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Anxiolytic Effect of Aqueous Root Extract of *Citrus aurantium* in Wistar Albino Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author MBY designed the study and wrote the protocol. Author BB managed the animals, collected all data, performed the statistical analysis and wrote the first draft of the manuscript. Author IJJ did the literature search and also wrote part of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Anxiety is a very common mental disorder among neurological disorders. *Citrus aurantium* decoction has been used to treat anxiety- like behaviour, by traditional medicine practitioners in Nasarawa State, Nigeria. The aim of this study was to evaluate the effect of aqueous root extract of *C. aurantium* AECA (50-200 mg/kg, p.o.), diazepam (2.5 mg/kg, i.p.), and normal saline 10 ml/kg on anxiety-like behaviour, spontaneous alternation behaviour. Locomotor and exploratory activity were evaluated in rats on hole-board, elevated plus maze (EPM), elevated zero maze (EZM), Y-maze, and open field apparatus, respectively. The results showed that AECA significantly ($p< 0.05$-0.001) and increased time spent on hole-board, open arm of elevated plus maze, EPM and open arm of elevated zero maze, EZM. The administration of AECA produced dose-dependent and

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significant (p< 0.05-0.001) increase in spontaneous alternation behaviour in rats. Locomotor and exploratory behaviour was significantly (p< 0.05-0.001) increased in the open field apparatus.
The present results provide evidence for anxiolytic effects of the AECA, hence may be developed as a safe alternative for continuous use in the therapeutic management of neurological disorders characterized by anxiety and amnesia.

Keywords: Anxiety; Citrus aurantium; spontaneous; alternation; behaviour; anxiolytic.

ABBREVIATIONS

AECA: Aqueous root extract of Citrus aurantium; mg/kg, po: milligram per kilogram of body weight per os (orally); mg/kg, i.p: milligram per kilogram of body weight intraperitoneal; EPM: elevated plus maze; EZM: elevated zero maze; OF: Open field; WHO: World Health Organisation; BZDs: benzodiazepines; $GABA_A$: Gamma-Aminobutyric acid; NVRI: National Veterinary Research Institute.

1. INTRODUCTION

Anxiety is a mental disorder characterised by apprehension and persistent fear that is not associated to any real danger or risk [1]. Hence, result in the feeling of uncertainty that may affect the normal routine of the life style of an individual. About 450 million people are reported to suffer from a mental or behavioural disorder including anxiety [2]. The increasing number of individuals suffering from anxiety and other neurological disorders has led to the rise in the use of antidepressant and anxiety drugs. The commonest, safest and widely used antidepressant drug are benzodiazepines (BZDs) used as anxiolytic agents which are known to act on receptors known as BZD-$GABA_A$ receptors [3]. However, some side effects such as violent behaviour, nervousness, loss of appetite and sexual dysfunction have been reported to be associated with anxiolytic drugs [1].

The utilisation of plants in the treatment of certain human diseases is evidence of man's ingenuity. This makes the knowledge of therapeutic, biochemical and molecular activities of medicinal plants used in traditional medicine, become necessary and led researchers to scientifically study the possible beneficial effects of medicinal plants. The search for a novel and alternative treatment from medicinal plants is still progressing. Some effective medicinal treatment for anxiety related disorders have been reported elsewhere [4,5].

Citrus aurantium which belongs to the family Rutaceae is widely distributed and most commonly found in the tropics, including Nigeria. The leaves and essential oil obtained from *C. aurantium* peel is reported to have anxiolytic and sedative effects in mice [6]. *Citrus aurantium* decoction has been used to treat anxiety related disorders in the traditional medicinal practice in Nasarawa State, Nigeria. This study is aimed at evaluating the effects of aqueous root extract of *C. aurantium* (AECA) on anxiety, spontaneous alternation behaviour, and locomotor activity in Wistar rats.

2. MATERIALS AND METHODS

2.1 Chemicals

Diazepam (Roche, Lagos, Nigeria), normal saline (0.9% sodium chloride solution), 70% methanol and distilled water were used in the current study.

2.2 Experimental Animals

Male Wistar albino rats weighing 100 to 120 g, obtained from National Veterinary Research Institute (NVRI) VOM, Plateau State, Nigeria were used in this study. The animals were grouped and housed in cages with four animals per cage and maintained under standard laboratory conditions of humidity (60±2%), temperature (25±1°C) and a controlled 12 h dark and light cycle. The animals were fed with standard vital diet supplied by Grand Cereals Ltd., Jos, Plateau State, Nigeria and fresh water ad libitum. All the animals were acclimatized to laboratory condition for one week before commencement of experiment. Food was withheld for 24 h prior to each experiment. All the guidelines of Institutional Animal Ethics Committee were followed while handling the animals.

2.3 Plant Material

The roots, leaves fruits and of *C. aurantium* were collected from Keffi (Nasarawa State, Nigeria) by a traditional medical practitioner, identified and authenticated by Mr Musa Mara Likita of Plant

Science and Biotechnology Unit, Biological Sciences Department, Nasarawa State University, Keffi and voucher specimen (NSH 312) was deposited at the herbarium. The roots were cut into small pieces, cleaned, air-dried at room temperature (35-37℃) to minimize loss of volatile constituents, and grounded into fine powder using mortar and pestle. The powder was stored in an airtight container and kept at 25℃ for subsequent use.

2.4 Extraction Method

Five thousand grams (100 g) of the powdered sample was then soaked in 300 ml of water for 48 h through infusion method at room temperature. The extract was then filtered twice through cotton wool, then through Whatman filter paper (NO1). The filtrate was allowed to evaporate to dryness using a water bath at 45℃ and dried under reduced pressure to obtain aqueous extract and percentage extraction yield was determined of *Citrus aurantium*.

2.5 Phytochemical Screening

Phytochemical studies of AECA were done using the standard method previously described by Tiwari et al. [7] with some modifications for detecting the presence of secondary metabolites: alkaloids, phenol, flavonoids, carbohydrates, triterpenes, saponin, and tannins. The values obtained are triplicates for all secondary metabolites determined.

2.6 Acute Toxicity (Ld50) Study

Acute toxicity study was carried out according to method described by Lorke, [8]. The study was carried out in two phases. In the initial phase, nine rats were randomized into three groups (three rats per group) and were orally administered with doses of 10, 100, and 1000 mg/kg/bw of AECA weight respectively. The animals were observed for 24 h after treatment and number of death were recorded, if any. They were also observed for 72 h for any sign of delayed toxicity. Absence of mortality in animals used for the first phase informed the choice of doses for the second phase, in which 2000, 4000 and 8000 mg/kg of the extract were administered orally to another fresh set of three rats in each group. The animals were also observed for signs of toxicity and mortality such as respiratory distress, paw-licking, stretching and death and its oral median lethal dose (LD_{50}) were established by taking the square root of the product of the lowest lethal dose (i.e., the geometric mean) of the consecutive doses for which 0 and 100% survival rates at the end of the second stage.

2.7 Behavioural Tests

All the behavioural procedures were carried out between 8:00 am and 12:00 pm in a temperature controlled room (25±1℃). The rats were grouped such that each group consisted of equal number of rats.

2.7.1 Hole-board

The hole-board test is an anxiety paradigm based on novelty and uncertainty on the animal behaviour. Head-dipping is generally considered to provide a measure of exploitation that was distinct from motor activity. Thirty-six rats were divided into six groups, six per group. The animals were placed on board (40 × 40) with 16 holes (symmetrically distributed in four rows) 1 h after oral administration of normal saline 10 ml/kg, standard reference drug diazepam (2.5 mg/kg, i.p.) and different doses of AECA (50, 100 and 200 mg/kg, p.o) were administered 30 minutes before the test. The frequency of head dips into the holes during 10 minutes was registered immediately after the administration of AECA at different doses used and the total locomotion (number of floor units entered-the floor of the squares crossed with all paws) was also recorded. They are represented as mean total number of head dips [9].

2.7.2 Elevated plus maze

This study was carried out according to the method described by Akanmu et al. [10] with slight modification. Thirty rats were divided into five groups of six rats each. The animals were administered the crude sample of AECA (50, 100, and 200 mg/kg, p.o.). The control group received normal saline; 10 ml/kg while standard reference drug diazepam (2.5 mg/kg, i.p.) was administered 30 minutes before the test. At the start of the session the each was placed at the centre of the maze with the head facing an open arm and allowed to explore the maze for 5 minutes. During the 5-minute observation period, the following measurements were recorded: the number of entries and the time spent in open and closed arms, and the exploratory behaviour (total number of arm entries). An entry with all feet with

the exception of the tail put into one arm is defined as an arm entry in this experiment. All the animals were also subjected to same apparatus was carefully wiped between tests with a cotton wool moistened with 70% methanol after each animal to prevent odour bias.

2.7.3 Elevated zero maze

This study was carried out according to the method described by Tijani et al. [5]. Rats were randomly divided into five groups of four rats each. One hour before this test, rats were treated with graded doses of AECA (50, 100, and 200 mg/kg, p.o.). The control group received 10 ml/kg of normal saline while standard reference drug diazepam (2.5 mg/kg, i.p.) was administered 30 minutes before the test. One hour after drug administration, each rat was placed at the centre of the open arm (facing toward the closed chamber). The times spent in both open and closed arms of the maze were manually recorded. The maze apparatus was thoroughly wiped between tests with a cotton wool moistened with 70% methanol after each animal to prevent odour bias.

2.7.4 Open-field test

The Open Field (OF) test was carried out using the method described by Herrera-Ruiz et al. [11]. The OF apparatus consists of a transparent glass box (45×45 cm). The floor was divided by lines drawn into 9 equally sized squares. Thirty-six rats were randomized into six groups of six rats each. An hour before test session, rats were treated orally with graded doses of AECA (50, 100, and 200 mg/kg, p.o.). Another group received diazepam (2.5 mg/kg, i.p.) while the control received 10 ml/kg of normal saline orally. One hour after extract and 30 minutes after diazepam administration, each rat was placed individually in the centre of the apparatus. The locomotor (number of squares crossed with four paws) and exploratory activities (indicated by frequency of rearing) was recorded manually for 5 minutes' period [12,13]. The apparatus was cleaned between tests with cotton wool moistened with 70% methanol after each animal to prevent odour bias.

2.7.5 Y-maze task

The Y-maze test was carried out using the method described by Tijani et al. [5] using six animals per group (five groups). Y-maze is used to measure immediate general locomotor activity, spatial working memory and stereotypic behaviour [14]. The Y-maze is made up of three-arm horizontal maze of equal size (45 cm long and 7 cm wide with walls 14 cm high) in which the floor of each arms consist of wood 5 cm wide. Rats were initially placed within one arm (A), and the arm entry sequence (e.g., ABC BCA, CAB, where letters indicate each arm codes) and the number of arm entries were mutually recorded for each rat over a 6-minute period. The maze arms were cleaned with cotton wool moistened with 70% methanol after each animal to prevent odour bias. The spontaneous alternation determined from successive entries into the three arms on overlapping triplet sets in which three different arms are entered. An actual alternation percentage was defined as entries into all three arms consecutively except for its tail (i.e., each arm is labelled A, B or C: ABC, A, B, CAB, BCA, B, A, ABC, B, C).

In the above example, the rat entered 18 arms, twelve of which were alternations. An entry was defined as placing all four paws within the boundaries of the arm. Hence, an hour before this test, rats were treated with graded doses of AECA (50, 100, and 200 mg/kg, p.o.). The control groups received 10 ml/kg of normal saline and diazepam (2.5 mg/kg, i.p.). The percentage spontaneous alternation was calculated. The number of maximum spontaneous alternation is therefore the total number of arms entered minus two and the percentage [15].

2.7.6 Statistical analysis

Values were expressed as mean ± SEM from six animals and mean ± SEM for four animals in elevated zero maze. Statistical analysis was carried out using one-way analysis of variance (ANOVA) using Smith Statistical Package (SSP). Student's t-test was used to calculate the significant difference if any at 95% probability level.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

Results of phytochemical screening of the aqueous root extract of *C. aurantium* (AECA) are shown in Table 1. As illustrated, the major phytochemical constituents of AECA are phenol, saponins and tannins.

Table 1. Results of phytochemical screening of the aqueous root extract of *Citrus aurantium* (AECA) the percentage extraction yield was 20%

Secondary metabolites	Inference
Alkaloids	+
Phenol	++
Flavonoids	+
Carbohydrates	-
Triterpenes	+
Saponin	++
Tannins	++

+ Slightly present, ++ present, - Absent

The phytochemical screening of the crude extract revealed the presence of alkaloids, phenol, flavonoids, triterpenes, saponin, and tannins. The therapeutic potential of medicinal plant extracts used as traditional remedies have been attributed to a combination of active secondary metabolites which might be involved in inhibiting some mediators that could cause anxiety [15]. The alkaloids, erysodine and erysothrine are reported to possess anxiolytic-like effects in mice [16]. Montanine, anisoquinoline alkaloids are reported to have anxiolytic, antidepressant and anticonvulsant-like effect in mice [17]. The behavioural effect of carvacrol, a monoterpenic phenol was reported [18]. However, flavonoids have the ability to bind to the benzodiazepine site of $GABA_A$ receptor [19]. Salgueiro et al. [20] reported anxioselective effects of two flavonoids including chrysin and apigenin on central benzodiazepine receptors. Moreover, triterpene rich fractions were shown by Chen et al. [21] to possess anxiolytic-like effects. Saponins have been shown to possess anxiolytic properties [22]. Tannins may also be responsible for the observed neurological effect of this plant due to its possible effect against many CNS disorders [23].

3.2 Effect of AECA on Hole-Board Model

The result showed that the extract (50, 100 and 200 mg/kg) significantly ($p < 0.05$-0.001) increased the frequency of head dips on hole-board model as dose decreases. Diazepam showed no significant difference when compared to the control, hence more potent in increasing the frequency of head dips on the hole-board than the AECA (Fig. 1). The decrease observed in rats administered with AECA is said to be dose-dependent on this model. The frequency of the head dips decreases as the concentration increases.

The results of the behavioural studies revealed that the extract (at doses 50 and 100 mg/kg) produced significant anxiolytic effect in rats on hole-board, elevated plus maze, elevated zero-maze, and spontaneous alternation behaviour on the open field as well as locomotor and exploratory behaviour of rats on Y-maze.

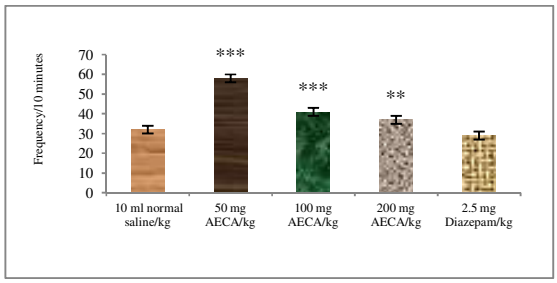

Fig. 1. Effect of AECA on frequency of head dips in hole-board model

***, *** Significantly different from the control at p<0.01 or p<0.001 respectively*

The anxiolytic activities of some agents have been assessed by using the hole-board test [9]. However, increased number of head dips into the holes on the board means reduced anxiety state. In this study, hole-board paradigm showed that exploratory behaviour decreased with increase in AECA dose. Therefore, further investigations on the effects of AECA on anxiety state using the elevated-plus maze may be necessary.

3.3 Effect of AECA on EPM

The extract (50 and 100 mg/kg) significantly ($p<0.05$-0.001) decreased the time spent in the close arm of the EPM. However, 200 mg/kg of AECA significantly increased the time spent in the closed arm of the EPM. Diazepam was more potent in reducing the time spent in closed arm of the EPM than the AECA except at concentration of 200 mg (Fig. 2).

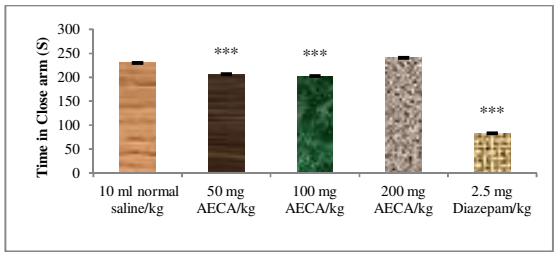

Fig. 2. Effect of AECA on time spent in close arm of the EPM

**** Significantly different from the control at p<0.001*

AECA significantly ($p<0.001$) increased the time spent in the open arm of the elevated plus maze except for 200 mg/kg AECA which decreased time spent in the open arm of elevated plus maze significantly, while diazepam increased time spent on open arm of the maze (Fig. 3).

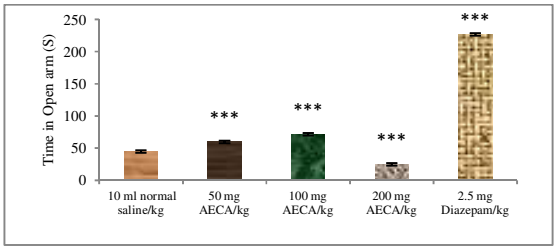

Fig. 3. Effect of AECA on time spent in opened arm of the EPM

**** Significantly different from the control at p<0.001*

The EPM test represents one of the most widely used animal models for screening anxiolytics [9,24]. This test is able to reproduce anxiolytic or anxiogenic effects in animals such that anxiolytics produce increase in the number of entries into the open arms of the maze and the time spent there, while anxiogenic activity of extract at doses of 50 and 100 mg/kg significantly increased the time spent in the open drugs produces the opposite effect [9,24]. In this study, *C. aurantium* aqueous root arm of the elevated plus-maze. However, the extract produced a significant increase in time spent in the closed arm of the maze at 200 mg/kg. This study is consistent with standard anxiolytics behaviour similar to benzodiazepines, a metabolite of diazepam with anxiolytic effect at low doses and anxiogenic or sedative effect at higher doses [4]. Diazepam, a benzodiazepine (receptor agonist) binds to $GABA_A$ receptors to increase the frequency of chloride channel openings resulting in hyperpolarization [25]. It increases the frequency of open-arm entries and the time spent in the open arms confirming its anxiolytic effects [26]. However, at higher dose, the decreased activity was concluded to have a sedative effect. The *C. aurantium* aqueous root extract had similar effects on these parameters at 200 mg/kg body weight whereas administration of lower doses (50-100 mg/kg body weight) confirmed its anxiolytic effect.

3.4 Effect of AECA on Elevated Zero-Maze

The aqueous extract significantly ($p<0.001$) decreased the time spent in the closed arm of the elevated zero maze except for 200 mg AECA/kg where significant increase in time spent in the open arm was observed (Fig. 4).

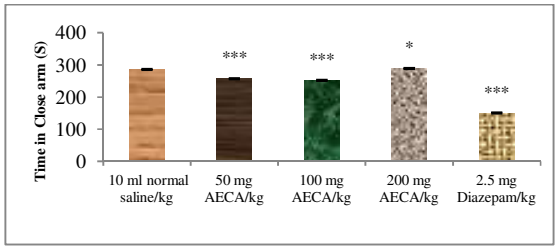

Fig. 4. Effect of AECA on time spent in closed arm of zero-maze

, * Significantly different from the control at p<0.05, or p<0.001 respectively*

AECA significantly ($p<0.001$) increased the time spent in the open arm of the elevated zero maze except for 200 mg AECA/kg which decreased time spent in the open arm of elevated zero maze significantly, while diazepam increased time spent on open arm of the maze (Fig. 5).

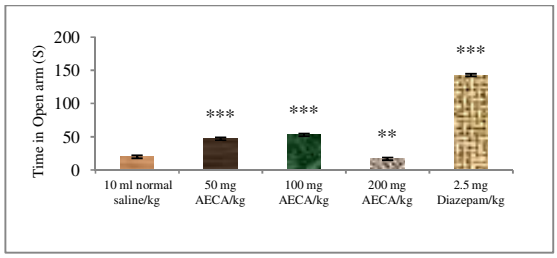

Fig. 5. Effect of AECA on time spent in open arm of zero-maze

***, *** Significantly different from the control at p<0.01 or p<0.001 respectively*

The anxiolytic effect of AECA was further confirmed by the results obtained from the use of the elevated zero-maze. The zero-maze has two major advantages over the elevated plus maze: lack of ambiguity associated with the interpretation of the time spent in the central area of the elevated plus maze and allowance of uninterrupted exploration [27]. The extract at 50 and 100 mg/kg produced anxiolytic-like effect which is clearly defined by the increased time spent in the open quadrant of the zero-maze apparatus. However, at 200 mg/kg body weight, the extract exerted anxiogenic-like effect in the various test methods used in the study. This might be due to the extreme spectrum of anxiolytic sedative effects characterized by sedation like behaviour [5]. This is consistent with the effect of sedative-anxiolytics.

3.5 Effect of AECA on Total Locomotor Activity

The extract at 50 and 100 mg/kg significantly ($p<0.05$-0.001) increased the total locomotive activity of rats in open field apparatus (Fig. 6). However, upon administration of 200 mg/kg of AECA a significant decrease in the locomotive activity of rats was observed while diazepam increased time spent on open arm of the maze (Fig. 6).

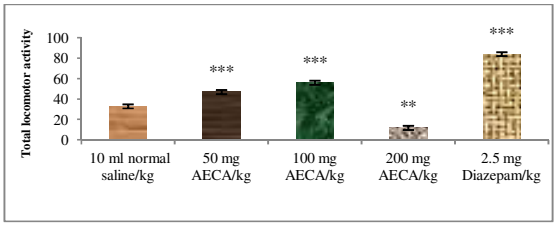

Fig. 6. Effect of AECA on total locomotive activity on open field apparatus

***, *** Significantly different from the control at p<0.01 or p<0.001 respectively*

The open-field apparatus provides information on anxiety-related behaviour characterized by natural aversion of animals to an open brightly lit area [25]. Animals express their anxiety and fear of the centre and spend more time in the protective corners and in freezing state [5]. Anxiolytics increase total locomotor activity resulting in a reduction of time spent in corners, and increased time spent in the centre. Anxiolytics also decrease time spent in freezing state hence decreasing the exploratory behaviour. The extract at 50 and 100 mg/kg body weight increased total locomotive activity and increased frequency of rearing in treated rats. This study further confirmed the anxiolytic potential of AECA. Natural products of plant origin may elicit anxiolytic effects via interaction with some endogenous mediators such as GABAergic and serotonergic pathways in the body [25]. The present study showed that AECA possesses potent anxiolytic effects as evidenced in the results obtained from the various models used.

3.6 Effect of AECA on Rearing

The extract significantly ($p<0.05$-0.001) increased the frequency of rearing at dose concentration of 50 and 100 mg/kg except for 200 mg/kg AECA which decreased the frequency of rearing while diazepam increased the frequency of rearing (Fig. 7).

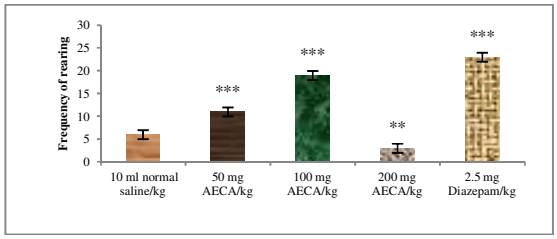

Fig. 7. Effect of AECA on rearing

***, *** Significantly different from the control at p<0.01 or p<0.001 respectively*

3.7 Effect of *C. aurantium* Extract on Spontaneous Alternation Behaviour of Rat in Y-maze

The extract (50 and 100 mg/kg) at $p<0.01$-0.001 increased the spontaneous alternation behaviour in rats compared with the control except for the 200 mg/kg dose which showed no significant effect. However, diazepam showed a significant increase in the spontaneous alternation behaviour (Fig. 8).

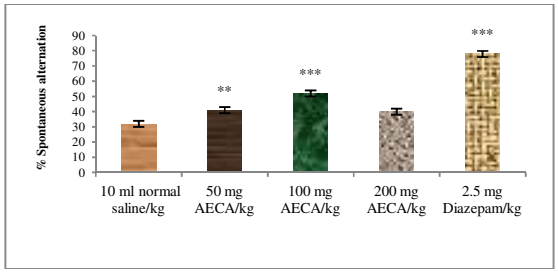

Fig. 8. Effect of AECA on spontaneous alternation behaviour

***, *** Significantly different from the control at p<0.01 or p<0.001 respectively*

Spontaneous alternation behaviour determined using Y-maze test is regarded as a measure of spatial short-term memory in animals including rats [28]. A rat must remember the least recently visited arm in order to alternate the arm choice [29]. The extract at 50 and 100 mg/kg body weight administered to healthy rats produced significant increase in spontaneous alternation behaviour.

Secondary metabolites such as tannins and saponins present in AECA may be responsible for the observed anxiolytic-like effects in treated rats. Similarly, tannins are reported to possess a significant effect against Alzheimer's disease [30] and epilepsy [31].

4. CONCLUSION

The present results provide evidence for anxiolytic effects of AECA in rats which might be possible through BZD-$GABA_A$ mechanism. This activity may be attributed to the presence of the secondary metabolites presence thus providing rational scientific evidence for its continuous use in the therapeutic management of neurological disorders characterized by anxiety, and amnesia. Further studies on identifying the specific metabolite(s) responsible for the observed pharmacological potential and possible mechanism(s) of actions of these bioactive compounds should be investigated.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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