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Central Inhibitory and Mechanism of Anxiolytic Potential of Ethanol Leaf Fractions of *Milicia* Excelsa C. C. Berg (Moraceae) in Mice

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Abstract

Milicia excelsa has been demonstrated to possess anxiolytic potential in earlier study. This study aimed to investigate the central inhibitory and anxiolytic effects of its fractions in mice. The n-hexane (HF), ethyl acetate (EF), n-butanol (BF) and aqueous (AF) fractions of the ethanol leaf extract were investigated using novelty-induced behaviours (rearing and locomotion) while the anxiolytic effects were assessed using hole board test (HBT), elevated plus maze (EPM) and staircase models (SCM) at 250, 500 and 1000 mg/kg in mice. The neural mechanism of anxiolytic effect of the most active fraction was also investigated using flumazenil (3 mg/kg, i.p.) and cyproheptadine (0.5 mg/kg, i.p.). The EF and AF significantly ($P < 0.05$) reduced rearing and locomotion compared to control suggestive of central inhibitory actions. Similarly, EF and AF significantly ($P < 0.05$) reduced head dip in HBT compared to control indicative of sedative effects. All the fractions significantly ($P < 0.05$) reduced open arm avoidance indices (OAAI) compared to control consistent with anxiolytic potentials. The AF significantly ($P < 0.05$) reduced the number of rearing in SCM compared to control suggestive of anxiolytic property. Flumazenil significantly ($P < 0.05$) reversed the anxiolytic effect of AF implicative of the involvement of GABAergic pathways in its anxiolytic actions. However, cyproheptadine did not show any significant ($P < 0.05$) reversal of the anxiolytic effect of AF suggesting noninvolvement of serotonin in the anxiolytic effect of AF. This study therefore, concluded that the fractions of *Milicia excelsa* leaf extract may possess central inhibitory and anxiolytic effects and its mechanism may involve $GABA_A$ benzodiazepine pathway.

Keywords: Aqueous fraction, central inhibitory effect, anxiolytic, $GABA_A$ benzodiazepine receptor.

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Introduction

Anxiety is one of the most leading mental illnesses in the world (Piech *et al.,* 2025). An estimated rise in global anxiety burden constitutes threats to human health and quality of life (Quek *et al.,* 2019). It is a negative emotion which is characterized by symptoms such as feeling of fear, restiveness, excessive worry, which often results in muscle tension, elevated heart rate and difficulty in concentrating (Gupta *et al.,* 2025). It negatively affects quality of life because of the disability (including educational and occupational) associated with this disorder (Dryman *et al.,* 2016; Moraiti *et al.,* 2024).

Treatments of anxiety with benzodiazepines (BZDs), barbiturates and tricyclic antidepressants (TCA's) are associated with serious undesirable effects (Ito *et al.,* 1996; Yeung *et al.,* 2018; Rodulfo *et al.,* 2021; Jahani *et al.,* 2022). For instance, cognitive impairment, rebound anxiety, dependence and discontinuation syndrome are some of the side effects associated with the use of benzodiazepines (Jahani *et al.,* 2022), while barbiturates are associated with narrow range of safety, physical dependence, addiction and withdrawal syndrome (Ito *et al.,* 1996; Rodulfo *et al.,* 2021). The TCA's have in addition, seizure and sexual dysfunction among others as side effects associated with them (Yeung *et al.,* 2018). These range of side effects have necessitated interest in the identification of new anxiolytic drugs from botanicals or alternative therapy with fewer side effects profile (Thippeswamy *et al.,* 2011). But the major impediment to the incorporation of herbal medicine into medical practice is the presence of insufficient scientific data and lack of better understanding of the efficacy and safety of the herbal medicines (Thippeswamy *et al.,* 2011). Hence, studies on how to find alternative therapy from plant sources with more specific anxiolytic effects are pertinent (Rabbani *et al.,* 2008).

Milicia excelsa (Welw.) C.C. Berg Moraceae popularly known as African teak is a large deciduous tree 30 to 50 m high occurring naturally in humid forests of West Africa (Agyeman *et al.,* 2009). Its various parts are used in African folkloric medicines in the preparation of ethnomedicines for the treatment of: mental derangement (Ibrahim et *al.,* 2006), rheumatism (Ndah *et al.,* 2013) and convulsion (Wahab, 2015), among other diverse folkloric uses.

Phytochemical estimations revealed that the leaf extract of *Milicia excelsa (M. excelsa)* contained alkaloids, flavonoids, tannins and phenols (Akinpelu *et al.,* 2020a). The ultraviolet-visible and fourier transform-infrared spectrum further corroborated the presence of alkaloids, flavonoids and phenols, among other phytocompounds in *M. excelsa* leaf (Akinpelu *et al.,* 2019a).

Pharmacologically, the antipsychotic (Akinpelu *et al.,* 2018a), antidiarrheal (Adebayo *et al.,* 2019), antistress (Akinpelu *et al.,* 2019b); sedative-hypnotic (Akinpelu *et al.,* 2020b), antihypoxic (Akinpelu *et al.,* 2020a); and anticonvulsant (Akinpelu *et al.,* 2018b; Akinpelu *et al.,* 2023) effects of the plant have been documented. Therefore, as a follow up to the anxiolytic effect of the crude leaf extract earlier reported in literature (Sofidiya *et al.,* 2021), this study aimed to evaluate the anxiolytic potentials of the fractions of *Milicia excelsa* leaf and carry out the probable neural mechanism of its anxiolytic potential using receptor antagonists in mice.

Materials and Methods

Plant Identification and Authentication

Milicia excelsa leaves were collected within the campus of Obafemi Awolowo University (OAU), Ile Ife. It was identified and authenticated by Mr. G. A. Ademoriyo of the Herbarium Unit, Department of Botany, Faculty of Sciences, OAU, Ile-Ife and herbarium number IFE 17482 was obtained.

Preparation of Plant Materials

The ethanol leaf extract of *Milicia excelsa* and its fractions were prepared as earlier documented in literature (Akinpelu *et al.,* 2018a). Briefly, the leaves of *Milicia excelsa* were air dried at room temperature. The dried leaves were pulverized and extracted with 3 liters of seventy percent (70%) ethanol for 72 h, concentrated *in vacuo* and freeze dried to yield the crude extract. Sixty gram (60 g) of the crude extract was successively partitioned into n-hexane, ethylacetate, n-butanol and aqueous fractions yielding HF, EF, BF and AF respectively. The HF, EF, BF were separately concentrated in vacuo and subsequently freeze dried while AF was also freeze dried. The fractions were freshly prepared by dissolution in 2% Tween 20 in normal saline on each day of the experiments.

Laboratory animals

Adult albino mice of both sexes (18–25 g) were obtained from the Animal House, Department of Pharmacology, Faculty of Pharmacy, OAU, Ile-Ife. The animals were kept in clean cages under 12 hr light/ 12 hr darkness natural cycles. They were maintained on standard animal pellets and clean drinkable water *ad libitum*. The experimental procedures adopted in this study followed the approved institutional Animal Ethical Committee guidelines vide the approval number IUO/ETHICS/22/012. The animals were fasted overnight prior to the experiments. The experiments were carried out between 9.00 am and 3.00 pm.

Materials and chemicals

Ethanol, n-hexane, ethyl acetate, n-butanol, separating funnel, open field apparatus, elevated plus maze, staircase apparatus, and hole board.

Drugs

Cyproheptadine (CYPRO), Tween 20 (Sigma Aldrich USA), Diazepam (DZP) (Roche, Basel, Switzerland), Flumazenil (FLU) (Hikma Pharmaceutical, Portugal, S.A.) and normal saline (Unique Pharmaceutical Limited, Lagos, Nigeria). The drugs were freshly prepared on each day of the experiments.

General Experimental Design

Animals were randomly selected into 5 groups (n = 6). Group I served as the control which received the physiological saline (2% Tween 20 in normal saline, 10 mL/kg) only. Test groups II–IV were orally treated with HF at the doses of 250, 500 and 1000 mg/kg respectively, while group V (positive control group) received DZP (1 mg/kg) via intraperitoneal (i.p.) route. These experimental procedures were repeated for EF, BF and AF at the same doses as HF.

Pharmacological Experiments

Novelty-Induced Behaviours

The scoring of rearing and locomotion behaviours were performed as earlier described (Olurankinse *et al.,* 2023). Each mouse was placed inside plexiglas's cage and observed for rearing and locomotion activities for 20 minutes preceding 1 hour of pre-treatment with 2% Tween 20 in normal saline, or the extract (250, 500 and 1000 mg/kg) or 30 minutes following intraperitoneal injection of diazepam (1 mg/kg). The number of squares crossed with all the fore and hind limbs were counted as locomotion. Rearing was determined as the number of times the animal places its fore paws against the wall of the cage or in the free air.

Hole Board Test

Head-dips were performed as previously described (Aiyelero *et al*., 2023; Sarkar *et al*., 2024). The animals were placed individually on top of a wooden box with 16 evenly spaced holes 1 h after oral administration 2% Tween 20 in normal saline or HF or 30 minutes after diazepam (1 mg/kg, i.p.) administration. The number of times each animal dipped its head into the holes in 5 minutes were counted and recorded. Seventy percent (70%) ethanol was used to clean the hole board after each experiment with a mouse and allowed to dry before continuing with other animals. The procedures were repeated for EF, BF and AF.

Elevated Plus Maze

The elevated plus maze (EPM) test used to evaluate the anxiolytic potentials of the fractions was as earlier reported (Adnan *et al*., 2020; Akinpelu *et al*., 2024). One hour following oral administration of 2% Tween 20 in normal saline or HF at graded doses or 30 minutes following intraperitoneal injection of diazepam (1 mg/kg). Each mouse was placed in the central square of the EPM facing an open arm and allowed to freely explore the maze for 5 minutes. During the exploration time of 5 minutes, the frequency of entry and time spent in the open arm of the EPM were noted and recorded.

The open arm avoidance index interpreted as level of anxiety (Trullas and Skolnick, 1993; Akinpelu *et al*., 2019c) was calculated as:

$$\frac{(100 - (\%\text{ time in open arm} + \%\text{ entries into open arm})}{2}$$

These procedures were repeated for EF, BF and AF. Seventy percent (70%) ethanol was used to clean the EPM after the expiration of 5 minute's time by each mouse. The EPM was allowed to dry before putting another mouse into it.

Staircase Model

The staircase test was carried out by the method earlier described (Shanbhag *et al*., 2022). Thirty minutes after intraperitoneal injection of diazepam (2 mg/kg, s.c.) or 1 hour following oral treatment with 2% Tween 20 in normal saline or test substances of HF, each mouse was gently placed on the floor of the box with its back facing the staircase. The number of steps climbed and the number of rearing responses were recorded for each mouse for 5 minutes. The staircase was wiped with 70% ethanol and allowed to dry between tests to remove any olfactory cues which might modify the behavior of next animal. These procedures were repeated for EF, BF, and AF.

*Determination of Neural Mechanism of Action of Aqueous Fraction***,**

To evaluate the involvement of GABAergic pathway in the mode of action of AF, mice were pretreated with flumazenil ($GABA_A$ receptor antagonist) at 3.0 mg/kg, i.p. or cyproheptadine (5-HT receptor antagonist) at 0.5 mg/kg, i.p., 15 minutes later, mice were given the most active dose of the most active fraction AF (1000 mg/kg, p.o.). One hour after oral treatment with AF (1000 mg/kg), mice were observed for 5 minutes on EPM (Adegbuyi *et al*., 2024). The EPM parameters of open arm entry and open arm duration were noted and recorded during 5 minutes of free exploration as done under EPM above.

Statistical Analysis

Results are expressed as mean ± standard error of mean (S.E.M), n=6. Experimental data were subjected to one way analysis of variance (ANOVA), followed by Dunnett's post hoc analysis. The GraphPad InStat® Biostatistics software (GraphPad Software, Inc., La Jolla, USA) was used for the statistical analysis and the level of significance for all tests was set at $P < 0.05$.

Results

Effect of the Fractions on Novelty-Induced Rearing Behaviours

The EF and AF (250 and 500 mg/kg) significantly ($P < 0.05$) decreased rearing activities compared to control while AF at all the tested doses and standard drug diazepam (1 mg/kg, i.p) significantly ($P < 0.05$) decreased rearing compared to control group [Figure 1A]. The EF and AF significantly ($P < 0.05$) decreased locomotion behaviour compared to control. However, HF at 500 mg/kg significantly ($P > 0.05$) increased locomotion behaviour compared to control. Diazepam (1 mg/kg, i.p.) significantly ($P < 0.05$) decreased locomotion behaviour compared to control (Figure 1B).

Effect of the Fractions on Head Dip in Mice

The HF (500 and 1000 mg/kg), BF (250 mg/kg) and AF (250 and 500 mg/kg) significantly ($P < 0.05$) reduced the episodes of head dip on HBT compared to control. Diazepam (1 mg/kg, (i.p) significantly ($P < 0.05$) increased the incidence of head dip compared to control (Figure 2).

Effect of the Fractions on Open Arm Activities.

All the fractions at all the tested doses and diazepam (1 mg/kg, i.p.) significantly ($P< 0.05$) increased the percentage entry into the open arm compared to control. (Figure 3A). Similarly, EF, BF and AF at all tested doses significantly ($P < 0.05$) increased the percentage time spent in the open compared to control (Figure 3B). All the fractions at all the tested doses and diazepam (1 mg/kg, i.p.) significantly ($P < 0.05$) decreased the anxiety indices measured by open arm avoidance indices compared to control (Figure 3C).

The AF at all the tested doses and diazepam (1 mg/kg, i.p.) significantly ($P < 0.05$) reduced rearing compared to control (Figure 4A). The AF (250 and 1000 mg/kg) and diazepam (1 mg/kg, i.p.) significantly ($P < 0.05$) reduced number of steps climbed compared to control (Figure 4B).

Effect of Receptor Antagonists on the Anxiolytic Activity of the Aqueous Fraction.

Pretreatment with flumazenil before oral administration of AF significantly ($P < 0.05$) reversed the frequency in open arm entry (Figure 5A) and the time spent in the open arm (Figure 5B) of the elevated plus maze compared to AF group. Similarly, flumazenil pretreatment before oral administration of AF significantly ($P < 0.05$) increased the open arm avoidance index on EPM as well (Figures 5A, B, C). However, pretreatment with cyproheptadine before AF administration did not significantly ($P > 0.05$) reverse the anxiolytic parameters compared to AF treated group (Figures 5A, B, C).

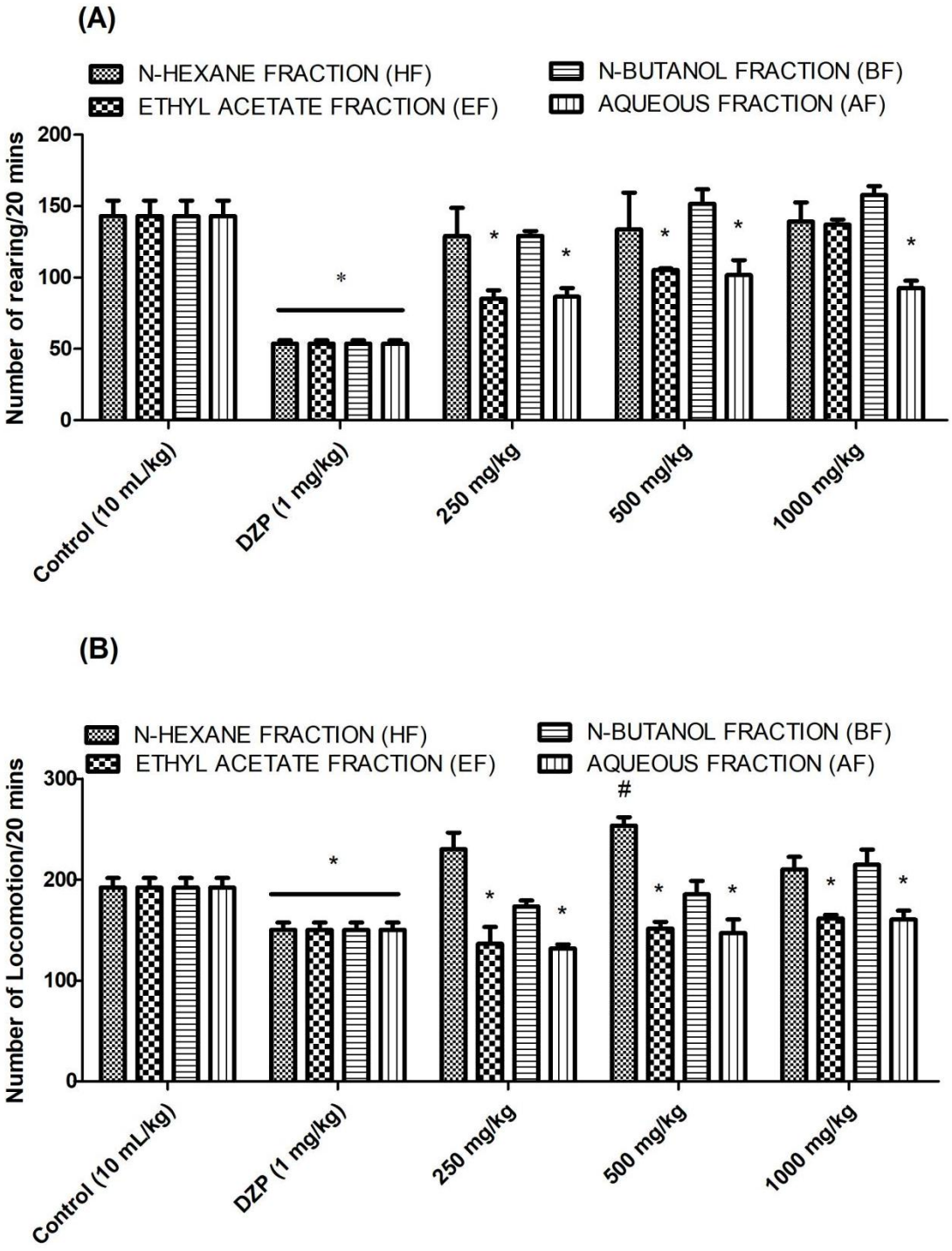

Figure 1: Effect of the fractions on novelty-induced rearing (A) and locomotion (B) behaviour in mice. Each bar connotes mean ± S.E.M, n = 6. *P < 0.05 decreased compare to the control, #P < 0.05 increased compared to control.

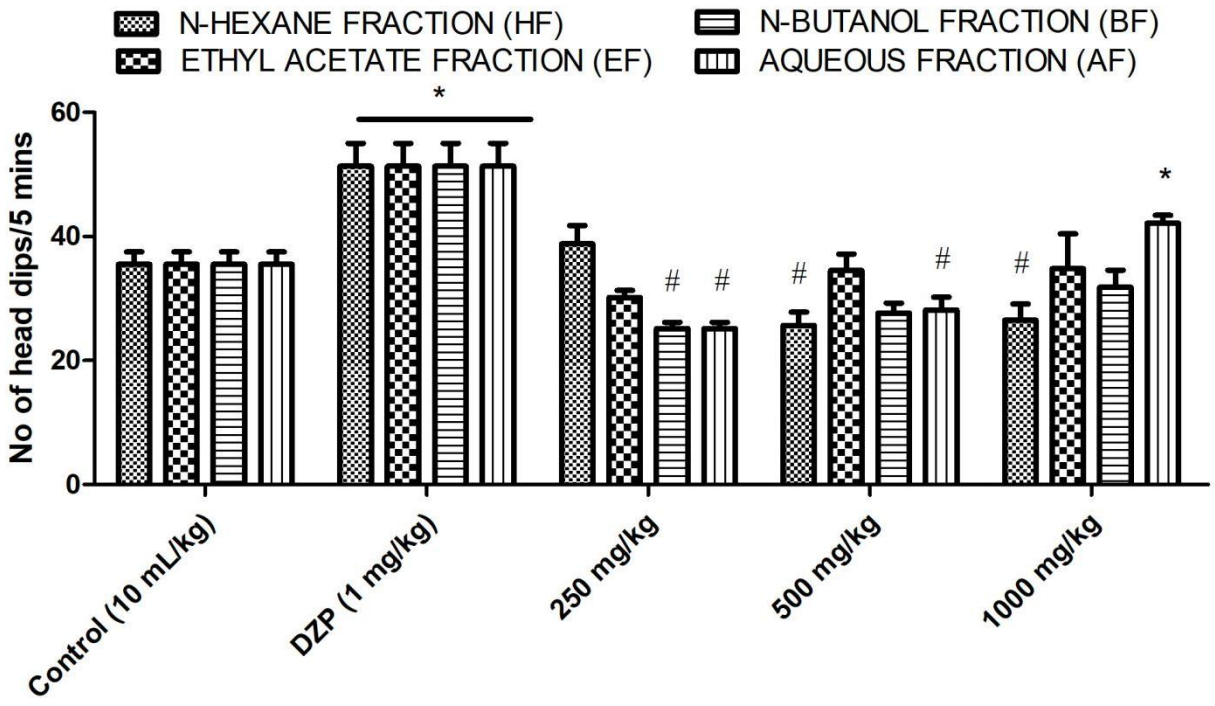

Figure 2: Effect of the fractions on number of head dips in hole board test. Each bar connotes mean ± S.E.M, n=6. *P < 0.05 increased compared to the control, #P < 0.05 decreased compared to control.

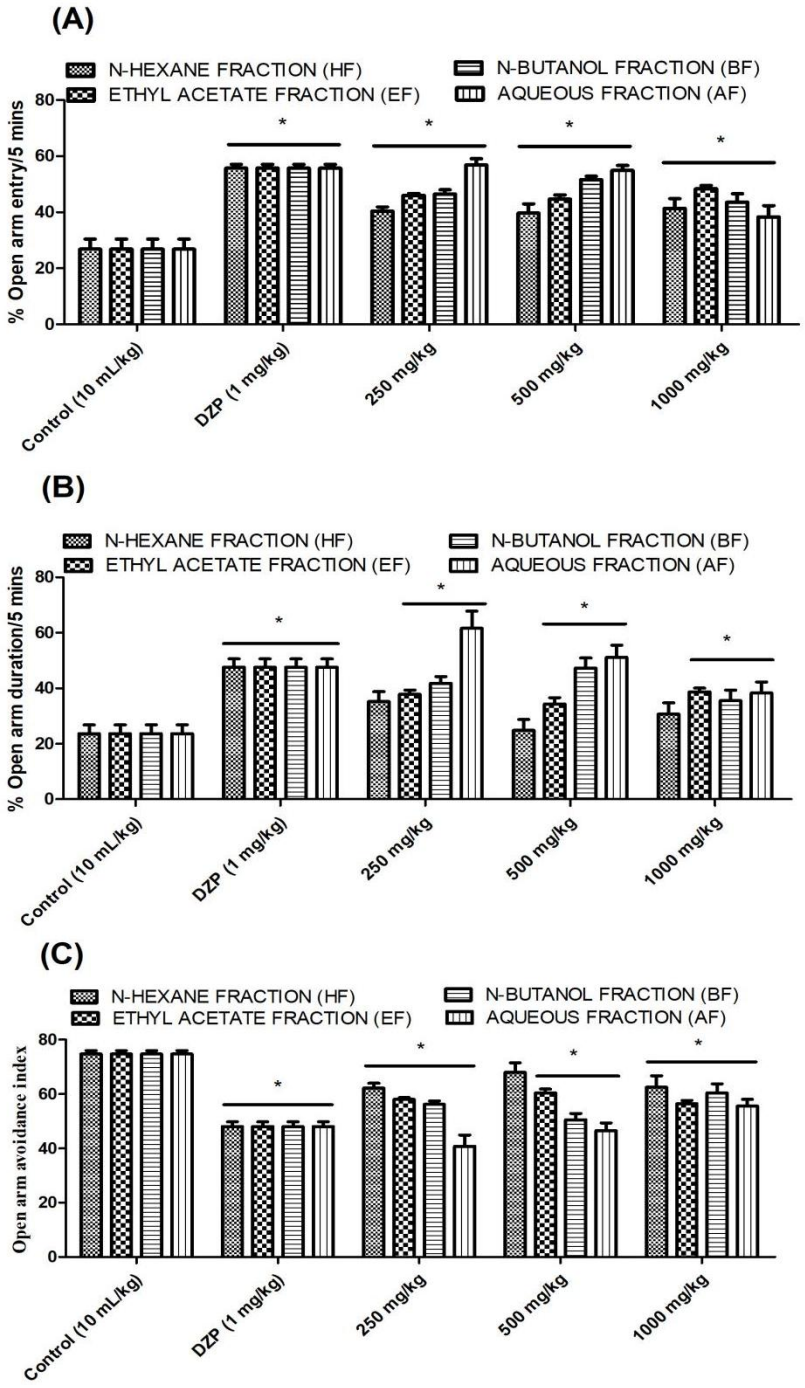

Figure 3: Effect of fractions on percentage number of open arm entries (A), percentage duration (B) and open arm avoidance index (C). Each bar connotes mean ± mean S.E.M, n=6. *P < 0.05 compared to control.

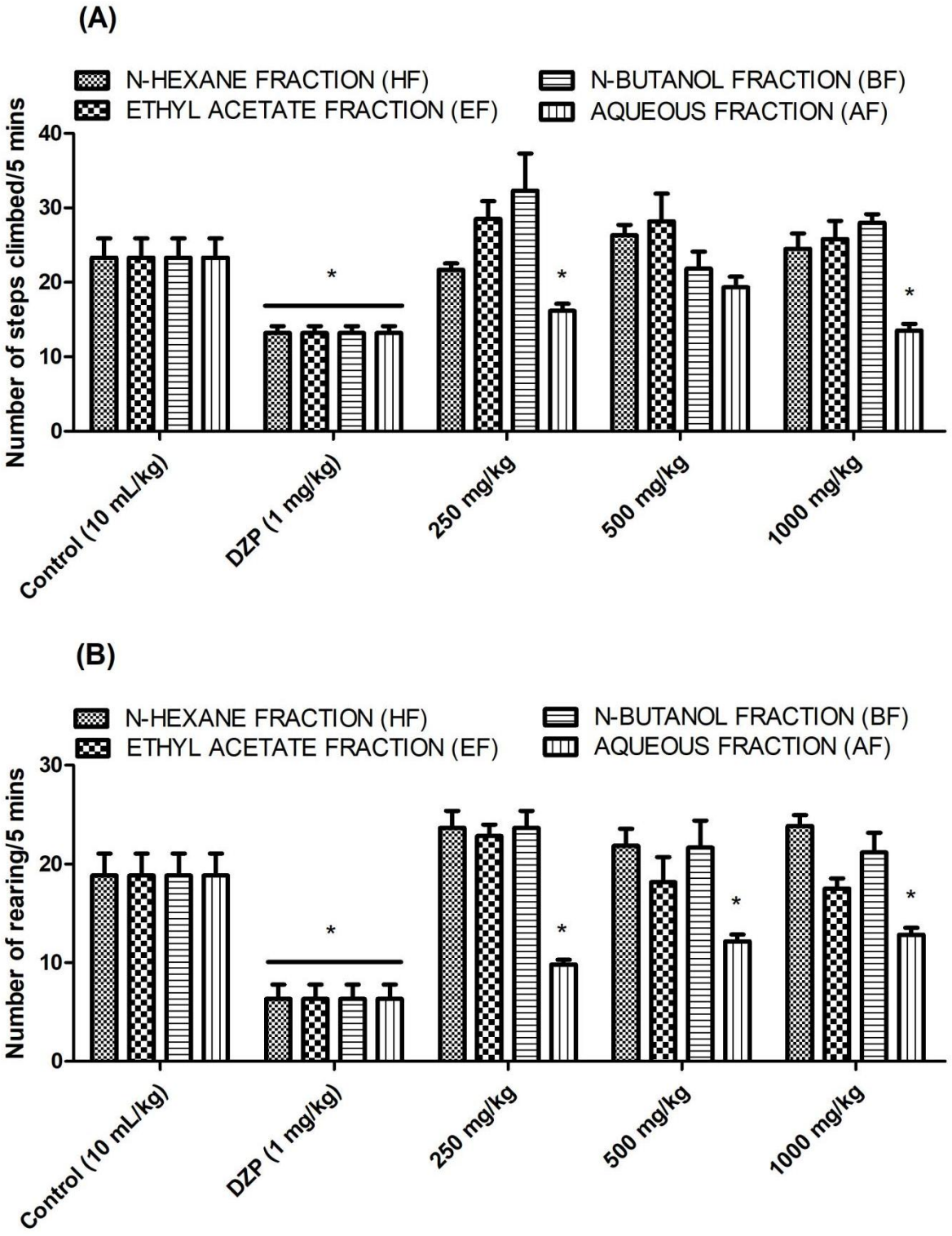

Figure 4: Effect of the fractions on number of steps climbed (A) and number of rearings (B) in staircase model. Each bar connotes mean ± S.E.M, n=6. *P < 0.05 compared to control.

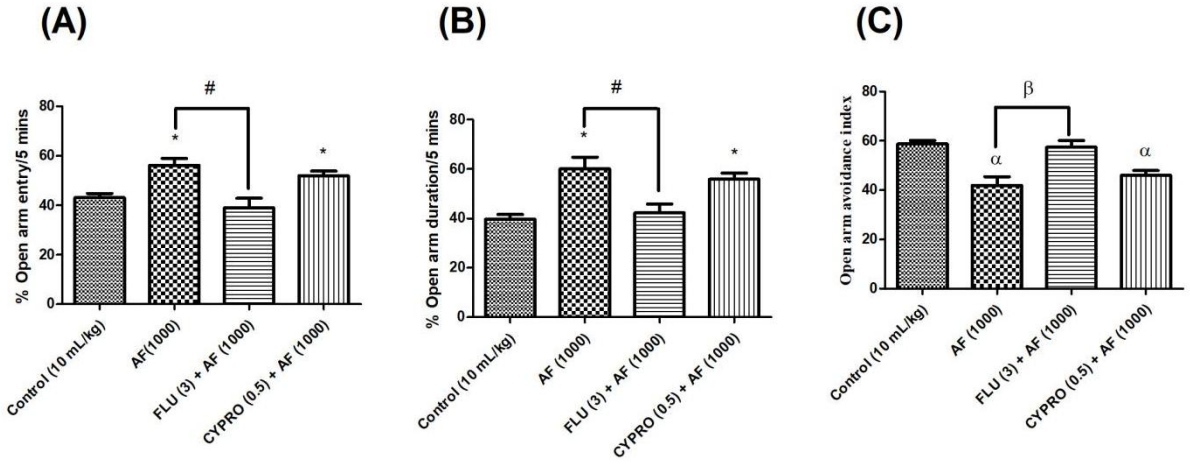

Figure 5: Effects of pretreatment with flumazenil and cyproheptadine on the anxiolytic potential of aqueous fraction of *Milicia excelsa* leaf extract. Each bar connotes mean ± S.E.M, n=6. $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to AF; $^{\alpha}P < 0.05$ compared to control and $^{\beta}P < 0.05$ compared to AF.

Discussion

This study investigated the central nervous system depressant and anxiolytic effects of n-hexane (HF), ethyl acetate (EF), butanol (BF) and aqueous (AF) fractions of ethanol leaf extract of *Milicia excelsa* in mice.

Earlier scientific investigation has reported the median lethal dose (LD_{50}) of the HF, EF, BF and AF to be greater than or equal to 5000 mg/kg (Akinpelu *et al.,* 2018a). Hence, 1/20, 1/10 and 1/5th of the LD_{50} of 5000 mg/kg which corresponded to 250, 500 and 1000 mg/kg were selected for the fractions and considered as low, medium and high doses respectively (Ali *et al.,* 2008) for the different behavioural investigations.

The reduction in novelty-induced rearing and locomotion suggests that EF and AF may possess central nervous system depressant activities. A number of reports have shown that agents that depress the central nervous system (CNS) inhibited rearing and locomotion in mice (Olurankinse *et al.,* 2023; Aiyelero *et al.,* 2023). For instance, it was reported that the administration of leaf extract of *Lagenaria breviflora* and *Solanum incanum* fruits respectively in mice reduced both rearing and locomotion and the authors suggested that the observed reduction might be due to CNS depressant effects (Akanmu *et al.,* 2021; Olurankinse *et al.,* 2023). Since, EF and AF reduced both rearing and locomotion, it can then be inferred that they may possess CNS depressant activities. These reductions might therefore, be due to their central inhibitory action on excitatory neural systems such as glutamatergic and dopaminergic systems among others or their possible potentiation of the central inhibitory systems such as γ-aminobutyric acid (Akanmu *et al.,* 2021).

Numerous reports have demonstrated that HBT can be employed as a model for the evaluation of psychotic, sedative and anxiety conditions in animals (Akkol *et al.,* 2020; Karpuz Ağören *et al.,* 2024). The decrease in head dip by HF, BF and AF suggest sedative potentials (Karpuz Ağören *et al.,* 2024). This behavioural observation agrees with earlier documented reports of reduced head poking in hole board by *Capparis sicula* aerial part extract and *Opuntia ficus indica* fruit extract and these two extracts were suggested to possess sedative properties (Akkol *et al.,* 2020; Karpuz Ağören *et al.,* 2024). It would therefore, not be out of place to suggest likewise that these fractions may possess sedative effects in this model.

Drugs or substances that increase open arm entries and duration are considered as anxiolytics and vice versa for anxiogenics (Yadav *et al.,* 2008; Akindele *et al.,* 2012). The increase in percentage open arm entry and concomitant increase in percentage time spent in the open arm of the EPM is indicative of anxiolytic effects. This observation is in line with earlier reports of medicinal plants that increased entry into and time spent in the open arm of EPM and were reported to possess anxiolytic effects (Amali *et al.,* 2020; Akinpelu *et al.,* 2024).

The reduction in the indices of open arm avoidance index interpreted as anxiety index (Trullas and Skolnick, 1993), which had factored in simultaneously the percentages number of entry and duration of stay in the open arm of EPM in its calculation of anxiety status of each mouse further showed that HF, EF, BF and AF may possess anxiolytic effects. AF reduced the number of steps climbed and number of rearing in staircase model, indicating that they may have sedative and anxiolytic effects. Anxiolytics

have been found to reduce rearing at doses which did not reduce the number of steps climbed (Aliyu *et al.,* 2014) or increase the numbers of steps climbed and decreased rearing (Guraji *et al.,* 2018). Our finding therefore, agrees with earlier published data of medicinal agents that decreased rearing or decreased both rearing and steps climbed and were suggested to possess anxiolytic effect (Aliyu *et al.,* 2014; Guraji *et al.,* 2018). It is important to say also that reduction in number of steps climbed in AF treated mice like diazepam could also be ascribed to sedative potentials while reduction in rearing responses by these two agents could also be due to anxiolytic potentials in mice. Of all the fractions, AF produced consistent anxiolytic actions in all the models used except in hole board test where it showed sedative effects. Thus, the probable neural mechanism of anxiolytic effect was investigated with AF at the highest and most effective dose of 1000 mg/kg using flumazenil ($GABA_A$ benzodiazepine receptor antagonist) and cyproheptadine (5HT-receptor antagonist) in mice.

The findings showed that AF might be acting via $GABA_A$-benzodiazepine receptor neurotransmission in its anxiolytic effect, since pretreatment with flumazenil ($GABA_A$-benzodiazepine receptor antagonist) abolished its anxiolytic activity. This observation is in accordance with reports of medicinal plants found in literature to be acting via $GABA_A$- benzodiazepine receptor pathways (Pitsikas and Tarantilis, 2020; Olurankinse *et al.,* 2023).
The non reversal of the anxiolytic potential of AF following its pretreatment with cyproheptadine may suggest non involvement of serotonergic pathways in its anxiolytic effects.

Conclusion

This study concluded that fractions of *Milicia excelsa* leaf extract may possess anxiolytic effects. The mechanism of anxiolytic effect may involve $GABA_A$ benzodiazepine receptor neurotransmission. Further study is hereby recommended to isolate and elucidate the anxiolytic bioactive principle(s) that may be present in these fractions.

Conflict of interest

We declare no conflict of interest

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