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PRELIMINARY STUDIES ON THE BEHAVIOURAL EFFECTS OF THE METHANOL EXTRACT OF *LEONOTIS NEPETIFOLIA* LINN STEM IN MICE

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Abstract

Background: *Leonotis nepetifolia* Linn (Lamiaceae) is used in traditional medicine for its calming (tranquilizing) effects. The aim of this study was to determine whether there is any scientific justification for this use. To achieve this purpose, we investigated the behavioural effects of the methanol extract of *Leonotis nepetifolia* stem (37.5, 75 and 150 mg/kg) in mice.
Methods: Acute toxicity studies were carried out on the methanol stem extract of *Leonotis nepetifolia* to determine the LD_{50}. The behavioural tests employed were diazepam-induced sleep onset and duration, hole board assay for exploratory activity, mouse beam walk assay for motor coordination, and the staircase test for the detection of anxiolytic compounds. Preliminary phytochemical screening was also carried out on the extract.
Results: The intraperitoneal LD_{50} value was found to be 3.8 g/kg. The results showed that the extract significantly prolonged the duration of diazepam-induced sleep at the highest dose (150 mg/kg). There was no observable effect on exploratory activity and motor coordination at the doses tested (37.5, 75 and 150 mg/kg). The extract, however, at 150 mg/kg elicited a significant decrease in the number of rearings in the staircase test, an effect also observed in the group of mice injected with an anxiolytic dose of diazepam. The preliminary phytochemical screening revealed the presence of alkaloids, saponins, glycosides and triterpenoids.
Conclusion: The results obtained suggest that the crude methanol extract of *Leonotis nepetifolia* stem possesses some biologically active constituents with potential anxiolytic activity and thus may justify its traditional use as a tranquilizer.

Keywords: behavioural; exploratory; *Leonotis nepetifolia*; motor coordination; anxiolytic

Introduction

Leonotis nepetifolia Linn also known as 'Christmas candle stick' and 'Lion's ear' belongs to the mint family known as Lamiacae. The plant is a robust annual herb, about 1-2 m tall, inflorescence globose whorled at upper nodes with orange flowers. The plant is found in waste lands and around villages. It occurs throughout the West African region from Senegal to West Cameroon, and widely dispersed elsewhere in tropical Africa and worldwide (Burkill, 1995). *Leonotis nepetifolia* is used in the folkloric treatment of certain ailments such as asthma (Clement et al., 2005), rheumatism, rickets, headaches and wounds (Burkill, 1995, Dhawan et al., 2013). The plant is also used in ethnomedicine as a tranquiliser in mental illness and children (Burkill, 1995).

The plant has been scientifically evaluated for antibacterial (Narayan, 2012), antioxidant, antiproliferative (David et al., 2007; Sobolewska et al., 2012; Veerabadran et al., 2013), analgesic (Makambila-Koubemba et al., 2011), anti-inflammatory (Parra-Degaldo et al., 2004), antidiabetic (Gungurthy et al., 2013), antidiarrheal (Gakunga et al., 2013), antitumor (Gurunagarajan and Pemaiah, 2010), and wound healing (Nithya et al., 2011) activities. To our knowledge, there is very little scientific evidence to support the ethnomedical claim of its tranquilising effects. In previous studies, we evaluated the anticonvulsant and behavioural effects of the methanol extract of the capitulum (flower head) of *Leonotis nepetifolia* (Ayanwuyi et al., 2009a; Ayanwuyi et al., 2009b). In the present study, we sought to evaluate, using behavioural experiments, the sedative and anxiolytic properties of the stem of this plant.

Materials and Methods

Plant material

The stem of *Leonotis nepetifolia* was collected from Dakaci village of Zaria local government area, Kaduna state, Nigeria in March, 2008. The plant was identified and authenticated at the herbarium in the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, and a voucher specimen (2309) was preserved at the herbarium for future reference.

Preparation of the extract

The stem of the plant was cleaned, air dried at room temperature for 7 days and reduced to a coarse powder using pestle and mortar. The powdered material was then subjected to Soxhlet extraction over 24 h using methanol. The extract was concentrated over a

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water bath at a low temperature of 60˚C using a vacuum rotary evaporator. This extraction procedure gave a yield of 3.28%. The extract was then stored in a desiccator and was reconstituted into a fresh aqueous solution prior to each experiment.

Preliminary phytochemical screening

Preliminary phytochemical screening of *Leonotis nepetifolia* stem was carried out on the methanol stem extract using standard methods of analysis (Trease and Evans, 2002).

Test animals

Swiss albino mice (16-21 g) of either sex were used for the study. The animals were matched for weight and sex for each experimental group. The animals were obtained from the animal facility in the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. They were housed in standard animal cages at room temperature and maintained on a standard diet and water *ad libitum*. All experimental procedures were approved by the University ethics committee. The experiments were conducted in a quiet room between the hours of 9h00-17h00.

Drugs

All test doses were administered via the intraperitoneal route in a volume of 10 ml/kg (aqueous solution). Diazepam (Roche, Pakistan) was used for the sleep potentiation experiments as well as the standard reference drug in the other experiments. Normal saline (10 ml/kg) was used as negative control for each experiment.

Acute toxicity study

The median lethal dose (LD_{50}) was determined according to the method of Lorke (1983). In the first phase, mice were divided into three groups with three mice in each group and were administered the methanol stem bark extract at doses of 10, 100 and 1000 mg/kg body weight respectively via the intraperitoneal route. The mice were observed for signs of toxicity and death for 24 h. In the second phase, three mice were injected with more specific doses (5000, 2900, and 1600 mg/kg) of the extract and observed for signs of toxicity and death for 24 h. The final LD_{50} was calculated as the geometric mean of the lowest dose that caused death and the highest dose at which the animal survived.

Diazepam-induced sleep test

The method of Rakotonirina et al., (2001) was adopted. Sleep time potentiation by the extract was studied with three groups of 6 mice per group. The first group served as the negative control; the second, third and fourth groups were injected with 37.5, 75 and 150 mg/kg of the extract, respectively. Thirty minutes later (post administration), diazepam (20 mg/kg) was administered via the same route to all the groups. The onset and duration of sleep for each mouse, as indicated by the loss and regain of righting reflex, respectively, was noted and recorded.

Hole Board assay for exploratory activity

The hole-board test as described by File and Wardill (1975) and Magaji et al., (2008) was used to measure exploratory behaviour. The apparatus consisted of a wooden board measuring 40×40 cm with 16 evenly spaced holes each of 1 cm diameter and 2 cm depth. Five groups of 6 mice each were used for the study. The first group was injected with normal saline; the second, third and fourth groups were injected with 37.5, 75 and 150 mg/kg of the extract respectively and the fifth group was injected with diazepam at the dose of 2 mg/kg. Thirty minutes post treatment, each mouse was placed singly at a corner on the board. The number of times the mice dipped their heads into the holes during a five-minute trial was counted and recorded.

Staircase test for anxiety

The method of Simiand et al., (1984) was used. This study employed a staircase with five steps (2.5×10×7.5 cm) enclosed in Plexiglas. The apparatus was 45 cm in length, with the height constant along the whole length of the staircase. Five groups of 6 mice each were used for the study. The first group was injected with normal saline (10 ml/kg); the second, third and fourth groups with 37.5, 75 and 150 mg/kg of the extract respectively, and the fifth group was administered diazepam at the dose of 0.5 mg/kg. Testing was done in a quiet room illuminated by red light. Each mouse (experimentally naïve) was placed individually on the floor of the box with its back to the staircase, and the behaviour was recorded using a video camcorder which was scored at a later time. The rearings and number of steps climbed by each mouse was counted for 3 min. A step was considered to be climbed only if the mouse placed all four paws on the step.

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The number of steps descended was not counted. Rearing was recorded when the mouse rose on its hind legs either on the step or against the wall. 70% ethyl alcohol was used to clean the staircase after each trial to avoid modification in behaviour due to olfactory cues.

Beam walking assay for motor coordination

The method described by Stanley et al., (2005) was used for this study. Mice were trained to walk from a start platform along a ruler (80 cm long, 3 cm wide) elevated 30 cm above the bench by a metal support to a goal box. Three trials were performed for each mouse. Only the mice that showed no neurological deficit and walked successfully along the ruler were admitted into the study and grouped into 5 groups of 6 mice each. Mice in the first group were injected with normal saline; second, third and fourth groups with 37.5, 75 and 150 mg/kg of the extract respectively, and the fifth with diazepam at the dose of 0.5 mg/kg. Thirty minutes post treatment, each mouse was placed at one end of a beam (60 cm long, 8 mm in diameter and elevated 30 cm above the bench by a metal support) and allowed to walk to the goal box at the other end. The number of foot slips, which is a sensitive measure of motor coordination deficit (Stanley et al., 2005), was recorded for each mouse using a tally counter.

Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). The data obtained from each experiment were tested for normality using the Shapiro-Wilk test. Due to lack of normality, Kruskal-Wallis test followed by Dunn's test for multiple comparisons were used to analyse the onset of sleep (diazepam-induced sleep experiment), steps climbed (staircase test) and foot slips (beam walking experiment) data. One way Analysis of Variance (ANOVA) followed by Dunnet's *post hoc* test was used to analyse the rest of the data. Results were regarded as significant at $P < 0.05$.

Results

Acute toxicity studies

The intraperitoneal median lethal dose (LD_{50}) of the methanol extract of LNS was found to be 3.8 g/kg body weight in mice.

Diazepam-induced sleep test

ANOVA showed that there was a significant effect of the extract on the duration of sleep [$F_{(3, 20)} = 3.48$; $p<0.05$]. *Post hoc* analysis revealed that LNS significantly prolonged ($P<0.05$) the duration of diazepam-induced sleep at the dose of 150 mg/kg (**Figure 1**).

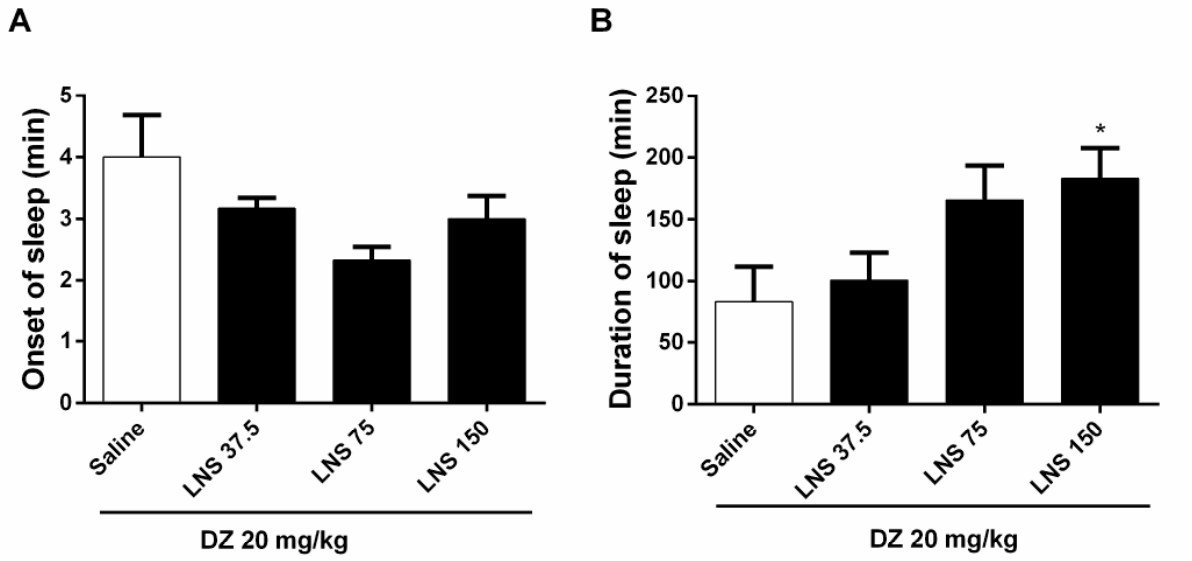

Figure 1: Effect of the methanol extract of *Leonotis nepetifolia* stem (LNS) in mg/kg on the onset (A) and duration (B) of diazepam-induced sleep in mice. Data are expressed as mean (±S.E.M.) time in minutes (n=6 per group). Route of administration was via intraperitoneal injection. *$p<0.05$,significant difference from negative control (Saline, 10 ml/kg).

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Hole board assay for exploratory activity

There was a significant effect of drug treatment on the number of head dips [$F_{(4, 25)} = 4.49$; $p<0.01$]. *Post hoc* analysis showed that diazepam (2 mg/kg) elicited a decrease ($P<0.05$) in the number of head dips. LNS did not affect head-dipping behaviour (**Figure 2**).

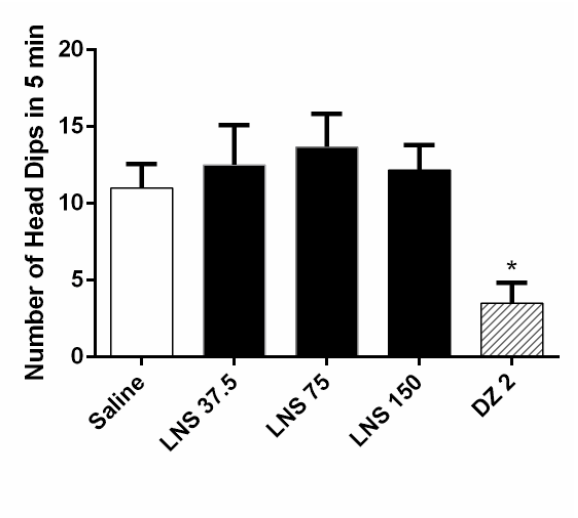

Figure 2: Effect of the methanol extract of *Leonotis nepetifolia* stem (LNS) and diazepam (DZ) in mg/kg on the number of head dips in 5 minutes. Data are expressed as mean (±S.E.M.; n=6 per group) number of head dips. Route of administration was via intraperitoneal injection. **p<0.05* significant difference from negative control (Saline, 10 ml/kg).

Staircase test for anxiety

Statistical analysis showed that there was a significant effect of treatment on the number of rearings in the staircase test [$F_{(4, 25)} = 4.67$; $p<0.01$]. *Post hoc* analysis revealed that LNS dose-dependently decreased the number of rearings which was significant ($P<0.01$) at the highest dose of 150 mg/kg compared to the negative control group. In a similar fashion, diazepam (0.5 mg/kg) significantly ($P<0.01$) decreased the number of rearings. There was also an effect of treatment on the number of steps climbed [$H=14.76$; $p<0.01$]. *Post hoc* analysis showed that diazepam increased ($P<0.05$) the number of steps climbed, an effect that was not elicited by LNS (**Figure 3**).

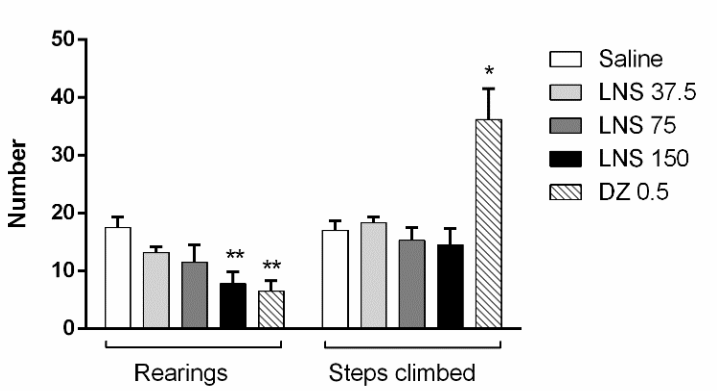

Figure 3: Effect of the methanol extract of *Leonotis nepetifolia* stem (LNS) and diazepam (DZ) in mg/kg on the number of rearings and steps climbed in the staircase test. Data are expressed as mean (±S.E.M.) number (n=6 per group). Route of administration was via intraperitoneal injection. **p<0.01* significant difference from negative control (Saline, 10 ml/kg).

Beam walking assay for motor coordination

There was a significant effect of treatment on foot slips in the beam walking assay [$H=17.28$; $p<0.01$]. *Post hoc* analysis revealed

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that diazepam (0.5 mg/kg) elicited a significant increase ($P< 0.05$) in the number of foot slips compared to the negative control group indicating motor coordination deficit. The extract, however, showed no significant motor deficit compared to the negative control group (**Figure 4**).

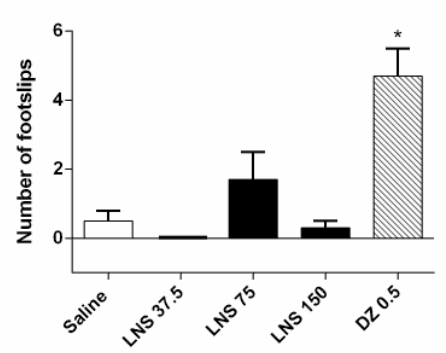

Figure 4: Effect of the methanol extract of *Leonotis nepetifolia* stem (LNS) and diazepam (DZ) in mg/kg on the number of foot slips in the beam walking assay. Data are expressed as mean (±S.E.M.; n=6 per group). Route of administration was via intraperitoneal injection. *$p<0.05$ significant difference from negative control (Saline, 10 ml/kg).

Preliminary phytochemical screening

The preliminary phytochemical screening of LNS revealed the presence of alkaloids, saponins, triterpenoids and glycosides.

Discussion

The methanol extract of LNS increased the duration of diazepam-induced sleep, suggesting a sedative property of the extract (Rakotonirina et al., 2001; Musa et al., 2008). This effect was also found with the capitulum of the plant (Ayanwuyi et al., 2009b). Diazepam, a benzodiazepine is a sedative-hypnotic drug, exerting its actions via allosteric modulation of $GABA_A$ receptors (Trevor and Way, 2012). LNS potentiated the sedative property of diazepam suggesting a possible interaction with GABA, a major inhibitory neurotransmitter in the central nervous system.

In the hole board experiment, LNS did not affect head dipping behaviour whereas diazepam (2 mg/kg) decreased this motor activity. The hole board experiment is a measure of exploratory behaviour in laboratory animals (File and Wardill, 1975) and is accepted as an experimental model for the evaluation of sedative and anxiety conditions in animals. A decrease in the number of head dips (exploratory activity) suggests sedative activity (File and Pellow, 1985; Magaji et al., 2008). In contrast to the sedative drug diazepam, LNS did not elicit a decrease in the number of head dips. This suggests that LNS does not produce marked CNS depression at the doses tested. The staircase test has been proven to be a relatively simple, reliable and efficient experiment for the screening of anxiolytic agents (Ago et al., 2007). LNS was found to decrease the number of rearings in the staircase test without altering the number of steps climbed. Similarly, the benzodiazepine, diazepam at a low dose (0.5 mg/kg) decreased the number of rearings but, interestingly, also enhanced step climbing behaviour. The number of steps climbed and rearings in the staircase test are behavioural measures of exploratory/locomotor activity and anxiety respectively (Weizman et al., 2001; Milman et al., 2006). Some studies have shown that anxiolytic drugs including benzodiazepines decrease the number of rearings without suppressing step climbing behaviour (Weizman et al., 2001; Milman et al., 2006, Mesfin et al., 2014). The ability of LNS to decrease the number of rearings without affecting step climbing behaviour is suggestive of an anxiolytic effect. The further enhancement of exploratory behaviour by diazepam as observed in this study may be explained as likely due to an extension of the anti-anxiety properties of the drug that increased exploratory activity of the mice (Ago et al., 2007, Gnanasekar et al., 2014). The inability of LNS to also increase step climbing behaviour was probably as a result of the doses used or may reflect anxiolytic action via a different mechanism compared to that of the benzodiazepines. The anxiolytic activity observed in this experiment could be linked to its reported antioxidant properties (Takeda et al., 1999; David et al., 2007) as oxidative stress has been implicated in anxiety (Bouayed et al, 2009).

It was also found that while the anxiolytic dose of diazepam increased the number of foot slips in the beam walking assay, LNS did not elicit any effect on this parameter. The beam walking assay is a test for pharmacologically-induced motor coordination deficits, and is predictive of sedative effects (Stanley et al., 2005). This assay has been found to detect benzodiazepine-induced motor coordination deficits, with the number of foot slips being a sensitive measure of motor coordination deficits (Stanley et al., 2005). The absence of observable effects on motor coordination by LNS suggests that, at the doses tested, it may produce its pharmacological effect without marked motor deficits in contrast to some clinically used tranquilisers (e.g. benzodiazepines). The absence of ataxia also suggests that the extracts are not likely to cause clinical sedation at the doses tested (Stanley et al., 2005).

The phytochemical constituents present in the plant extracts may be responsible for their observed pharmacological activities. Alkaloids, which were observed to be present in LNS are reported to possess sedative and anxiolytic actions (Elisabetsky and Costa-Compos, 2006). Triterpenoids have also been shown to have CNS depressant properties (Amos et al., 2002). Phenylethanoid glycosides obtained from the stem of the plant were found to demonstrate antioxidant properties (Takeda et al., 1999), which may also be linked to its anxiolytic effect.

The result of this preliminary investigation suggests that the methanol extract of *Leonotis nepetifolia* stem possesses modest sedative and anxiolytic properties in laboratory animals and thus may support the ethnomedical use of the plant as a tranquilizer. Further studies are required to isolate the bioactive constituent(s) responsible for this activity. Evaluating the effect of the extract on other neuro behavioural models may prove useful in the elucidation of the mechanism of action.

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