psychosomatic diseases. Among all mental diseases, the anxiety disorders, including panic disorder with or without agoraphobia, generalized anxiety disorder, social anxiety disorder, specific phobias, and separation anxiety disorder, are the most frequent.[2] There is a widespread opinion that anxiety is a characteristic feature of our modern times, and that the prevalence of anxiety disorders has increased due to certain political, societal, economical, or environmental changes.[3] Anxiety disorders can be treated with medication and psychological therapies, like cognitive behavioural therapy.[4] Only approximately two-thirds of the anxious patients respond to the currently available treatments but the magnitude of improvement is still disappointing, besides, they also produce various systemic side effects and exhibit dependence and tolerance on chronic treatment which now have become a major concern about the use of currently used medicines.[5] Anxiolytic substances mostly belonging to the benzodiazepines group are among the first line of anxiolytic drugs with well-known benefits. Their side effects are prominent, including sedation, muscle relaxation, anterograde amnesia and physical dependence. Hence, there is a need of drug which possesses greater efficacy, lesser undesirable effects with minimum or no tolerance and dependence. Plants are widely accepted sources of medicine, which play an important role in health care programme worldwide.[6] Only 10% of plants have been studied for their pharmacological properties.[7] *Bridelia micrantha* (Euphorbiaceae) is a semi-deciduous to

deciduous tree up to 20 m with an open spreading crown, and bare stem. *B. micrantha* occurs in savannah and secondary forest, in swamp forest, along forest edges in riverine woodland and in gallery forest. *Bridelia micrantha* has several applications in traditional medicine in Africa: the roots and bark decoction are crushed and used for treating stomach aches, tapeworms, diarrhoea, headaches, and sore joints. The leaf sap is used for sore eyes. The fruits are sweet, tasting like currants and are readily eaten by children. The bark is also mixed with milk and drunk as a tonic. The powdered bark is applied to burns to speed healing of stomach ache and is used for diseases of the central nervous system like epilepsy and insomnia in Cameroon.[8,9] Different plant parts, aqueous and organic extracts exhibited anthelmintic, antimicrobial, anticonvulsant and sedative, antidiabetic, antidiarrhoeal, antinociceptive, antioxidant, antiplasmodial, antischistosomal, hepatoprotective, insecticidal and β-lactamase inhibitory activities.[10,11,12,13,14,15] Multiple classes of phytochemicals including alkaloids, anthocyanidin, anthraquinones, carbohydrates, cyanogenic glycoside, essential oil, ester, flavonoids, oxalate, phenolic compounds, saponins, sterols, tannins, terpenoids as well as several minerals have been isolated from the bark, fruits, leaves and roots of *B. micrantha*.[16,17,18,19,20] The present study, aimed to evaluate the anxiolytic- like effects of the aqueous extract of *B. micrantha* bark on experimental models of Anxiety, using the elevated plus maze (EPM), open field (OF), hole-board and hyperthermia induced-stress in mice. The possible mechanisms of action of this extract were also investigated in regard of receptor systems involved in the anxiolytic-like effects.

MATERIAL AND METHODS

Material

Plant material

The barks of *B.micrantha* were collected in the immediate vicinity of Yaoundé (Nkombassi), Cameroon, during the dry season in July 2010. The plant materials were identified and authenticated (voucher specimen Nº 9678/SRF/Cam) at the National Herbarium of Cameroon in Yaoundé.

Animals

Swiss albino naive mice of either sex weighing approximately 20-30 g, aged about 2 to 3 months were used for experimental purpose. The animals were obtained from the animal house of the laboratory of Animal Physiology of the University of Yaoundé I. They were housed in standard cages with the temperature maintained at 25 ± 3°C, and 12 h alternating light and dark cycles. They were supplied with food and water *ad libitum*. All animal handling procedures were done in accordance with National Ethic Guidelines (FWA-IRB00001954), and the experiments were designed to minimize the number of animals used and to minimize their suffering.

Chemicals

All the chemicals products used in this study were as analytic grade. These chemicals included: Diazepam, Phenobarbital, Bicuculline, Flumazenil (RO151788) and N-méthyl- β-carboline-3-carboxamide (FG7142). All drugs were obtained from Sigma® (U.S.A.).

Methods

Preparation of the aqueous extract

The decoction and doses of *B. micrantha* were obtained based on the traditional medicine protocol. The collected barks of the plant were dried under room temperature. The dried and powdered bark (100 g) of *B. micrantha* was macerated for 1 h in 1L of distilled water. The mixture was boiled for 20 min. After cooling, the supernatant (decoction) was collected and filtered using Watt man filter paper Nº 1. The filtrate was then evaporated to dryness using an oven at 60°C giving aqueous extract with a 6.1% yield.

Pharmacological tests

Hyperthermia Induced-Stress in Group-Housed Mice

When mammals are faced with stressful situations, their body temperature rises, referred to as stress-induced hyperthermia (SIH). The group-housed animals version of SIH is based on the principle that, in a group of animals maintained together in the same cage, when removed from the cage at the regular interval of time, it observed the raise of body temperature from the first animal removed to the last one.[21] The equipment used to perform the SIH test is a thermometer (Harvard Apparatus) with a probe 2 mm in diameter and 2 cm in length. 24 h before all manipulations, animals were transferred from the animal room to the laboratory according to the protocol described by Bourin.[22] To perform the test, male mice were divided into six groups (n = 10). The first group, the positive control, received phenobarbital (20 mg/kg). The second group that received distilled water served as control. Four test groups were treated respectively with each of the following doses of *B. micrantha* aqueous extract: 30, 76, 152 or 305 mg/kg. Except for phenobarbital administered intraperitoneally (i.p), all the other substances were administered orally. One hour after treatment, the animals were removed one by one from the cage in the same order as the administration and the body temperature measured anally. The SIH (ΔT) was calculated by the method used by Borsini,[23] and represented the difference between the basal T1 (mean temperature of first three mice) and the end T2 (mean temperature of last three mice) of group-housed male mice.[23]

Elevated Plus Maze Test (EPM)

The plus maze consists of two open arms and two closed arms (50 × 10 × 40 cm each) elevated to a height of 50 cm. Animals were divided into 6 homogeneous groups of 5 animals each. They were treated with distilled water (10 ml /kg, po) for the negative control group, diazepam (3 mg /kg, ip) for the positive control group and the

different doses of the *B. micrantha*(30, 76, 152 and 305 mg/kg) for testing groups. After administration of different substances, the animals were returned to their original cages to reduce phobic responses due to the experimental environment.[24,25] One hour after administration of the various treatments, the animal was placed at the centre of the plus-maze with its nose in the direction of one of the closed arms. The following parameters were noted for 5 min. (1) number of entries in the open and closed arms and (2) time spent by the animal in the open and closed arms.[26] Finally, we compared the preference of the animals to open or enclosed arm, average time spent in open arm and the number of entries in open arm in each group. Every precaution was taken to ensure that no external stimuli, other than the height of the plus-maze could invoke mice anxiety. The apparatus was carefully cleaned with 10% ethanol solution after every test. All test sessions were taped by using a video camera (Panasonic V385, 2 Mega pixel).

Open Field Test

The open field has been considered to be a non-conditioned anxiety test based on the creation of a conflict between the exploratory drive of the mice and its innate fear to exposure in an open area.[27] The open field test has been employed to assess the spontaneous activity, general exploration and ambulation of the rodents. Animals were divided into 6 homogeneous groups of 5 animals each. They were treated with distilled water (10 ml /kg, po) for the negative control group, diazepam (0.3 mg /kg, ip) for the positive control group and the different doses of the *B. micrantha* (30, 76, 152 and 305 mg/kg) for testing groups. One hour after appropriate treatment, each mice was placed individually in the centre of the apparatus and observed for 5 minutes to record its locomotor activity (the number of line crossings), exploratory activity (indicated by frequency of rearing) and time spent in the centre.[28,29] The apparatus was carefully cleaned with 10% ethanol solution after every test. All test sessions were taped by using a video camera (Panasonic V385, 2 Mega pixel).

The hole-board test

The apparatus was composed of a gray wooden box (50 cm×50 cm×50 cm) with four equidistant holes 3 cm in diameter in the floor.[30,31] The centre of each hole was 10 cm from the nearest wall of the box. The floor of the box was positioned 15 cm above the ground and divided into squares of 10 cm ×10 cm with a water-resistant marker. After treatment administration, each animal was placed in the centre of the hole-board and allowed to freely explore the apparatus for 5 minutes. Mouse behaviour was continuously videotaped by a digital video camera (Panasonic V385, 2 Mega pixel). The total locomotor activity (numbers of squares crossed), and the number and duration of head dipping were recorded. A head dipping was scored if both eyes disappeared into the hole. The positive control group received diazepam (0.5 mg/kg).

Study of benzodiazepine site of GABA-A receptor complex on the anxiolytic properties of *B. micrantha* by using the β-carboline and Flumazenil

For the evaluation of the involvement of benzodiazepines site of the GABA-A receptor complex in the anxiolytic properties of *B. micrantha, g*roups of mice were treated with flumazenil (3 mg/kg, i.p.), a GABA benzodiazepine receptor antagonist or β-carboline (5 mg/kg, *ip*.) an inverse agonist of the receptor site of the GABA-A receptor complex of benzodiazepines, 30 minutes before the administration of *B. micrantha* extract (152 or 305 mg/kg, p.o). The dose of flumazenil and β-carboline administered has been found to block the GABA receptors.[32,33] The anxiety evaluation using elevated plus maze was carried out one hour after the administration of *B. micrantha* or vehicle. Between each trial, the maze was wiped with 10% ethanol to prevent olfactory cue from animals. Conventional and ethological parameters of EPM were observed and recorded for a period of 5 minutes. All test sessions were taped by using a video camera (Panasonic V385, 2 Mega pixel).

Study of the Involvement of Gaba Sites of GABA-A Receptors Complex on the Anxiolytic properties of *B. micrantha* by using Bicuculline

The involvement of GABA site of GABA-A receptor complex for anxiolytic properties by *B. micrantha* was evaluated. The bicuculline, a competitive antagonist of GABA site of GABA-A receptor complex were used in this study. Mice were treated with bicuculline (2.5 mg/kg; *i.p*.) 30 minutes before administration of the plant extract (152 and 305 mg/kg). One hour after administration of the test substances, the mice were placed one after the other in the centre of the elevated plus maze and conventional and ethological parameters were observed and recorded for a period of 5 minutes. All test sessions were taped by using a video camera (Panasonic V385, 2 Mega pixel).

Statistics

Values were expressed as mean ± SEM (standard Error of the Mean). All data were analysed by one way analysis of variance (ANOVA). Post hoc tests were then performed using Dunnet's or Turkey's test, with the level of significance set at $P<0.05$.

RESULTS

Effects of the aqueous extract of *B. micrantha* on the mean rectal temperature

As shown in figure 1, a significant ($P<0.001$) decrease of mean rectal temperature of mice treated with the aqueous extract of *B. micrantha* (30, 76, 152 and 305 mg/kg) or phenobarbital (20 mg/kg) as compared to the negative control group was observed. The mean rectal temperature in the negative control group from 33.50 ± 0.11°C decreased to 32.63 ± 0.05°C, 31.58 ± 0.15°C and 31.55 ± 0.05°C, respectively, in mice treated with *B.micrantha* at the doses of 30, 76, 152 and 305 mg/kg.

The mean rectal temperature in mice treated with phenobarbital was 31.21 ± 0.15°C.

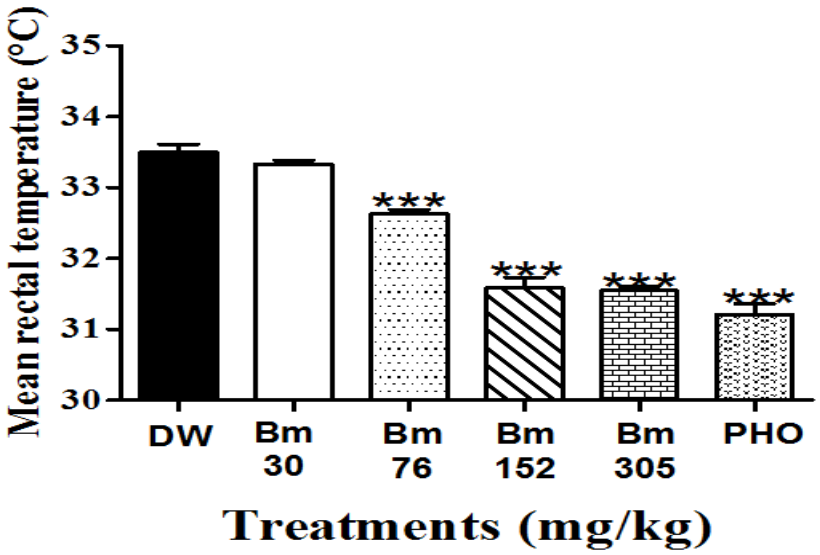


Figure 1: Effects of the aqueous extract of the *Bridelia micrantha* on mean rectal temperature.
Each bar represents the mean rectal temperature ± SEM, n= 10. *P* values for groups' comparison were obtained by one way ANOVA followed by Dunnet post-hoc test. ***p<0.001 significantly different with respect to distilled water treated group. DW: negative control treated with distilled water. Bm 30, 76, 152 and 305: mice treated with respective dose of 30, 76, 152 and 305 mg/kg of the aqueous extract of *B. micrantha*. PHO: positive control group treated with Phenobarbital (20 mg/kg).

Effects of the aqueous extract of *Bridelia micrantha* on stress-induced Hyperthermia (SIH)

The anti-pyretic properties of *B. micrantha* were assessed in mice upon stress-induced Hyperthermia experiment. *B. micrantha* aqueous extract evoked a significant (P<0.001) dose dependent decrease of stress-induced Hyperthermia (Figure 2). The mice treated with Phenobarbital (20 mg/kg) significantly reduced the value of SIH to 0.1°C (P<0.001). The mice treated with Phenobarbital 20 mg/kg also showed the same variation as the plant extract (P<0.001) with a HIS equal to 0.1°C.

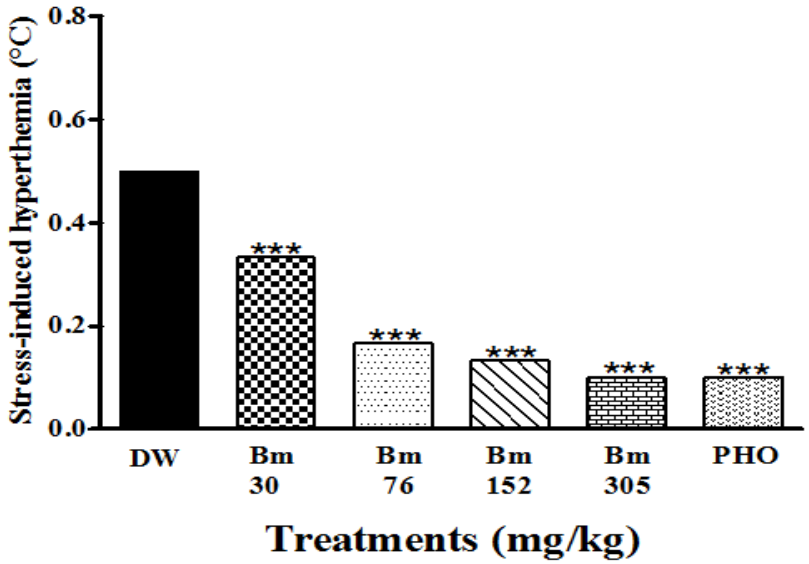


Figure 2: Effects of the aqueous extract of the *Bridelia micrantha*on stress-induced hyperthermia.
Each bar represents the SIH. n= 10. *P* values for groups' comparison were obtained by one way ANOVA followed by Dunnet's post-hoc test. ***p<0.001 vs distilled water treated group. DW: negative control treated with distilled water. Bm 30, 76, 152 and 305: groups treated with the aqueous extract of *B. micrantha* at the respective dose of 30, 76, 152 and 305 mg/kg. PHO: positive control treated with phenobarbital (20 mg/kg).

Effects of Single Administration of the aqueous extract of *Bridelia micrantha* on Elevated Plus Maze Parameters

As it is shown from figure 3A to figure3D, the single administration of aqueous extract of *B. micrantha* in mice at the doses 152 and 305 mg/kg resulted in a significantly higher number of entries into open arms (2.33 ± 0.49; P< 0.05 and 2.83 ± 0.40; P< 0.01 respectively), percentage of open arms entries (53.61%; P< 0.01 and 73.89%; P< 0.001 respectively), the time spent in open arms (129.8 ± 26.00s and 186.0 ± 12.25s; P< 0.001 respectively) and percentage of time spent in open arms (43.28%; P< 0.01 and 62.00%; P< 0.001) of the EPM compared to the negative control group (0.83 ± 0.16, 12.00%, 1.16 and 0.38%, respectively). However, the dose 76 mg/kg of the plant extract did not show a significant variation of these later parameter compared to the negative control group. As expected for a positive control group, a single administration of 3 mg/kg of diazepam via intra-peritoneal route also significantly (P< 0.001) induced an increased number of open arms entries, the percentage of entries into and time spent in the open arms and the percentage of time spent in the open arm of the EPM. As shown in figures 3E and 3F, the plant extract (76, 152 and 305 mg/kg) elicited a significant (P< 0.001) decrease of the numbers of rearing and head dipping in treated mice compared to the negative control group. The same trend was observed with the positive control group. Furthermore, both diazepam and aqueous extract of *B. micrantha* induced significant reduction in the number of closed arms entries, the percentage of entries into closed arms and the percentage of time in closed arms (data not shown).

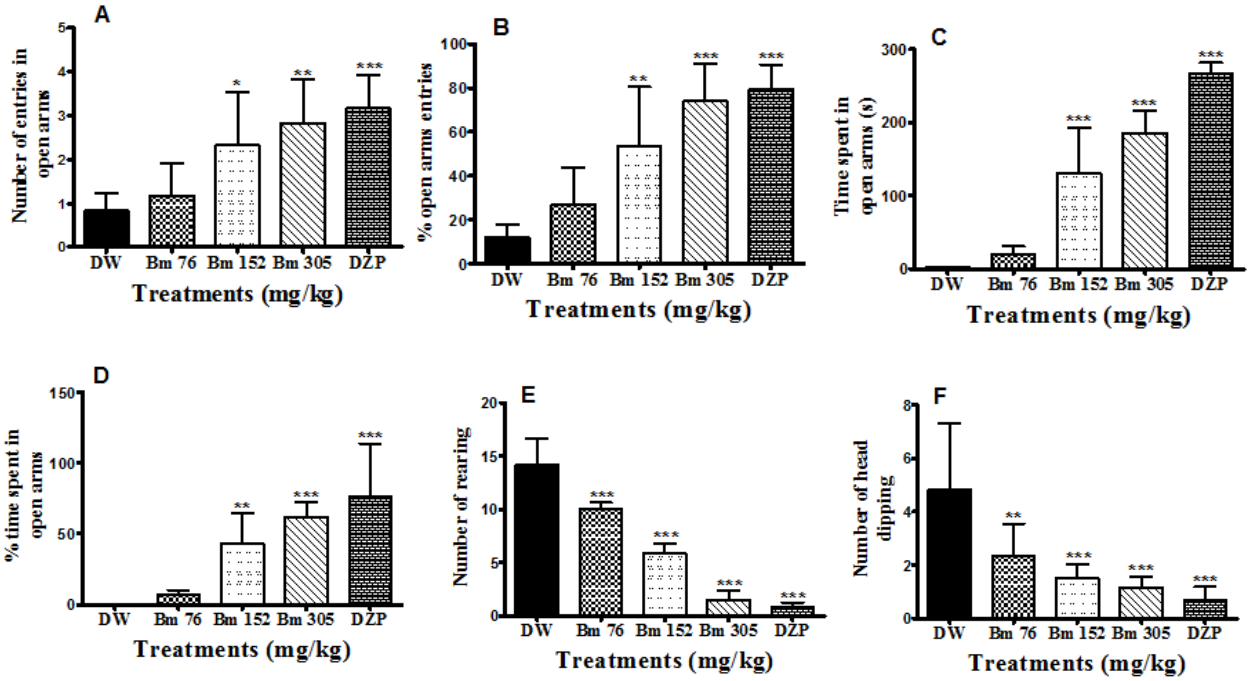


Figure 3: Effects of a single administration of the aqueous extract of *Bridelia micrantha* on the parameters of EPM. (A): open arms entries. (B): percentages of open arms entries. (C): Time spent in the open arms. (D): percentage of time spent on open arms. (E): Number of rearing. (F): Number of head dipping. Data are expressed as mean ± S.E.M, n = 6. *P* values for groups' comparison were obtained by one way ANOVA followed by Dunnet's post-hoc test. **P*< 0.05, ***P*< 0.01, ****P*< 0.001 significantly different as compared to the negative control. DW: negative control group treated with distilled water. 76, 152 and 305: groups treated with of the aqueous extract of *B. micrantha* at the respective dose of 76, 152 and 305 mg/kg, DZP: positive control treated with Diazepam (3 mg/kg).

Effects of single administration of aqueous extract of *B. micrantha* on Open Field Parameters

Figure 4 shows the variations of open field parameters in mice treated with graded doses of the aqueous extract of *B. micrantha*. As shown in figure 4A, the number of crossing significantly increased from 17.33± 0.55 in the negative control group to 30.50 ± 0.56 (P< 0.01), 42.5 ± 3.18 (P< 0.001) and 52.17 ± 2.90 (P< 0.001), in mice treated with the plant extract at the respective doses of 76, 152 and 305 mg/kg. This number was 65.17± 3.19 (P< 0.001) in the positive control group mice receiving 0.3 mg/kg diazepam. The number of grooming and the time spent in centre of the open field (OF) also significantly (P< 0.01) increased compared to the negative control group (Figure 4B & D). The number of rearing significantly (P< 0.001) decreased in mice groups treated with the aqueous extract of *B. micrantha* with peak effect produced at the dose 305 mg/kg with a

value of 3.16 ± 0.31 compared to 15.00 ± 1.46 for negative control. The single administration of different doses (76,152 and 305 mg/kg) of *B. micrantha* extract also elicited a significant (P< 0.001) decreased of the mass of fecal boli (0.13 ± 0.01g, 0.06 ± 0.02g, 0.03 ± 0.02g respectively) compared to negative control group (0.48 ± 0.09).

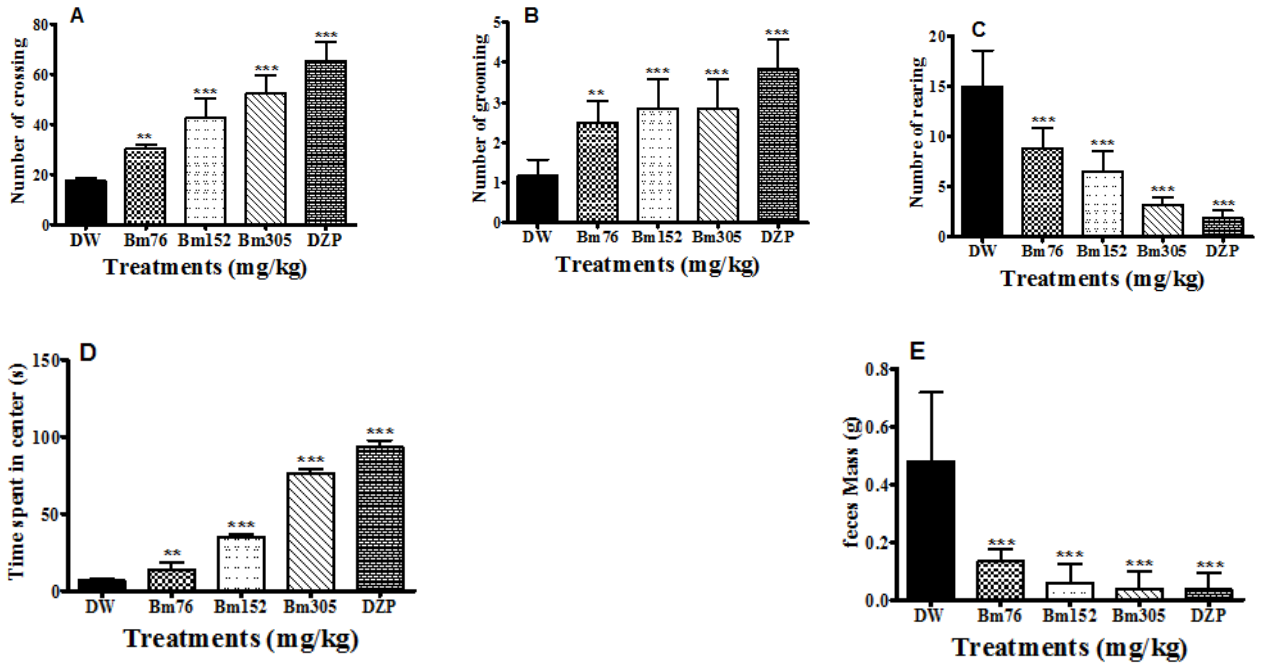
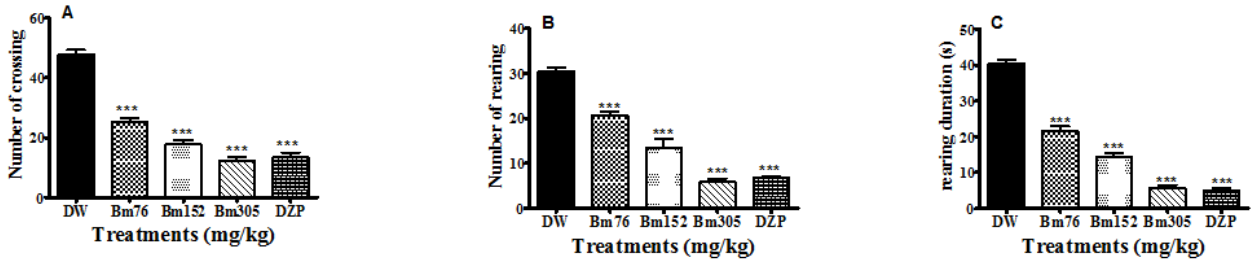


Figure 4: Effects of a single administration of the aqueous extract of *Bridelia micrantha* on Open Field Parameters.
Data are expressed as mean ± S.E.M, n = 6. *P* values for groups' comparison were obtained by one way ANOVA followed by Dunnet's post-hoc test. **P*< 0.05, ***P*< 0.01, ****P*< 0.001 significantly different as compare to the negative control. DW: negative control group treated with distilled water. Bm 76, 152 and 305: groups treated with the aqueous extract of *B. micrantha* at the respective dose of 30, 76, 152 and 305 mg/kg, DZP: positive control treated with Diazepam (0.3 mg/kg). (A): Number of crossing. (B): Number of grooming. (C): Number of rearing. (D): Time spent in center. (E): Feces mass.

B. micrantha aqueous extract significantly and dose-dependently decrease the number of crossing with values being 25.33± 0.55; 17.67 ± 0.61; 12.33 ± 0.42 respectively at dose of 76, 152 and 305 mg/kg, compared to value of negative control group. With the same doses of the plant extract, the number (20.67± 0.33; 13.50 ± 0.80; 5.83 ± 0.30 respectively), and duration of rearing (21.67 ± 0.49s; 14.17 ± 0.47s; 5.66 ± 0.33s respectively) as well as the head dipping first latency (25.83 ± 0.40s; 15.17 ± 0.30s; 8.00 ± 0.37s respectively) was significantly decreased as compare to the negative control group. The number and the duration of head dipping was significantly increased by the treatment with the same doses of *B. micrantha* extract and diazepam compared to the mice of negative control group as shown in figures 5D & E (P <0.01).



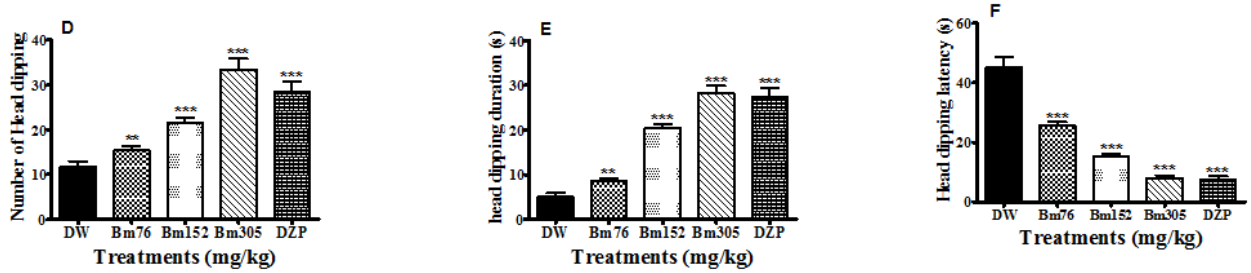


Figure 5: Effects of the aqueous extract of *Bridelia micrantha* on exploratory behavior in mice tested on the hole-board.

Data are expressed as mean ± S.E.M. n = 6. *P* values for groups' comparison were obtained by one way ANOVA followed by Dunnet's post-hoc test. ***P*< 0.01, ****P*< 0.001 significant different compared to the negative control. DW: negative control group treated with distilled water. Bm76, 152 and 305: groups treated with the aqueous extract of *B. micrantha* at the respective dose of 76, 152 and 305 mg/kg, DZP: positive control treated with Diazepam (0.5 mg/kg), (A): Number of crossing. (B): Number of rearing. (C): Rearing duration. (D): Number of head dipping. (E): Head dipping duration. (F): head dipping latency.

Involvement of GABA site of GABA-A receptor complex on the anxiolytic properties of *B. micrantha* in mice using bicuculline

Based on the dose-activity data in EPM from 3 doses (low, medium, and high) of the aqueous extract of *B. micrantha*, we used two of the most effective doses among the three, 152 and 305 mg/kg to investigate antagonism study. The administration of distilled water (DW) followed by 2.5 mg/kg of bicuculline (BIC) resulted in significant (P<0.001) decrease of number of open arms entries, time spent in open arm, percentage of open arms entries and percentage of time spent in open arm in mice, while a contrary effect was observed in mice treated with distilled water and 152 or 305 mg/kg of *B. micrantha* extract compared to the negative control (Table 1). However, the number of rearing and head dipping significantly increased in mice treated with DW+BIC, and decreased in mice receiving DW plus 152 or 305 mg/kg of *B. micrantha* extract compared to the negative control (P< 0.01). The anxiogenic effects induced by bicuculline (2.5 mg/kg) were significantly reversed by *B. micrantha* extract.

Table 1: Effects of bicuculline on anxiolytic properties of aqueous extract of *B. micrantha* in mice placed in EPM.

	DW+ DW	DW+BIC	DW+152	DW+DZP	DW+305	BIC+152	BIC+305	DZP+152	DZP+305
NOAE	4.4 ± 0.2	1.2 ± 0.2***	8.8 ± 0.4***c	10.0 ± 0.3***c	10.6 ± 0.4***c	7.6 ± 0.2***c	8.6 ± 0.4***c	16.8 ± 0.7***c	21.4 ± 1.0***c
NCAE	16.4 ± 1.0	28.0 ± 1.3***	3.6 ± 0.2***c	4.6 ± 0.2***c	1.6 ± 0.2***c	4.8 ± 0.2***c	3.8 ± 0.2***c	2.4 ± 0.2***c	1.0 ± 0.3***c
TSOA	46.0 ± 1.8	17.2 ± 1.2***	197.6 ± 1.2***c	181.2 ± 5.0***c	240.4 ± 6.1***c	162.2 ± 2.4***c	183.8 ± 7.6***c	244.0 ± 8.9***c	344.8 ± 14.2***c
TSCA	94.8 ± 3.5	162.2 ± 2.0***	27.0 ± 1.1***c	22.8 ± 2.0***c	14.2 ± 0.7***c	27.4 ± 2.7***c	35.6 ± 1.5***c	16.4 ± 0.5***c	7.8 ± 0.4***c
REARING	10.8 ± 0.4	15.0 ± 0.5***	6.4 ± 0.4***c	3.0 ± 0.3***c	2.8 ± 0.4***c	7.6 ± 0.5***c	5.0 ± 0.5***c	1.8 ± 0.2***c	1.0 ± 0.3***c
HEAD DIPPING	10.8 ± 0.4	15.2 ± 0.4***	7.2 ± 0.4***c	4.4 ± 0.4***c	4.2 ± 0.5***c	7.8 ± 0.2***c	6.2 ± 0.4***c	3.0 ± 0.3***c	1.0 ± 0.3***c
POAE	21.2 ± 0.8	4.2 ± 0.8***	71.0 ± 1.4***c	68.5 ± 1.4***c	87.1 ± 1.6***c	61.3 ± 1.5***c	69.3 ± 1.8***c	87.62 ± 0.7***c	95.5 ± 1.4***c
PCAE	90.2 ± 0.9	95.8 ± 0.8	28.9 ± 1.4***c	31.5 ± 1.4***c	12.9 ± 1.6***c	38.7 ± 1.5***c	30.7 ± 1.8***c	12.4 ± 0.7***c	4.5 ± 1.4***c
PTSOA	15.3 ± 0.6	5.7 ± 0.4***	65.9 ± 0.4***c	60.4 ± 1.7***c	80.1 ± 2.0***c	54.1 ± 0.8***c	61.3 ± 2.5***c	81.3 ± 3.0***c	114.9 ± 4.7***c
PTSCA	31.6 ± 1.2	54.1 ± 0.7***	9.0 ± 0.4***c	7.6 ± 0.7***c	4.7 ± 0.2***c	9.1 ± 0.9***c	11.9 ± 0.5***c	1.0 ± 0.1***c	0.3 ± 0.1***c

Values are expressed as mean ± SEM, n = 6. *p < 0.05, ***p<0,001 significant difference compared to negative control, a, b and c: significant difference with p <0.05, p <0.01 and p <0.001 respectively compared to bicuculline-treated mice (by one away ANOVA followed by Turkey's multiple comparison post hoc tests). DW+ DW: negative control group of mice treated with distilled water (DW). DW+BIC: mice group treated with distilled water and 2.5mg/kg of Bicuculline (BIC). DW+305: mice group treated with distilled water and 305mg/kg of *B. micrantha* extract. DW+152: mice group treated with distilled water and 152mg/kg of *B. micrantha* extract. BIC+305: mice group treated with and 2.5mg/kg Bicuculline and 305mg/kg of *B. micrantha* extract. BIC+152: mice group treated with 2.5mg/kg of Bicuculline and 152mg/kg of *B. micrantha* extract. NOAE: Number of open arms entries. NCAE: Number of closed arms entries. TSOA: Time spent in open arm. TSCA: Time spent in closed arm. POAE: Percentage of open arms entries. PCAE: percentage of closed arms entries. PTSOA: percentage of time spent in open arm. PTSCA: percentage of time spent in closed arm.

Involvement of Benzodiazepine site of GABA-A Receptor Complex on the anxiolytic properties of *B. micrantha* using FG 7142 and RO 151788

The action mechanism of anxiolytic properties of aqueous extract of *B. micrantha* was assessed, using the inhibition pathway of benzodiazepine site of GABA-A Receptor Complex on the anxiolytic properties of *B. micrantha* by the N-methyl-β-carboline-3-carboxamide (FG 7142) and Flumazenil (RO 151788) in mice placed in EPM. The results are shown in table 2. The administration of distilled water (DW) followed by 3 mg/kg of Flumazenil (RO 151788) or 5 mg/kg of N-methyl-β-carboline-3-carboxamide (FG 7142) induced the significant ($P< 0.001$) decrease of number of open arms entries, time spent in open arm, percentage of open arms entries and percentage of time spent in open arm in mice, while a contrary effect was observed in mice treated with distilled water and 152 or 305 mg/kg of *B. micrantha* extract compared to the negative control. However, the number of rearing and head dipping significantly ($P< 0.001$) increased in mice treated with DW+RO or DW + FG 7142, and decreased in mice receiving DW plus 152 or 305 mg/kg of *B. micrantha* extract compared to the negative control. The anxiogenic effects induced by Flumazenil or N-methyl-β-carboline-3-carboxamide were significantly reversed by *B. micrantha* extract. As shown in Table 2, the treatment of mice with the aqueous extract of *B. micrantha* (152 and 305 mg/kg) preceded by Flumazenil or N-methyl-β-carboline-3-carboxamide significantly increased the number of open arms entries, time spent in open arm, percentage of open arms entries and percentage of time spent in open arm, and decreased the number of rearing and head dipping in mice placed in EPM as compared to RO + DW or DW + β-carb mice groups.

Table 2: Effects of N-methyl-β-carboline-3-carboxamide (FG 7142), of Flumazénil (RO 151788) on anxiolytic properties of aqueous extract of *B. micrantha* in mice placed in EPM.

	DW+DW	DW + RO	DW+β-carb	DW+152	DW+305	RO+152	RO+305	β-carb+152	β-carb+305
NOAE	4.2 ± 0.2	1.2 ± 0.2***	1.8 ± 0.2***	7.0 ±0.3***cf	9.2 ±0.4***cf	5.8 ± 0.2*cf	7.0 ±0.6***cf	6.0 ± 0.3**cf	6.4 ±0.3***cf
NCAE	9.6 ± 0.2	28.8 ±1.7***	24.0 ± 1.6***b	1.6 ±0.2***cf	0.8 ±0.2***cf	2.4 ±0.2***cf	2.0 ±0.5***cf	2.4 ±0.2***cf	1.4 ±0.2***cf
TSOA	44.4 ±2.1	3.4 ± 0.5***	8.6 ± 0.3***	169.0 ±5.8***cf	227.8 ±7.2***cf	151.4 ±1.1***cf	172.8 ±8.7***cf	151.6 ±1.8***cf	171.4 ±2.7***cf
TSCA	60.6 ±2.6	157.8 ±3.1***	209.0 ± 7.1***c	25.6 ± 0.5***cf	9.4 ± 0.5***cf	33.4 ± 2.3***cf	18.2 ± 0.6***cf	32.8 ± 2.6***cf	22.4 ± 0.2***cf
REARING	8.4 ± 0.4	13.0 ± 1.1***	13.0 ± 1.1***	2.2 ± 0.4***cf	1.6 ± 0.2***cf	7.0 ± 0.3cf	4.4 ± 0.2***cf	7.2 ± 0.4cf	6.0 ± 0.3cf
HEAD DIPPING	9.40 ± 0.24	15.40 ±1.50***	13.00 ± 1.27*	1.40 ±0.40***cf	0.60 ±0.24***cf	5.20 ± 0.37**cf	2.40 ± 0.0***cf	5.40 ± 0.24*cf	2.20 ±0.58***cf
POAE	30.4 ±0.9	4.1 ± 0.7***	7.3 ± 1.0***	81.6 ± 2.6***cf	92.1 ± 2.0***cf	76.4 ± 2.4***cf	78.3 ± 4.2***cf	71.5 ± 2.2***cf	82.1 ± 2.9***cf
PCAE	65.0 ± 1.7	95.9 ± 0.7***	92.9 ± 1.0***	18.4 ± 2.6***cf	7.9 ± 2.0***cf	34.1 ± 1.7***cf	21.7 ± 4.2***cf	32.3 ± 1.7***cf	17.9 ± 2.9***cf
PTSOA	14.8 ± 0.7	1.1 ± 0.2***	2.9 ± 0.1***	56.3 ± 1.9***cf	75.9 ± 2.4***cf	50.5 ± 0.4***cf	57.6 ± 2.9***cf	50.5 ± 0.6***cf	57.1 ± 0.9***cf
PTSCA	20.2 ± 0.9	52.6 ± 1.0***	69.7 ± 2.4***c	8.5 ± 0.2***cf	3.1 ± 0.2***cf	11.1 ± 0.8***cf	6,1 ± 0,2***cf	10.9 ± 0.9***cf	7.5 ± 0.1***cf

Values are expressed as mean ± SEM, n = 9. *p < 0.05,P<0.01 ***p<0,001 significant difference compared to negative control, values of p <0.05, p <0.01 and p <0.001 respectively (a, b, c) compared to Flumazenil treated group; (d, e, f) compared to the batch treated with N-methyl-β-carboline-3-carboxamide (by one away ANOVA followed by the Turkey's multiple comparison post hoc tests). DW+ DW: negative control group of mice treated with distilled water (DW). DW+RO: mice group treated with distilled water and 3 mg/kg of Flumazénil (RO 151788). DW+ β-carb: mice group treated with distilled water and 5 mg/kg of N-methyl-β-carboline-3-carboxamide. DW+305: mice group treated with distilled water and 305mg/kg of *B. micrantha* extract. DW+152: mice group treated with distilled water and 152mg/kg of *B. Micrantha* extract. RO+305: mice group treated with and 3 mg/kg of Flumazénil (RO 151788) and 305mg/kg of *B. micrantha* extract. RO + 152: mice group treated with 3 mg/kg of Flumazénil (RO 151788) and 152mg/kg *B. micrantha* extract. β-carb + 305: mice group treated with and 5 mg/kg of N-methyl-β-carboline-3-carboxamide and 305mg/kg of *B. micrantha* extract. β-carb + 152: mice group treated with and 5 mg/kg of N-methyl-β-carboline-3-carboxamide and 152mg/kg of *B. micrantha* extract. NOAE: Number of open arms entries. NCAE: Number of closed arms entries. TSOA: Time spent in open arm. TSCA: Time spent in closed arm. POAE: Percentage of open arms entries. PCAE: percentage of closed arms entries. PTSOA: percentage of time spent in open arm. PTSCA: percentage of time spent in closed arm.**

DISCUSSION

The present study was aimed to assess the anxiolytic like effects of the aqueous extract of *B. micrantha* barks in mice and the possible involvement of GABA-A receptors complex. In the current work we examined, for the first time, the anxiolytic effects of the aqueous extract of *B. micrantha* using the hyperthermia induced-stress, the elevated plus maze test (EPM), the open field test (OF) and the hole-board test. Stress-induced hyperthermia test is often used to identify benzodiazepines like anxiolytic agents well known for their phenobarbital sleep potentiating effects.[34] Therefore, the observed effects of the aqueous extract of *B. micrantha* in the dose finding experiments could as well be due to their modulating effects on the benzodiazepine site of GABA receptors. This plant extract would thus act on the barbiturates receptor sites by extending the opening of voltage-dependent chloride channel to produce the anxiolytic effect.[35,36] Previous studies demonstrated a significant relationship between the anxiolytic drugs and the reduction of body temperature.[36,37] The anxiolytic properties of *B. micrantha* extract were assessed using elevated plus maze (EPM) test, hole-board and open field tests. Elevated plus maze and open field tests are based on spontaneous anxiety and are widely used for screening of anxiolytic activity. It is based on the strong conflict between rodents' proclivity toward dark, enclosed alleys (approach) and an un-conditioned fear of brightly lit areas, heights or open spaces (avoidance). In the present study there is a significant increase in number of entries into, percentage of entries into, time spent into and percentage of time spent in open arms.

The plant extract also significantly elicited the decrease of the numbers of rearing and head dipping in mice compared to the negative control group ($P< 0.001$). The same trend was observed with the positive control group. These results are similar to the findings published by Manavi and Masoumeh, where they demonstrated the anxiolytic properties of *Plumeria rubra* and *Coriandrum sativum* extract respectively using EPM.[24,38,39] In the open field test, forced confrontational situations induce anxiety behaviour in rodents. In such a situation, rodents spontaneously prefer the periphery of the apparatus and enter less in the central parts of the open filed. Indeed, mice and rats walk close to the walls, a behaviour called thigmotaxis.[21] An increase in central locomotion or in time spent in the central part of the device without modification of total locomotion is interpreted as an anxiolytic effect.[27,28] In the present study, *B. micrantha* extract significantly increased the number of crossing and grooming and the time spent in centre like in diazepam treated animals. Moreover, like in the EPM test, the rearing number significantly decreased in mice groups treated with the aqueous extract of *B. micrantha* and diazepam compared to the negative control group ($P< 0.001$). The hole-board test is useful for modelling anxiety in animals, in this test an anxiolytic-like state may be reflected by an increase in head dipping behaviours.[30,31] Our results showed that aqueous extract of *B. micrantha* increased the head dipping corroborating the anxiolytic-like effect previously shown in the EPM and OF tests. Taken together, all these finding suggest the anxiolytic properties of aqueous extract of bark of *B. micrantha*.[34,40] Previous studies on the chemical constituents of *B. micrantha* revealed the presence of alkaloids, anthocyanidin, anthraquinones, carbohydrates, cyanogenic glycoside, essential oil, ester, flavonoids, oxalate, phenolic compounds, saponins, sterols, tannins, terpenoids.[17,18,19,20] The anxiolytic properties of *B. micrantha* may be related to the presence of certain of these compounds in the extracts such as flavonoids, alcaloids, phenolic compounds, saponins and tannins that activate barbiturates, benzodiazepines and/or GABA receptors in the GABA-A receptor complex[41,42,43] and therefore reflect a decrease anxiety.[44,45] Flavanoids have been recently implicated for various pharmacological activities and they have been identified as a new type of ligand with *in vivo* anxiolytic properties. Some natural and synthetic flavonoids have been found to bind specifically and competitively to benzodiazepine receptors and to possess anxiolytic effects.[46,47,48] The GABA-A receptor is known to be a mediator of unconditioned anxiety. In this study, the involvement of GABA receptor in the anxiolytic activity of *Bridelia micrantha* was assessed using Bicuculline as antagonist. Bicuculline-sensitive GABA receptors are part of the super family of Cys-loop pentameric ligand-gated ion channel receptors that include nicotinic acetylcholine, glycine and $5HT_3$ receptors.[49] Bicuculline acts as a competitive antagonist at $GABA_A$ receptors in the fact that it competitively inhibits GABA binding to these receptors.[50] The inhibition of GABAergic neurotransmission way is known as causing anxiety effect while its stimulation leads to the anxiolytic effect.[36,37] Our findings show that anxiogenic activities of bicuculline (decrease of number of open arms entries, time spent in open arm, percentage of open arms entries and percentage of time spent in open arm; increased in the number of rearing and head dipping in mice) were inhibited by the aqueous extract of *B. micrantha* **(**152 and 305 mg/kg). These results suggest that the plant extract would act on Bicuculline-sensitive GABA receptors to induce anxiolytic effect in mice. Since the anxiogenic effect of Bicuculline was not completely reversed by the plant extract, we investigated the involvement in others GABA-Aergic receptors. Flumazenil, (RO 151788), a competitive antagonist of the receptor site of benzodiazepine of GABA-A receptor complex and β-carboline (FG 7142), an inverse agonist of the receptor site of the GABA-A receptor complex of benzodiazepines, have like bicuculline an anxiogenic effect in rodents undergoing the EPM test.[51] The inhibition of anxiogenic activities of these drugs by the plant extract in our study suggest a possible interaction between the GABAergic system and the anxiolytic activities of secondary metabolites of our plant extract.[52,53,54]

CONCLUSION

In summary, our findings show that a single administration of the aqueous bark extract of *B. micrantha* induces anxiolytic effects in the mouse hyperthermia induced-stress, elevated plus maze test, open field test and hole-board test. These anxiolytic effects are still significant in the presence of GABA-A receptor antagonists. Taken together, our results suggest that *B. micrantha* has anxiolytic-like effects mediated by regulation of the GABA-A Benzodiazepine receptor complex.

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