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ANXIOLYTIC ACTIVITY OF AQUEOUS EXTRACT OF *BRIDELIA MICRANTHA* (HOCHST) BAILL (EUPHORBIACEAE) IN MICE EXPOSED TO CHRONIC IMMOBILIZATION STRESS

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ABSTRACT

Anxiety disorders are a major public health concerning worldwide. The current anxiolytics are ineffectives and associated with major side effects. Thus, medicinal plants with better effects constitute source of new pharmaceutical drugs. This work aimed to assess the anxiolytic-like properties of aqueous bark extract of *Bridelia micrantha* on behavioral and neurochemical characteristics, using male mice subjected to chronic immobilization stress. The anxiolytic properties of *B. micrantha* were assessed in mice after repeated stress by immobilization. Mice received distilled water (10 ml/kg, p.o), diazepam (2 mg/kg, i.p) or *B. micrantha* extract (76, 152 or 305 mg/kg, p.o) one hour prior to daily exposure to stress by immobilization in the adapted plastic cone (3h/day) for 14 days. The behavioral parameters were evaluated using Elevated Plus Maze (EPM), Open Field (OF) and Hole-Board (HB) tests. The plasma levels of serotonin and corticosterone were determined by ELISA technique. Like diazepam, the extract increased ($p<0.001$) the number of open arms entries and the time spent in open arms of the EPM. The extract elicited the increase of crossing and grooming number, and the time spent in centre of the of. On the HB, the extract increased ($p< 0.001$) the number of head dipping and decreased the number of

crossing. The extract increased ($p<0.001$) plasma serotonin levels and decreased corticosterone levels. The chronic restraint stress-induced behavioral and neurotransmitters levels abnormalities were attenuated by the extract. These overall results suggest that aqueous bark extract of *B. micrantha* might have a promising therapeutic potential for stress-related disorders.

KEYWORDS: Chronic Immobilization Stress, Anxiety, *B. Micrantha*, Serotonin, Corticosterone.

INTRODUCTION

Acute and chronic stresses are characterized by the physiological changes that occur in response to novel or threatening stimuli. Chronic stress has been linked to the pathophysiology of mood including anxiety disorders and depression.[1] Mental Stress is defined as the non-specific response of the body to any demand imposed upon it.[2] Stress is known to alter the physiological homeostasis of the organism and complex mechanisms contribute to the breakdown in adaptation processes resulting in various visceral, endocrinal, and behavioral changes.[3] The neuroendocrine damages in response to acute and chronic stresses are mediated by both the sympathetic nerve system and the hypothalamus-pituitary-adrenal (HPA) axis, which lead to the regulation of the release of dopamine, norepinephrine, serotonin, glutamate, and corticotropin-releasing and adrenocorticotropic hormones in central nervous system (CNS) and the secretion of glucocorticoids in plasma.[4] Cortisol and corticosterone are thus often used as biomarkers for stress, depressive and anxiety disorders.[5] Stress plays the main role in pathogenesis of mental disorders.[6,7] A host of chronic psychiatric disease states like melancholic depression, anorexia nervosa, panic disorders, anxiety disorders and cognitive dysfunction have been reported to involve abnormality of stress axis.[8] Restraint stress can induce a series of dysfunctions of central nervous system, such as cognitive impairment, anxiety, depression, amnesia, and insomnia. A number of chronic stress models, including electric footshock (EF) stress, forced swimming, noise stimulus and immobilization, have been employed to induce anxiety disorders in the past decades.[9] However, elevated plus-maze is a widely used test, which is based on the natural aversion of rodents to heights and open spaces, and which has been validated to be suitable for the assessment of anxiety disorders for both mice and rats.[10] As another characteristic behavior of the anxiety state of animals, the open field and hole-board tests are also used as a screening model for the detection of anxiolytics.[10,11] Anxiety disorders in particular, affect 1/8 th of total population worldwide and have become one of important

research interest in psychopharmacology during this decade.[12] The hallmark of anxiety disorders is marked, persistent and excessive or unreasonable fear that is experienced to a degree that significantly interferes with everyday life.[13] Chronic stress and chronic stress-induced anxiety disorders have become increasingly more important public health concerns in recent years. However, the ranges of available pharmacotherapy like the benzodiazepines for the treatment of anxiety disorders induced by chronic stress are limited and suboptimal with regard to efficacy and tolerability.[14] A search for novel pharmacotherapy from medicinal plants for psychiatric illnesses has progressed significantly in the past decade. This is reflected in the large number of herbal preparations for which psychotherapeutic potential has been evaluated in a variety of animal models.[15] Several plants have proven anxiolytic-like effects in animal models.[16,17,18] *Bridelia micrantha* (Hochst.) Baill. is a small to medium sized tree belonging to the family Phyllanthaceae (formerly Euphorbiaceae), commonly known as mitzeerie or coastal golden leaf.[19] The following activities have been reported from *B. micrantha*: anthelmintic, antibacterial, anticonvulsant and sedative, antidiabetic, antidiarrhoeal, antifungial, anti-Helicobacter pylori, antimycobacterial, antinociceptive, antioxidant, antiplasmodial, antischistosomal, antiviral, hepatoprotective, insecticidal, β-lactamase inhibitor.[19] Thus it is not surprising that the bark, leaves and roots of *B. micrantha* are widely used as herbal medicines in tropical Africa.[19] The powdered bark is used for diseases of the central nervous system like epilepsy and insomnia in Cameroon.[19,20] 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*.[19,21] The aim of the present study was to evaluate the neuropharmacological activities of aqueous bark extract of *B. micrantha*, in an experimental model of chronic stress-induced anxiety disorders by investigating the behavioral parameters, using the elevated plus-maze, open field and hole-board tests, and determining its influences on the levels of serotonin and corticosterone in the plasma.

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 (voucher specimen Nº 9678/SRF/Cam) at the National Herbarium of Cameroon in Yaoundé.

Animals

Adult male Swiss albinos mice weighing 20-25g and about 2-3 months from our breeding stock (Animal house of the laboratory of Animal Physiology of the University of Yaoundé I) were used in this study. The animals were housed at 25±3°C with 12:12 h light and dark cycle. They had free access to food and water. The animals were acclimatized for a period of seven days before the study. All animal handling procedures were done in accordance with National Ethic Guidelines (FWA-IRB00001954), and the experiments designed to minimize the number of animals used and to minimize their suffering.

Chemicals

Diazepam was obtained from Roche (France). Serotonin and Corticosterone kits were purchased from IBL INTERNATIONAL GMBH (Hamburg, Germany).

Methods

Preparation of the aqueous extract

The aqueous extract 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 in 1l of distilled water for 1 hour. The mixture was boiled for 20 min. After cooling, the supernatant was collected and filtered using Whatman filter paper Nº1. In another experiment the filtrate was then evaporated to dryness using an oven at 45°C giving aqueous extract with a 6.1% yield.

Experimental Design

The aqueous extract of *Bridelia micrantha* or diazepam in distilled water was freshly prepared before being administered. On group made up of animals unstressed, received distilled water. All the treatments were given once daily for two weeks and the animals were subjected to immobilization stress on the first day (3 hours/day) until the fourteenth day. In this study all the animals were randomly divided into six groups of five mice for the each behavioral analysis. The following protocol was used: Group I: unstressed animals (normal control); Group II: stressed animals (negative control), daily oral administration of distilled water; Group III-IV-V: stressed animals, treated with the aqueous extract of *B. micrantha* at 76, 152 or 305 mg/kg respectively; Group VI: stressed animals, treated with standard reference drug diazepam (2 mg/kg, i.p).

Stress Procedure

The animals were subjected to stress by repeated immobilization which consisted of a complete immobilization of the mice inside the 50 ml conical tubes (cylinder 3 cm in diameter and 8 cm in height). The mice were restrained for 3 hours/day without access to water and food. Stress was applied to animals for 14 consecutive days. The different treatments were administered 1 hour before immobilization. On the fifteenth day, the mice were treated with drugs or extract, and subjected to behavioral studies on the elevated plus maze (EPM), Open field (OF) and Hole board (HB). One hour after behavioral completion, they were returned at their initial cage until the sacrifice. The blood was withdrawn from the jugular vein, collected in the EDTA tubes and centrifuged to separate plasma from the erythrocytes at 3,000 rpm for 20 min at 4°C. Plasma was transferred to 1.5 ml Eppendorf vials and kept at -20°C. Post-stress corticosterone and serotonin were measured in the plasma by an enzyme-linked immunosorbant assay using the corticosterone and serotonin ELISA kits in accordance with the manufacturer's protocol (IBL INTERNATIONAL GMBH, Hamburg Germany). The resulting concentration of plasma corticosterone and serotonin were expressed as ng/mL using prepared corticosterone and serotonin standards.

Behavioral analysis

Elevated plus-maze test

Anxiety was evaluated in an elevated plus maze. The wooden apparatus, consisted of two open arms (16×5cm each), two enclosed arms (16×5cm×10cm; each) and a central plat form (5×5cm) arranged in such a way that the two arms of each type were opposite to each other. The maze was elevated 50cm above the floor. The experimental procedure was similar to that described by Pellow.[22] After the induction of chronic immobilization stress (14 days), on the 15th day the mice were placed in the centre of the elevated plus maze facing one of the open arms. During the 5 min test period the number of open and closed arms entries, the times spent in open or closed arms, rearing and head dipping were recorded with stop watches. An entry was defined as all four feet into one arm. An increase in open arms entries and increase in time spent in open arms were accepted as the measures of potential anxiolytic activity. The apparatus was carefully cleaned with 10% ethanol solution after every passage of mouse. All test sessions were taped by using a video camera (Panasonic HC- V385, 100 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.[23] The open field test has been employed to assess the spontaneous activity, general exploration and ambulation of the rodents. After the induction of chronic immobilization stress (14 days), the 15th day each mouse was placed individually in the centre of the apparatus and observed for 5 min to record its locomotor activity parameters (the number of line crossings), exploratory activity (indicated by frequency of rearing) and time spent in the center.[24] The apparatus was carefully cleaned with 10% ethanol solution after every test. All test sessions were taped by using a video camera (Panasonic V385, 100 Mega pixel).

Hole-board test

The hole-board apparatus was used to determine a high-anxiety-like state in mice. 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. 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 the induction of chronic immobilization stress (14 days), the 15th day each animal was placed in the centre of hole board and allowed to freely explore the apparatus for 5 min. Mouse behavior was continuously videotaped by a digital video camera (Panasonic V385, 100 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-board.[25] The apparatus was carefully cleaned with 10% ethanol solution after every test.

STATISTICAL ANALYSIS

All the results were expressed as mean ± SEM. All statistical analysis was done using one way analysis of variance (ANOVA) followed by the Dunnett's post hoc test. $p<0.05$ was considered as significant when compared to their respective control group.

RESULTS

Effects of *Bridelia micrantha* on the CIS-induced anxiety in the Elevated plus maze (EPM): The results indicated that in the elevated plus maze **(EPM)**, the chronic restraint stress (3h/day for 14 consecutive days) induced a significant ($p <0.001$) reduction of the number of open arms entries, time spent in open arms, percentage of open arms entries and percentage of time spent in open arms as it is reported in Table 1. The post treatment of *B. micrantha* aqueous extract (76, 152 and 305 mg/kg) reversed the chronic restraint stress-

induced anxiety by increasing the number of open arms entries (6.80 ± 0.86; p<0.05, 10.40 ± 1.43; p<0.001 and 13.80 ± 1.86, p<0.001, respectively), the time spent in open arms (66.60 ± 4.37s ; 171.80 ± 6.85s and 209.60 ± 5.35s ; p<0.001, respectively), the percentage of open arms entries (55.63%; 64.76% and 79.90%; p<0.001, respectively) and the percentage of time spent in open arms (22.20%; 57.27%; and 69.87%; p<0.001) compared to the negative control group (1.40 ± 0.24; 2.40 ± 0.40s, 7.69% and 0.80%, respectively). Standard drug diazepam also increased (p<0.001) the number of open arms entries, time spent in open arms, percentage of open arms entries and percentage of time spent in open arms. However, 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 diazepam group. The aqueous extract of *B. micrantha* like diazepam significantly induced reduction (p<0.001) in the number of closed arms entries, time spent in closed arms, the percentage of closed arms entries and the percentage of time in closed arms (Table1).

Table. 1: Effects of *Bridelia micrantha* on the CIS-induced anxiety in the EPM.

	NC	CIS+ DW	CIS + 76	CIS + 152	CIS + 305	CIS + DZP
Noae	6.06 ± 0,51	1.40 ± 0.24$^{\mu}$	6.80 ± 0.86*	10.40 ± 1.43***	13.80 ± 1.86***	14.20 ± 1.32***
Ncae	9.80 ± 0.58	17.00 ± 0.71$^{\mu}$	5.80 ± 1.39***	5.40 ± 0.40***	3.20 ± 0.37***	2.80 ± 0.37***
Tsoa	68.60 ± 7.27	2.40 ± 0.40$^{\mu}$	66.60 ± 4.37***	171.80 ± 6.85***	209.60 ± 5.35***	177.60 ± 9.45***
Tsca	99.00 ± 4.30	153.40 ± 2.42$^{\mu}$	95.60 ± 3.22***	26.60 ± 0.93***	12.80 ± 0.66***	14.20 ± 0.97***
Rearing	8.20 ± 0.37	23.80 ± 0.58$^{\mu}$	11.60 ± 1.43***	7.20 ± 0.37***	2.80 ± 0.37***	3.40 ± 0.40***
Head diping	7.80 ± 0.58	27.00 ± 1.70$^{\mu}$	8.00 ± 0.55***	6.80 ± 0.73***	2.20 ± 0.80***	3.80 ± 0.37***
Poae	37.20 ± 1.24	7.69 ± 1.43$^{\mu}$	55.63 ± 9.40***	64.76 ± 4.12***	79.90 ± 3.83***	83.70 ± 0.80***
Pcae	62.80 ± 1.24	92.31 ±1.43$^{\mu}$	44.37 ± 9.40***	35.24 ± 4.12***	69.87 ± 1.79***	16.30 ± 0.80***
Ptsoa	22.87 ± 2.42	0.80 ± 0.13$^{\mu}$	22.20 ± 1.45***	57.27 ± 2.28***	69.87 ± 1.79***	59.20 ± 3.15***
Ptsca	33.00 ± 1.43	51.13 ± 0.87$^{\mu}$	31.87 ± 1.07***	8.87 ± 0.31***	4.27 ± 0.22***	4.73 ± 0.32***

All values are mean ± SEM, n = 6. *P* values for groups' comparison were obtained by one way ANOVA followed by Dunnett's post-hoc test. *p<0.05, ***p<0.001 significant difference compared to negative control **(CIS+DW)**; μp<0.001 vs. normal control. **NC**: normal control. **CIS+ DW**: negative control group of mice treated with distilled water (DW) and subjected to cis. **CIS+76**: mice group treated with 76mg/kg of *B. micrantha* extract and subjected to **cis**. **CIS+152**: mice group treated with 152mg/kg of *B. micrantha* extract and subjected to **cis** . **CIS +305**: mice group treated with 305mg/kg of *B. micrantha* extract and subjected to **cis**. **CIS+DZP**: mice group treated with 2mg/kg of diazepam and subjected to

cis. 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. **CIS:** chronic immobilization stress.

Effects of *Bridelia micrantha* on the CIS-induced anxiety in the Open Field (OF)

The chronic restraint stress led to a significantly ($p<0.001$) decrease of the numbers of crossing and grooming as well as the time spent in centre compared to normal control group (Fig 1 A, B & D). The stress was accompanied by an increase of the number of rearing and the mass of fecal boli (Fig 1 C & E). The administration of aqueous extract of *B.micrantha* (76, 152, and 305 mg/kg) attenuated the restraint stress effects on the open field behavior. As shown in figure 1A, the number of crossing significantly increased from 8.40 ± 0.51 in the negative control group to 17.60 ± 0.68 ($p< 0.05$), 20.20 ± 1.02 ($p<0.01$) and 74.00 ± 1.79 ($p<0.001$), in mice treated with the plant extract at the respective doses of 76, 152 and 305 mg/kg. This number was 70.60 ± 2.77 ($p<0.001$) in the positive control group mice receiving 2 mg/kg of diazepam. At the dose 152 and 305mg/kg, the number of grooming (2.20 ± 0.20; $p<0.01$, 3.40 ± 0.24; $p<0.001$ respectively) and the time spent in centre (62.80 ± 2.50s; $p<0.001$, 90.80 ± 1.66s; $p<0.001$) of the open field (OF) also significantly increased compared to the negative control group (1.00 ± 0.00; 4.20 ± 0.37) (Fig 1B & D). The chronic restraint stress also resulted to a significant increase ($p<0.05$) of the number of rearing and feces mass. The number of rearing significantly decreased in mice groups treated with the aqueous extract of *B. micrantha* (13.20 ± 0.58; $p<0.01$, 13.20 ± 0.58; $p<0.01$, 3.20 ± 0.37; $p<0.001$ respectively at the dose 76,152 and 305 mg/kg). The diazepam caused a more significant decrease in the number of rearing (2.00 ± 0.32; $p<0.001$) compared to negative control (16.20 ± 1.02) (Fig 1C). The chronic administration of *B. micrantha* aqueous extract (76,152 and 305 mg/kg) also elicited a significant decreased of the mass of fecal boli (0.57 ± 0.01g; 0.53 ± 0.01g; $p<0.01$, 0.09 ± 0.02g; $p<0.001$, respectively) compared to negative control group (0.64 ± 0.02g). Similarly, diazepam significantly ($p<0.001$) decrease the mass of fecal boli in stress induced groups compared to distilled water treated mice (Fig 1E).

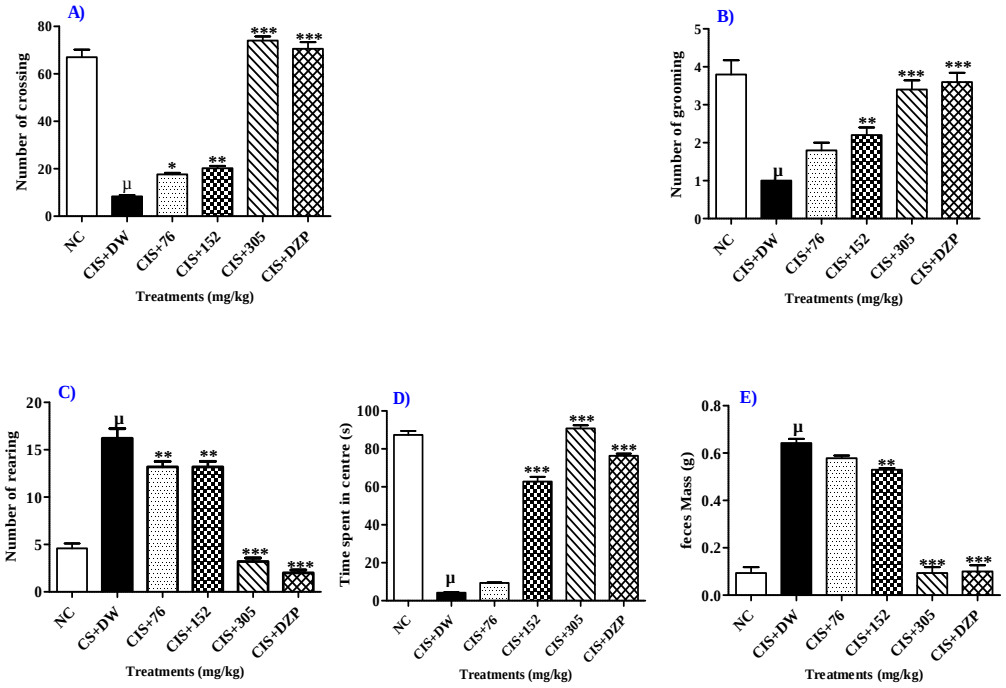

Figure. 1: Effects of *Bridelia micrantha* on the CIS-induced anxiety in the Open field Data are expressed as mean ± S.E.M, n = 6. *P* values for groups' comparison were obtained by one way ANOVA followed by Dunnet's post-hoc test. NC: Normal control.

CIS+DW: Negative control group of mice treated with distilled water (DW) and subjected to cis; **CIS+76:** mice group treated with 76 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+152:** mice group treated with 152 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+305:** mice group treated with 305 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+DZP:** mice group treated with 2mg/kg of diazepam and subjected to cis. **µ**: $p<0.001$ vs **NC**; ** $p<0.01$, ******* $p< 0.001$ significant difference compared to the negative control (CIS+DW). **CIS**: chronic immobilization stress**).** (A): number of crossing. (B): number of grooming. (C): number of rearing. (D): time spent in center. (E): feces mass.

Effects of *Bridelia micrantha* on the CIS-induced anxiety in the hole-board

Restraint stress significantly ($p<0.001$) decreased the number of head dipping, the duration of head dipping and increased the head dipping first latency, the number of crossing, the number and duration of rearing in stress-induced mice group when compared to normal groups. Post administration of *B. micrantha* aqueous extract or diazepam (2mg/kg), attenuated the restraint stress effects on the hole-board behavior. The aqueous extract of *B. micrantha* significantly and dose-dependently decreased the head dipping first latency with values being 25.20 ± 0.86s; ($p<0.01$), 15.20 ± 0.86s; ($p<0.001$) and 6.20 ± 0.73s ($p<0.001$) respectively at dose of

76, 152 and 305 mg/kg, against (32, 40 ± 2,50s) for the negative control group. With the same respective doses of plant extract, the number (25.40 ± 0.75; ($p<0.05$), 17.40 ± 1.07; ($p<0.001$), 7.60 ± 0.51; ($p<0.001$)) and duration (40.60 ± 1.36s; ($p<0.05$), 13.80 ± 0.97s; ($p<0.001$), 4.20 ± 0.73s ($p<0.001$)) of rearing, as well as the number of crossing (43.80 ± 1.77; 24.00 ± 1.41; ($p<0.001$), 8.40 ± 0.75($p<0.001$) was significantly decreased compared to the negative control group (29.80 ± 1.59; 44.60 ± 1.63s; 46.60 ± 2.16). The number (10.20 ± 0.58; ($p<0.01$), 15.40 ± 1.12; ($p<0.001$), 35.60 ± 2.30; ($p<0.001$)) and the duration of head dipping (6.40 ± 0.75s, 12.40 ± 0.93s, 31.20 ± 1.11s; ($p<0.001$)) significantly increased with the graded doses of *B. micrantha* extract compared to the mice of negative control group (4.80 ± 0.37; 3.80 ± 0.37s) as shown in figures 2B &C.

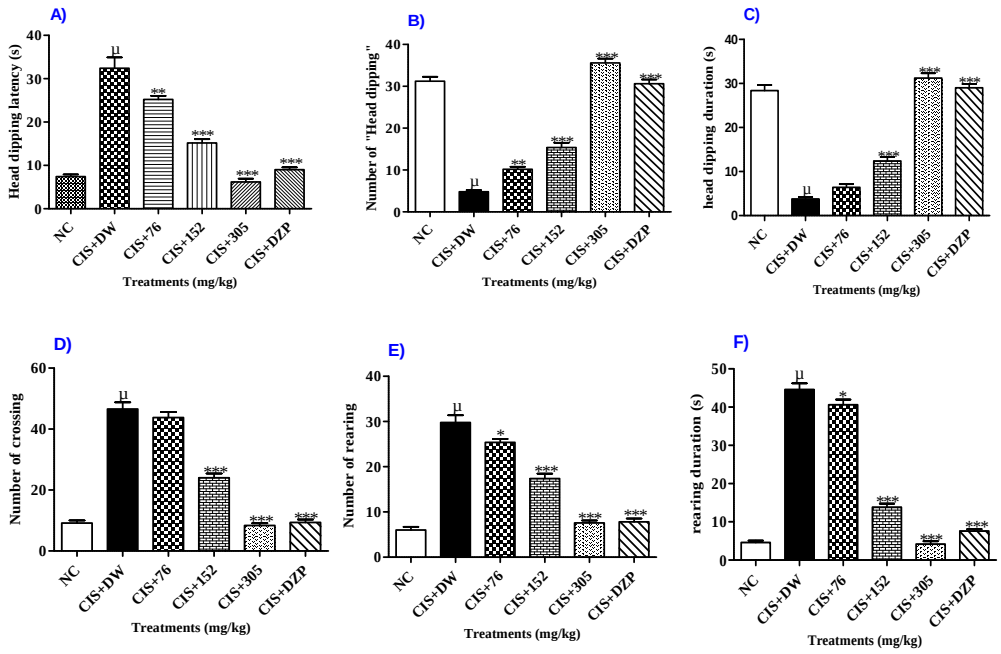

Figure. 2: Effect of chronic treatment of *Bridelia micrantha* on CIS-induced anxiety in the hole-board.

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. **NC**: Normal control; **CIS+DW:** Negative control group of mice treated with distilled water (DW) and subjected to cis; **CIS+76:** mice group treated with 76 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+152:** mice group treated with 152 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+305:** mice group treated with 305 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+DZP:** mice group treated with 2mg/kg of diazepam and subjected to cis. **µ**: $p<0.001$ vs

NC; *p<0.05, ** p<0.01, ******* p< 0.001 significant difference compared to the negative control (CIS+DW). **CIS**: chronic immobilization stress. (A): head dipping first latency. (B): number of head dipping. (C): head dipping duration. **(**D): number of crossing. (E): number of rearing. (F): rearing duration.

Effects of aqueous extract of *Bridelia micrantha* on the plasma levels of serotonin (A) and corticosteron (B) in mice induced immobilization stress

Restraint stress group showed significant (p<0.001) decrease in the level of plasma serotonin when compared with normal control group. Groups treated with *Bridelia micrantha* (76, 152 and 305 mg/kg) and diazepam (2 mg/kg) along with the restraint stress showed significant (p<0.001) increase in the levels of serotonin (299.1 ± 47.3 ng/ml, 342.7 ± 55.0 ng/ml and 370.4 ± 15.08ng/ml, respectively for graded doses of the extract; and 358.5 ± 31.4 ng/ml for Diazepam) when compared with restraint stress group (68.84 ± 5.71 ng/ml). The higher level of corticosterone in negative control group (163.7 ± 19.9 ng/ml) compared to normal control (92.18 ± 8.5 ng/ml) (p<0.001) was significantly (p<0.001) reduced in mice groups that received *B. micrantha* (108.0 ± 6.0 ng/ml and 92.3 ± 5.1 ng/ml for doses 152 and 305 mg/kg respectively) and diazepam (90.6 ± 6.1 ng/ml) along with the restraint stress (figures 3A & B).

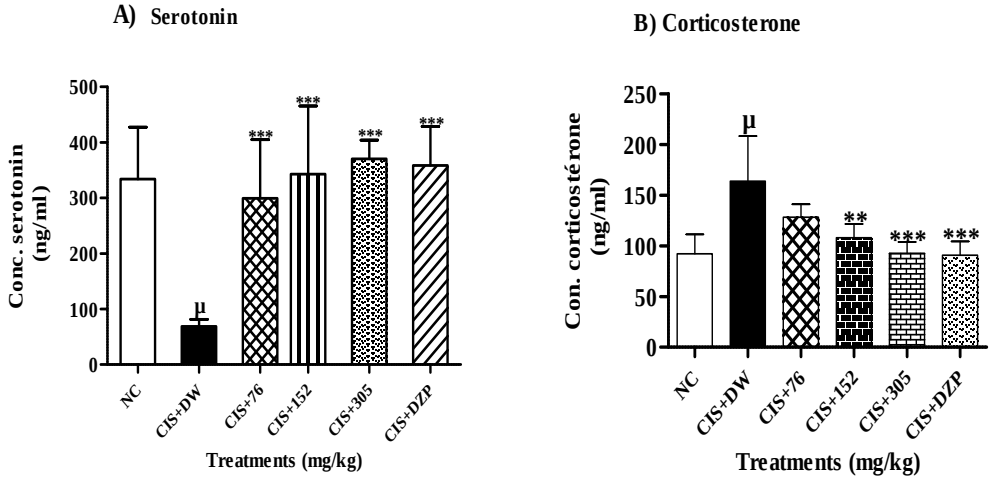

Figure. 3: Effects of aqueous extract of *Bridelia micrantha* on the plasma levels of serotonin (A) and corticosteron (B) in mice induced immobilization stress.

Results are expressed as mean ± S.E.M; n=6. *P* values for groups' comparison were obtained by one way ANOVA followed by Dunnett's post-hoc test. **NC**: Normal control; **CIS+DW:**

Negative control group of mice treated with distilled water (DW) and subjected to cis; **CIS+76:** mice group treated with 76 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+152:** mice group treated with 152 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+305:** mice group treated with 305 mg/kg of *B.micrantha* extract and subjected to cis; **CIS+DZP:** mice group treated with 2mg/kg of diazepam and subjected to cis. **µ**: $P^{< 0.001}$ vs. NC; ** p<0.01, ******* p< 0.001 significant difference compared to the negative control (CIS+DW). **CIS**: chronic immobilization stress.

DISCUSSION

The increase of corticosterone levels in the stressed mice indicates that, stress is able to activate the HPA axis and change the catecholamine, GABA and serotonin levels. Stress thereby involves activation of HPA axis.[26] It is well known that, the exposition to chronic immobilization stress of animals or psychological stress in humans is implicated in the pathophysiology of anxiety and mood disorder.[27] Immobilization stress, both acute and chronic, has affected motor activity, anxiety-like behavior, and depression-like behavior in animals.[28] The present findings demonstrated that mice exposed to CIS for 14 days showed anxiety-like behavior and exhibited an increase in corticosterone and a decrease in serotonin. The behavioral changes observed in this study might be due to the alterations in the brain regions that control motor activity and anxiety-like behavior. Aqueous extract of *Bridelia micrantha* (76, 152 or 305 mg/kg) provided significant protection against immobilization stress. The HPA axis activity can be regulated by monoamines.[29,30] Generally the monoamieric level varies among brain regions such as the hippocampus, hypothalamus, prefrontal cortex and amygdale in stress conditions.[31,29,30] In this study, for the further precise anxiolytic functions of aqueous extract, the effects of *Bridelia micrantha* on chronic immobilization stress-induced anxiety disorders were investigated by evaluating the elevated plus-maze, open field and hole board tests, as well as the levels of serotonin and corticosterone in mice as an animal model system. The elevated plus-maze test is used to evaluate anxiety disorders, utilizing the natural fear of rodents for open and elevated places.[22,32] Mice normally prefer to spend much of their allotted time in the closed arms of the model system (as a more secure location), reflecting an aversion toward the open arms, that is generated by a fears of the open spaces.[22,10] Our results indicate that in the open arms, the number of entries, the time spent and their respective percentages significantly increased in the chronic restraint stressed mice, in the presence of the grade doses (76,152 and 305 mg/kg) of aqueous extract of *Bridelia micrantha* and were comparable to the effects of

diazepam a recognized anxiolytic. On the contrary the aqueous extract of *Bridelia micrantha* significantly decreased the percentage of closed arms entries and time spent. Any increased activity in open arms indicates a decreased anxiety level.[15,32,33] Also, a decrease of these behavioral parameters in the closed arms indicates a reduction of stress level.[32,34,35] These results show the anxiolytic-like activity of the aqueous extract of *Bridelia micrantha*.[15,32] Diazepam is referred to as an anxiolytic in humans and causes decrease in anxiogenic-like. Several studies have reported that diazepam at anxiolytic dose facilitates exploratory behavior which is expressed as increased locomotion in the elevated plus maze.[15,34,36] Similarly in the open field test, restraint stress induced behavioral alterations as evidenced by increase in number of rearing and decrease in number of crossing and time spent in centre.[15,34,37] Post treatment with aqueous extract of *Bridelia micrantha* (76, 152, and 305 mg/kg) and diazepam (2mg/kg) produced reversal behaviors of stress, standard drug and plant extract decreased in number of rearing and increased in number of crossing and time spent in centre. These results corroborate with the anxiolytic activity observed in the elevated plus maze (EPM).The anxiolytic-like activity was also observed in the Hole-board test indicated that the head-dipping behavior was sensitive to changes in the emotional state of the animal, and suggested that the expression of an anxiolytic state may be reflected by an increase in head-dipping behavior.[15,34,37] In our study, aqueous extract of *Bridelia micrantha* increased the numbers and duration of head dipping poking compared to the stress induced group.[15] In order to further corroborate the anxiolytic activity observed in the elevated plus maze, open field and hole-board tests, we also assessed the stress markers levels. Our results showed that stressed mice exhibited anxiogenic behavior associated to reduction of plasma serotonin concentrations and the increased plasma corticosterone levels. This reveals that the mice underwent stress and the alteration observed is similar to clinically related pathophysiology of anxiety.[38,39,40,41] Administration of the aqueous extract of *Bridelia micrantha* during stress period restored the exploratory behavior of mice. The results showed that stressed mice treated with the aqueous extract of *Bridelia micrantha* had corticosterone level significantly reduced almost to the normal values.[32,42] The HPA axis is made up of an assembly of stress responses mediated by the brain, pituitary, and adrenal gland. The endocrine activity of the hypothalamus causes the production of the corticotrophin releasing factor (CRF), a compound that stimulates the production of adrenocorticotropic hormone (ACTH). ACTH is liberated into the circulatory system, and causes the adrenal cortex to secrete corticosteroid hormones, particularly cortisol. Cortisol increases the availability of refueling the body with substances necessary for the body's response to stress.[43] These

substances levels were reversed and were returned to more normal value in aqueous extract of *Bridelia micrantha* treated stressed mice, suggesting that the aqueous extract of *Bridelia micrantha* showed anxiolytic properties as it was demonstrated in our previous work.[15] The present study also revealed that, CIS resulted in a significant decreased of serotonin levels when compared to the normal control and treated groups. These findings are similar to the results obtained by MairairaVeronique[32] in the analogous studies with *Senna. singuena.* The aqueous extract of *Bridelia micrantha* is rich in polyphenols, flavonoids, tannins, tripertenes [19], or other secondary metabolites that may support the anxiolytic activity of the plant.[44] The increase of serotonin suggests that this mediator can suppress the anxiety-like behavior of mice via attenuation of glutamineric transmission in increment of the release of GABA with a decrease in excitation of glutamatergic projections.[15,34]

CONCLUSION

The present study demonstrated that the aqueous extract of *B. micrantha* has a stress-attenuating effect in mice subjected to CIS. This effect may be mediated at least in part by its ability to decrease corticosterone and increase serotonin. Further experimentations are needed to better understand the action mechanism of this plant extract on chronic stress and to provide benefits for its effective use in therapeutic purposes.

AUTHORS' CONTRIBUTIONS

DMZM (Ph.D student), JPOO (Ph.D), AKK (Ph.D), FNT (Ph.D) wrote and revised the article. ENB and TD were responsible for the project concept and supervision of the study, as well as the writing and review of the manuscript.

CONflICTS OF INTEREST

The authors declare no conflict of interest.

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