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The Anxiolytic Properties of *Vernonia Amygdalina* (Asteraceae) in Laboratory Mice

*Onasanwo S.A, Aitokhuehi N.G, Ajayi T.O & Faborode O.S

Neurosciences and Oral Physiology Unit, Department of Physiology, University of Ibadan, Ibadan, Nigeria.
Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria

ABSTRACT

Anxiety is a state of excessive fear and is characterized by motor sympathetic hyperactivity, apprehension and vigilance syndromes. *Vernonia amygdalina* commonly called bitter leaf, belongs to the family *Astaraceae*, and has been reported to be used locally in the treatment of psychiatric challenges. However, no work has been reported on pharmacological potentials of ethyl acetate fraction of *Vernonia amygdalina* on anxiety. This study was therefore designed to investigate the effect of ethyl acetate fraction *Vernonia amygdalina* (EAVA) on anxiety status in mice. The air-dried leaves of *Vernonia amygdalina* (VA) pulverized and macerated in methanol for 72 hours, before the extract was partitioned into n-hexane and ethyl acetate fractions. The anxiolytic-like effect of EAVA was investigated using the elevated plus maze (EPM), elevated zero maze (EZM) and light-dark test (LDT). The results obtained were expressed as mean ± S.E.M. Data were analyzed using One-way Analysis of Variance (ANOVA), followed by Newman– Keuls' multiple comparisons test, $P<0.05$. *Vernonia amygdalina* showed anxiolytic-like effect in mice, 50- 100mg/kg were significantly different from control by the time spent in the open arms in elevated plus maze and elevated zero maze, and the time spent in light chamber in the light-dark test. In conclusion, this study has shown that ethyl acetate fraction from *Vernonia amygdalina* possess anxiolytic-like effects. However, further work need to be done to ascertain its mechanism of action.

Keywords: *Vernonia amygdalina,* Anxiety**,** elevated plus maze, Elevated zero maze, Light-dark test

Author for correspondence: E-mail: samphil2002@yahoo.com; *Tel:* +234-8055264769

INTRODUCTION

Anxiety is a state of excessive fear and is characterized by motor sympathetic hyperactivity, apprehension and vigilance syndromes. The most common observation is an acute stress response characterized by a state of abnormal or exaggerated arousal or fear (Ninan, 2001). Generally, anxiety is an adaptive response to supposedly dangerous stimuli, which may perturb homeostasis. However, when it becomes disproportional in intensity, chronic and/or irreversible, or not genuine, it manifests as debilitating anxious state presenting itself in form of phobia, panic attacks, post-traumatic stress disorder, social anxiety disorder or generalized anxiety disorder.

Moreover, anxiety states are controlled by both inhibitory and facilitatory mechanisms that either counter or favor anxiety states. Regional brain networks involved in such stress, anxiety, and anxious behaviors may be appropriate targets for actions of anxiolytics. Drug development in these directions also aims to generate new pharmacological agents with action at specific neurotransmitters and neuropeptides, their re-uptake and metabolism. The ultimate objective is to develop substances that are as effective as benzodiazepines, which have been the traditional treatment for anxiety for over 40 years. Anxiolytic drugs increase the level of neurochemicals like serotonin, dopamine, noradrenaline and gamma-aminobutyric acid (GABA) at the synaptic cleft. Yet, there are few major setbacks with conventional anxiolytic drugs such as slow onset of action and negative side effects. Hence, there is a growing interest in alternative medicine and to tap the untapped source of compound that might serve as a template for the development of anxiolytic drugs.

Vernonia amygdalina (family: *Asteraceae*), is a small shrub that grows in the tropical Africa. *Vernonia amygdalina* typically grows to a height of 2-5 m. The leaves are elliptical and up to 20 cm long. Its bark is rough (Ijeh and

Ejik, 2011). *V. amygdalina* is commonly called bitter leaf in English because of its bitter taste. Locally, *Vernonia amygdalina* (VA) is called *ewuro* in Yoruba; onugbu in Igbo; chusar-doki in Hausa; oriwo in Edo (Kokwaro and John 2009; Egedigwe, 2010).

Study showed that VA possessed antioxidant properties and contains phytochemicals such as flavonoids, saponins, terpenes and phenolic acids (Erasto *et al*, 2007). Also, there is evidence that VA has anxiolytic-like potentials using elevated T-maze and hole-board apparatus (Imoru *et al.,* 2014). In an attempt to identify the fraction and ultimately, the compound(s) responsible for this anxiolytic-like potential; this study, therefore, was designed to study the effects of ethyl acetate fraction of *Vernonia amygdalina* on anxiety status in laboratory mice using the elevated plus maze, elevated zero maze and light-dark test models.

MATERIALS AND METHODS

Plant preparation and extraction: Fresh leaves of *Vernonia amygdalina* (VA) were collected at University of Ibadan, Ibadan, Oyo State, Nigeria. The identification and authentication of the plant was done at the herbarium section of the Forest Reserve Institute of Nigeria, (FRIN) Ibadan, Oyo State. These were air-dried and pulverized. The pulverized *V. amygdalina* (1.2kg) was soaked in 4.5 liters of methanol for 72 hours. The mixture was filtered and the filtrate was concentrated using a rotary evaporator at a maximum temperature of 45ºC to obtain the crude aqueous extract of the plant which was further fractioned into n-hexane and ethyl acetate fractions, and kept in the refrigerator at 4^0C for further use. The ethyl acetate fraction of VA was subsequently reconstituted in normal saline at appropriate concentration, and administered orally.

Experimental animals and Treatment: Mice weighing 20–25g were used in this study and were obtained from the Animal House, College of Medicine, University of Ibadan, Ibadan, Nigeria. The animals were kept under a conducive environmental condition, and fed with standard rodent pellet and water *ad libitum*. Animals were divided into four groups and treated as follows: Group 1 (normal saline, 10ml/kg), Group 2-3 (ethyl acetate extract of *Vernonia amygdalina* (EAVA), 50mg/kg and 100mg/kg) and Group 3 (diazepam, 1mg/kg) before subjecting them to their respective models. The experimental procedures adopted in this study were in accordance with the United States National Institutes of Health Guidelines for Care and Use of Laboratory Animals in Biomedical Research (NIH, 1985).

Anxiety Experimental Models
Elevated Plus Maze: The standard elevated plus maze test described by Montgomery (1958) is used to assess anxiety-like behavior in laboratory animals. The apparatus is composed of two open and two closed arms that radiated from a central platform to form a plus sign that is elevated to a height of 40 cm above the floor level. Animal was placed on the center of the platform, facing an open arm. The number of entries and the time spent in the open and closed arms was recorded during a 5-minute test period. The apparatus was

cleaned thoroughly between trials with damp and dry towels to remove any residue or unpleasant odor.

Elevated Zero Maze: The elevated-O-maze (EZM) is a modification of the plus-maze and displays the advantage of lacking the ambiguous central area of the elevated plus-maze. As in the elevated-plus maze, this test is based on two conflicting innate tendencies: exploring a novel environment and avoiding elevated and open spaces constituting situations of predator risk. The apparatus consisted of two open (stressful) and two enclosed (protecting) elevated arms that form a zero or circle. Time spent in exploring enclosed versus open arms indicates then the anxiety level of the animal. When placed into this apparatus, naïve mice by nature tend to explore less the open arms due its natural fear of heights and open spaces. In this context, anxiolytics generally increased the time spent exploring the open arms and anxiogenics have opposite effect, increasing the time spent into the closed arms. Animal was placed on the center of the platform, facing an open arm. The number of entries and the time spent in the open and closed arms was recorded during a 5-minute test period. The apparatus was cleaned thoroughly between trials with damp and dry towels to remove any residue or unpleasant odor.

Light-Dark Test: The apparatus consisted of a Plexiglas box with two compartments (20cm × 20cm each), one of which was illuminated with a white light while the other remained dark. Each animal was placed at the junction of the light dark, facing the illuminated compartment. The time spent in illuminated chamber, as well as the number of entries in each space was recorded for 5minutes (Young and Johnson 1991). After each test, the box was carefully clean up with a wet tissue paper (10% ethanol solution).

Statistical Analysis: The results obtained were expressed as mean ± S.E.M. Variance was analyzed using One-way Analysis of Variance (ANOVA), followed by Newman–Keuls' multiple comparisons test. Statistical significance was set at $P<0.05$. All statistical analyses were done using (Graph-Pad Prism Software, San Diego, CA, USA)
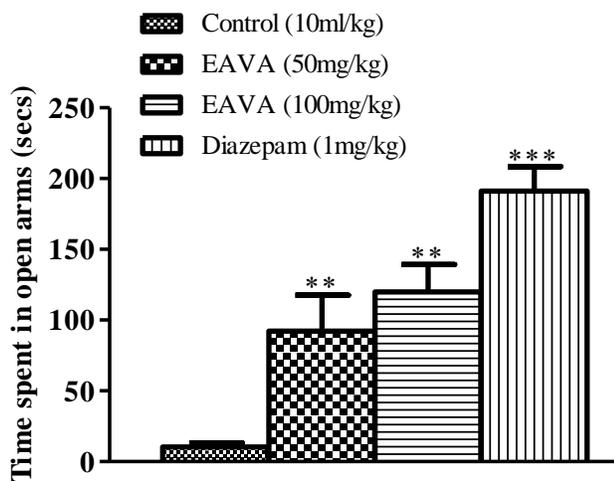
RESULTS

Effect of ethyl acetate fraction of Vernonia amygdalina in mice on number of open arms entries in Elevated Plus Maze: In Figure 1, ethyl acetate fraction of Vernonia amygdalina (50mg/kg, 100mg/kg) and diazepam 1mg/kg showed significant increase (***p<0.001, **p<0.01and ***p<0.001 respectively) in the number of open arms exploration in Elevated Plus Maze when compared with the control as shown by One-way ANOVA.

Effect of ethyl acetate fraction of Vernonia amygdalina on time spent in open arms in Elevated Plus Maze: Figure 2 shows that ethyl acetate fraction of Vernonia amygdalina (50mg/kg, 100mg/kg) and diazepam (1mg/kg) produced significant increase (**p<0.01, **p<0.01 and ***p<0.001 respectively) in the time spent in the open arms of the Elevated Plus Maze when compared with the control.
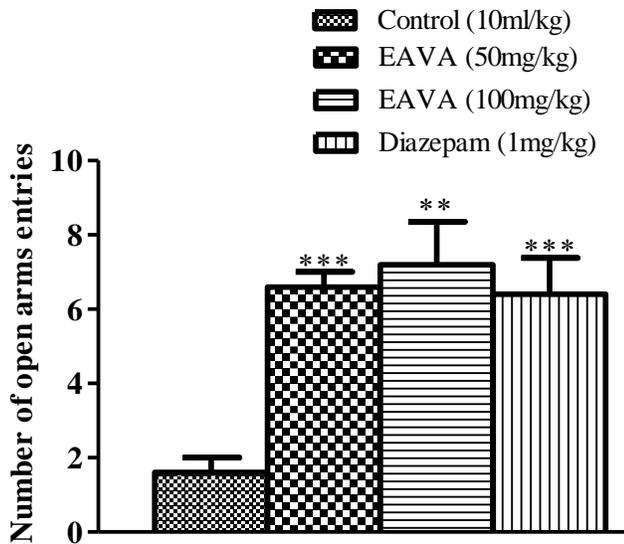
Fig. 1:
Effect of EAVA administration on number of open arm time entries in Elevated Plus Maze (EPM). The results were expressed as means ± SEM (n=6). Data were analyzed using One-way ANOVA followed by Newman-Keuls' post hoc test. ***p<0.001, **p<0.01 compared with the control.

and **p<0.01 respectively) in time spent in open arms in Elevated Zero Maze when compared with the control as shown by One-way ANOVA.

Fig. 3:
Effect of EAVA administration on number of open arms entries in Elevated Zero Maze (EZM). The results are expressed as means ± SEM (n=6). Data were analyzed using One-way ANOVA followed by Newman-Keuls' post hoc test. **p<0.01 compared with the control
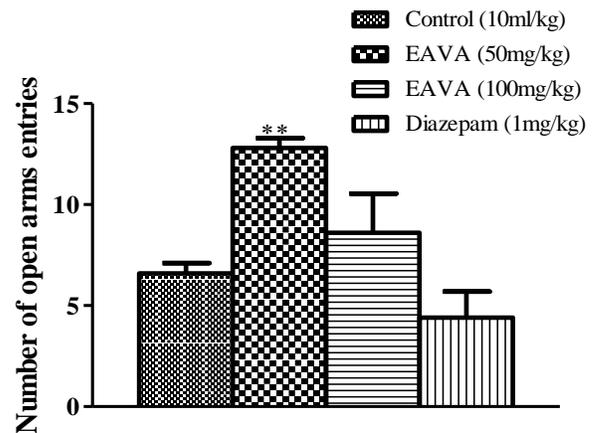

Fig. 2:
Effect of EAVA administration on time spent in open arms of Elevated Plus Maze (EPM). The results are expressed as means ± SEM (n=6). Data were analyzed using One-way ANOVA followed by Newman-Keuls' post hoc test. **p<0.01, ***p<0.001 compared with the control

Fig. 4:
Effect of EAVA administration on time spent in open arms in Elevated Zero Maze (EZM). The results are expressed as means ± SEM (n=6). Data were analyzed using One-way ANOVA followed by Newman-Keuls' post hoc test. *p<0.05, **p<0.01 compared with the control.
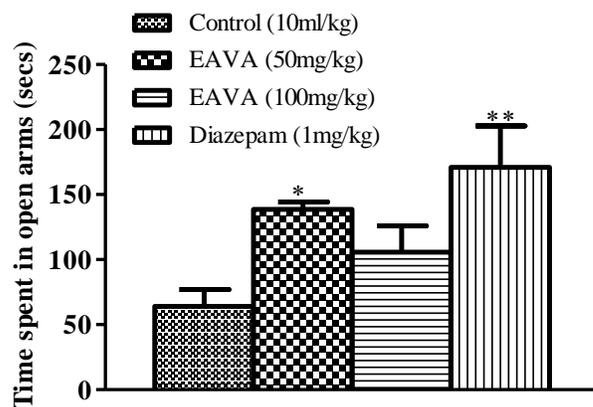
Effect of ethyl acetate fraction of Vernonia amygdalina on number of open arms entries in Elevated Zero Maze: As shown in Figure 3, ethyl acetate fraction of Vernonia amygdalina (50mg/kg) showed significant increase (**p<0.01) in the number of open arms exploration in Elevated Zero Maze when compared with the control as revealed by One-way ANOVA.

Effect of ethyl acetate fraction of Vernonia amygdalina on time spent in open arms of Elevated Zero Maze: In Figure 4, ethyl acetate fraction of Vernonia amygdalina (50mg/kg) and diazepam (1mg/kg) showed significant increase (*p<0.05

Effect of ethyl acetate fraction of Vernonia amygdalina on number of entries in light chamber in Light-dark Test : Figure 5 shows that only diazepam (1mg/kg) was able to increase significantly (*p<0.05) the number of light chamber exploration as when compared with the control.

Effect of ethyl acetate fraction of Vernonia amygdalina on time spent in light chamber in Light-dark Test: Figure 6 shows that Vernonia amygdalina (50mg/kg) and diazepam (1mg/kg) produced significant increase (*p<0.05) in the time spent in light chamber when compared with the saline-treated group
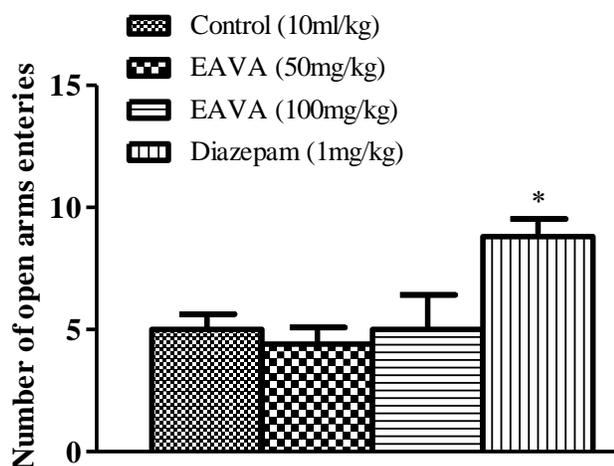
Fig. 5:
Effect of EAVA administration on number of entries in light chamber in Light-dark Test (LDT). The results are expressed as means ± SEM (n=6). Data were analyzed using One-way ANOVA followed by Newman-Keuls' post hoc test. *$p<0.05$ compared with the control
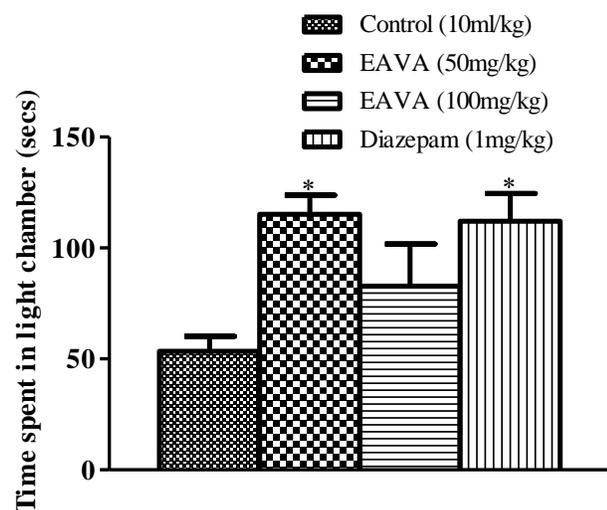
Fig. 6:
Effect of EAVA administration on time spent in light chamber in Light-dark Test (LDT). The results are expressed as means ± SEM (n=6). Data were analyzed using One-way ANOVA followed by Newman-Keuls' post hoc test. *$p<0.05$ compared with the control

DISCUSSION

This study was carried out to investigate the effects of ethyl acetate fraction of *Vernonia amygdalina* (VA) on anxiety in mice. Compounds or drugs that inhibit the re-uptake (or increases the efficacy) of Gamma Aminobutyric Acid (GABA), decrease the excitability of neurons, or reduce the communication between neurons (which brings about a calming effect) has been reported to be clinically effective in treating anxiety disorder

Behavioral studies have been shown to play an important part in the evaluation and development of anxiolytic drugs (Pellow *et al.,* 1985). Elevated plus maze and light-dark chamber are the most frequently used behavioral models for anxiolytic compound with the modification of elevated plus

maze to zero maze. Exposure of animals to novel maze subjected to fear, freeze, become immobile, defecate and show fear-like movement which also may reflect in humans in anxious state. The plasma cortisol level has been reported to increase in animals in these anxiolytic models as a true reflection of anxiety and these fear-like behaviors is reduced by anxiolytic agents like diazepam and newer anxiolytic drugs (Kulkarni *et al.,* 2009). Findings from this study showed that VA is rich in flavonoids and alkaloids and these two constituents of VA are rich in antioxidant activity. The naturally occurring flavonoids have been recently reported to selectively bind with high affinity to the central benzodiazepine receptor, and to exert powerful anxiolytic and other benzodiazepine-like effects in rats (Salgueiro *et al.,* 1997). The elevated plus-maze (EPM) is currently one of the most frequently used animal models of anxiety (Hogg, 1996). In elevated plus-maze, rodents display an avoidance of exposed open arms of the maze and take preference for the closed arms. The parameter of anxiety in this model is the number of open arm entries and time spent in the open arms which are sensitive to agents thought to act through the GABA-A receptor complex, justifying the use of diazepam as a positive control in this study. In accordance with previously published reports, diazepam increased the percentage of open arm entries and the time spent in the open arms (Crawley and Goodwin, 1980), confirming its anxiolytic effects. In this study, VA increase the number of open arms entries and the time spent in open arms entries, therefore, indicating its anxiolytic-like properties.

The elevated zero maze (EZM) was created to eliminate the center region of the Plus, and has also been pharmacologically validated with anxiolytic drugs (Shepherd *et al.,* 1994). Percent times spent in open and closed areas are the acceptable scoring variables used to reflect anxiety status and elevated zero maze (Rodgers *et al.,* 1997). Increases in time in open areas are interpreted as decreased anxiety. In this study VA was able to increase time spent in open arms, and it can therefore be said that VA potentiate anxiolytic-like activities using the zero maze model.

The anxiolytic effect was also tested in the light–dark test. As with the EPM test, it is also useful for modeling anxiety, and it has been developed for predicting the potency of clinically used compounds for treating this disease. It has been assumed that the time mice spent in the illuminated side of the box is the most useful and consistent parameter of anxiety (Young and Johnson, 1991). Administration of VA and diazepam produced a significant response in the light dark test of anxiety, as the time spent by animals in the light chamber increased in comparison with their saline-treated counterpart, indicating an anxiolytic-like activity of the extract.

In conclusion, this study has shown that ethyl acetate fraction of *Vernonia amygdalina* possess anxiolytic-like effect in elevated plus maze, elevated zero maze and light-dark test model. However, further work needs to be done to ascertain its mechanism(s) of action.

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