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ORIGINAL RESEARCH ARTICLE

An Appraisal of the Interactions between Hydroalcholic Leaf Extract of *Cnidoscolous acontifolius* and Benzodiazepine-$GABA_A$ Receptor Complex

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ABSTRACT

The *Cnidoscolous acontifolius* hydroalcholic leaf extract (CAHLE) exerts sedative and anxiolytic effects in mice. This study was designed to study the interactions that may exist between CAHLE and benzodiazepine receptor sites on $GABA_A$ macromolecular complex. Swiss albino mice (18-22g) were randomly allotted to eight groups (n=5 each). Group 1 received 10 mL/kg of normal saline and groups 2-7 received 400 mg/kg of CAHLE only, 1 mg/kg of diazepam, 200mg/kg, 400mg/kg and 800mg/kg of CAHLE plus 1 mg/kg of flumazenil each and 1mg of flumazenil respectively. Group 8 received normal saline plus 1mg/kg of flumazenil.All drugs and test compounds were administered via intraperitoneal route. Data analysis was by one factor analysis of variance (ANOVA) followed by post hoc analysis using Student Newman Kuels multiple comparison tests. Statistically significant inhibition of the mean frequency of rearing and total number of fine and basic movements on the elevated plus maze by CAHLE/flumazenil combination compared to flumazenil/ normal saline treated groups was observed. The study concluded that CAHLE interacted with the benzodiazepine receptor site on $GABA_A$receptor complex and may explain one mechanism by which CAHLE exerts sedation and anxiolysis in mice.

Key words: Neurotransmitters, Receptors, anxiolytic, sedative and central nervous system.

INTRODUCTION

Cnidoscolous acontifolius was taxonomically recognized by I. M. Johnston in 1923 [8] and it is known as “tree spinach” or “chaya” by natives of Mexico and Central America [16]. It is a large, fast growing leafy perennial shrub, native to the Yucatán Peninsula of Mexico [16]. *Cnidoscolous acontifolius* has been a highly domesticated plant for centuries and was relatively unknown outside of Central American region [16]. It was introduced to the West African sub region in 1977 through Ghana, from an agricultural research station in Puerto Rico [13]. It spread to the neighbouring countries in the ensuing years including Nigeria where it has become widely domesticated [13]. It has soft stems which exude a milky sap when cut. It can grow to be 3-6 meters tall. It is quite popular in western Nigeria among the Yoruba speaking people, cookedas a leafy vegetable and eaten as soup [19]. The edible parts of chaya plant, which taste like spinach when cooked, provide important nutritional sources for protein, vitamins (A and C), minerals (calcium, iron, and phosphorus), niacin, riboflavin, and thiamine [15]. Although *Cnidoscolous acontifolius’* (chaya) main use in its original area of domestication, was a valued food source, and continues to be an important medicinal plant [16]. Much of the recent spread of chaya into new areas can be attributed to its medicinal value [16]. A wide variety of claims have been made as to the medical efficacy of chaya as a treatment for numerous ailments, ranging from the ability to strengthen fingernails and darken greying hair strengthen fingernails [16], it has also been used as a cure for alcoholism [4], insomnia [18], venereal disease [11], gout [14], scorpion stings [17], and as an improvement of brain function and memory [9]. A published study on its anti diabetic properties indeed found a significant drop in blood sugar levels in diabetic rabbits fed increasingly higher quantities of chaya [10]. A published study has shown CAHLE to possess sedative, anti nociceptive and anxiolytic

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actions [20].These demonstrated neuro pharmacologic activities of hydroalcholic extract of *Cnidoscolous acontifolius* (CAHLE) may be exerted via the interactions of CAHLE with neurotransmitter systems in the brain.The neuroanatomical circuits that support anxiety behaviour are modulated by a variety of chemical neurotransmitter systems [6]. These include the peptidergic neurotransmitters, CRH, neuropeptide Y (NPY), and substance P, the monoaminergic transmitters, NE, serotonin (5-hydroxytryptamine or 5-HT), and dopamine (DA), and the amino acid transmitters, GABA and glutamate [6]. The observed biologic effects in animal studies are a sum of different chemical reactions between active constituents of test compounds and cellular components. Bioactive principles present in plant extracts, through additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with physiological processes may be responsible for the observed effects [5]. One of these possible target sites at which CAHLE may exert itself is the $GABA_A$ receptor complex. This is because the BZD (benzodiazepine) and gamma amino butyric A ($GABA_A$) receptors form parts of the same macromolecular complex and although they constitute distinct binding sites, they are functionally coupled and regulate each other in an allosteric manner [7]. Central BZD-receptor agonists potentiate and prolong the synaptic actions of the inhibitory neurotransmitter, GABA, by increasing the frequency of GABA-mediated chloride channel openings [7]. Agents with sedative and anxiolytic effects modulate the function of the $GABA_A$/BZD-receptor–chloride ionophore complex.This study purposed to investigate the possible interactions between CAHLE and this receptor site using behavioural paradigms such as open field test and elevated plus maze.

Fig 1: *Cnidoscolous acontifolius* Leaf [8].

MATERIALS AND METHODS

Plant Material:

The leaves of *Cnidoscolous acontifolius*were collected in November 2008 during the dry season in backyard garden of a family at Owode estate Apata, Ibadan, southwest Nigeria. The leaves were identified and authenticated by Mr. T.K. Odewo, a taxonomist in the Forestry Research Institute of Nigeria (FRIN), Ibadan where a voucher specimen (FHI; 107727) of the plant already exist.

Extract Preparation:

The leaves of *Cnidoscolous acontifolius* were air-dried at room temperature (28^0C) powdered and weighed (200g). The powdered leaves were cold extracted with methanol and water (80:20) over a 72 hour period. The marc leaves were sieved using the whatman No. 1 filter paper and the residue collected. The residue was concentrated in vacuo at 60^0C and air dried at room temperature until all the methanol/water had been removed. The residue was weighed to be 10.2g. The yield was 5.1% w/v. The dried extract was dissolved in normal saline (1: 50 w/v) to obtain a stock solution of 800 mg/kg which was further diluted to 200 mg/kg and 400 mg/kg.These doses were employed in our study to determine the interactions between *Cnidoscolous acontifolius* hydroalcholic leaf extract (CAHLE) and benzodiazepine receptor complex.

Drugs:

Diazepam (Roche, Switzerland) and Flumazenil(Sigma Chemicals, USA).

Experimental Protocol:

Male and female Swiss albino mice weighing 18-22g obtained from the animal house of the Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria were purchased for this study. All animals were kept at room temperature (28 ± 1^0C, 70-80% humidity, 12-hr light/dark cycle) in the animal holding unit of the laboratory of the department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife for one week before the commencement of the study. All experiments were conducted in a quiet testing room with overhead fluorescent lights between 8 am and 3 pm at an ambient temperature of 27° to 28°C to avoid the potential confounding variable of circadian rhythm. Thirty minutes before testing mice were randomly allotted to eight groups of five mice each (n=5). Groups 1 received 10 mL/kg of normal saline and groups2-7 received 400 mg/kg of CAHLE only, 1 mg/kg of diazepam, 200mg/kg, 400mg/kg and 800mg/kg of CAHLE plus 1 mg/kg of flumazenil each and 1mg of flumazenil respectively. Group 8 received normal saline plus 1mg/kg of flumazenil.All drugs and CAHLE were administered intraperitoneally.

After injections, the mice were returned to their cages to reduce any confounding influences or exposure to any additional unfamiliar environments

Open Field Test:

The CAHLE interaction at the benzodiazepine receptor site on the $GABA_A$ receptor was tested by the group receiving flumazenil with CAHLE, a known benzodiazepine receptor antagonist.Each mouse was placed in an open field box (68 × 68 × 45 cm) equipped with a video camera. Observations over a 30 minutes period were recorded for assessment later. The spontaneous motor (exploratory) activities of the mice were measured. Three parameters namely locomotion (line crossing), rearing and grooming were recorded. Before the introduction of each animal into the open field box, it was cleaned with 70% alcohol to eliminate possible bias due to odour that might have been left behind by the previous subjects.Locomotion was defined as the number of floor units entered or crossed with all paws; frequency of grooming was counted as the number of body cleaning with paws, picking the body, pubic area and face washing actions. The number of times the animal stands on its hind legs with the forearms free or against the wall of the observation cage was regarded as the frequency of rearing [3].

The elevated plus-maze test:

Each animal after thirty minutes of administration of CAHLE was placed in the center of the EPM, facing an open arm, and behavioural responses were evaluated by the EPM for 5 minutes. The EPM was networked with Motor Monitor software (Hamilton-Kinder, Poway, California) with laser sensors integrally attached to the EPM, which tracked the number of total basic and fine motor movements. Basic motor movements are the simple count of beam breaks in the EPM. Each time a photo beam is interrupted the basic movement count is increased. These movements reveal a gross measure of locomotion but do not distinguish what type of activity is being performed. Fine motor movements are a compilation of small animal movements such as grooming, rearing head weaves, or bobs. The experiment was carried out in a quiet laboratory to ensure that no external stimuli other than the height of the plus maze could invoke maze anxiety. Before the introduction of each animal into the elevated plus maze it was cleaned with 70% alcohol to eliminate possible bias due to odour that might have been left behind by the previous subjects.

Ethical Considerations:

This study was conducted in line with standard practices, procedures, international protocols and accepted principles for laboratory animal use and care as found in the guiding principles for the care and use of animals in research and teaching (Helsinki declaration 2010 update).

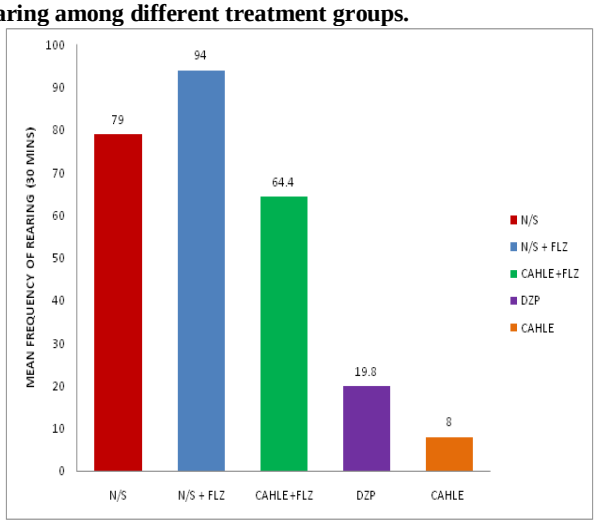
Statistical Analysis:

The test doses were compared with control by one factor analysis of variance (ANOVA) followed by post hoc analysis using Studentized Newman Kuels multiple comparison tests. Test doses were also compared with the standard drug (diazepam). The results were analyzed using the Graph Pad Prism 4.0 statistical software. All results were expressed as mean ± standard error of mean (S.E.M.).Values of P less than 0.05 were taken as significant (i.e. $P<0.05$).

RESULTS

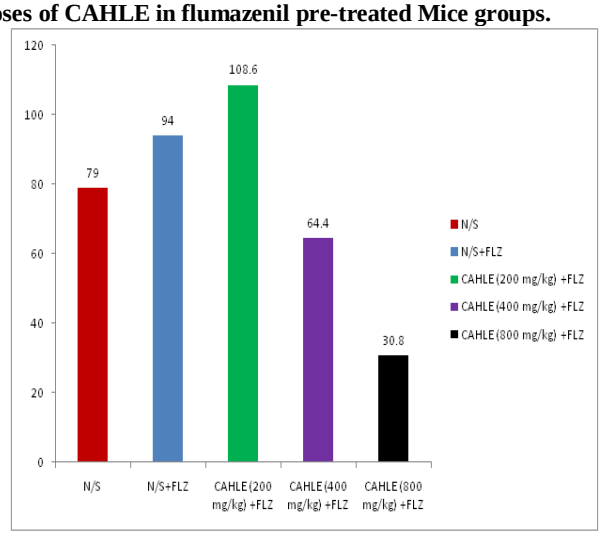
The study revealed a statistically significant reduction in the mean frequency of rearing in the group treated with a combination of flumazenil and CAHLE compared to normal saline and flumazenil/normal saline treated groups. Diazepam and CAHLE only, also showed significantly lower mean frequency of rearing compared to normal saline and flumazenil/normal saline treated groups.CAHLE only group, showed statistically significant reduction in rearing compared to the CAHLE+ flumazenil group (**Fig 2 & Table 1**). The study further revealed a dose dependent reduction in the mean frequency of rearing as the dose of CAHLE was increased from 200 mg/kg through 400 mg/kg to 800 mg/kg while keeping the dose of flumazenil constant at 1 mg/kg (**Fig3 & Table 2**).Analysis of the basic movements on the elevated plus maze revealed a significant reduction in basic movements by CAHLE, CAHLE/flumazenil combination and diazepam compared to control (normal saline +flumazenil) (**Table 3**).CAHLE and diazepam showed significantly greater decrease in basic movements when compared to CAHLE/flumazenil combination. The fine movement on the elevated plus maze showed a similar picture in which CAHLE/flumazenil combination, CAHLE and diazepam all significantly reduced the number of fine movements made (**Table 4**). CAHLE and diazepam treated groups showed significantly greater reduction in fine movements compared to CAHLE/flumazenil treated group.

Fig 2: A graphical representation of the mean frequency of rearing among different treatment groups.



N/S –Normal saline; N/S + FLZ – Normal saline/ Flumazenil Co-administration; CAHLE + FLZ- CAHLE/FlumazenilCo-administration; DZP- Diazepam; CAHLE- Cnidoscolous acontifolius hydro alcholic leaf extract.

Fig.3.The graph of mean frequency of rearing against varied doses of CAHLE in flumazenil pre-treated Mice groups.



N/S –Normal saline; N/S + FLZ – Normal saline/ Flumazenil Co-administration; CAHLE + FLZ- CAHLE/ Flumazenil Co-administration

Table 1: showing mean frequency of rearing, standard error of the mean and the level of significance of different treatment groups compared with normal saline and normal saline/flumazenil combination

Treatment Groups	Mean±SEM	P<0.05 (compared with N/S)
Normal Saline	79±2.67	
Normal Saline + Flumazenil	94±1.41	YES
CAHLE+ Flumazenil	64.4±2.48*	YES
Diazepam	19.8±1.28*	YES
CAHLE	8.0±0.84*	YES

P<0.05(compared with N/S +flumazenil)

Table 2: Table showing mean frequency of rearing, standard error of the mean and the level of significance of graded doses of CAHLE/flumazenil combination compared with normal saline and normal saline/flumazenil combination.

Treatment Groups	Mean±SEM	P<0.05(compared with N/S)
Normal Saline	79±2.67	
Normal Saline + Flumazenil	94±1.41	YES
CAHLE (200mg/kg)+ Flz	108.6±2.48*	YES
CAHLE (400mg/kg)+ Flz	64.4±2.48*	YES
CAHLE (800mg/kg)+ Flz	30.8±0.84*	YES

P<0.05(compared with N/S +flumazenil)

Table 3: Showing mean basic movement, standard error of the mean and the level of significance by different treatment groups on the elevated plus Maze.

Treatment Groups	Mean±SEM
Control(flumazenil+Normal Saline)	258.6±2.62
CAHLE+FLZ	169.4±2.43*
CAHLE	106.2±2.51*
Diazepam	120.4±2.49*

P<0.05 (compared with normal saline/flumazenil combination)

Table 4: Showing mean frequency of fine movement, standard error of the mean and the level of significance by different treatment groups on the elevated plus Maze.

Treatment Groups	Mean±SEM
Control(flumazenil+Normal Saline)	119.2±1.41
CAHLE+FLZ	98.6±2.06*
CAHLE	9.0±1.64*
Diazepam	19.8±1.28*

DISCUSSION

Rearing is a parameter that is measured under the open field test alongside grooming and locomotion and they are all controlled by central nervous system in rodents [1]. These parameters are affected by chemical events that affect the neurotransmitters and their receptors. These parameters (i.e rearing, grooming and locomotion) are increased or decreased by drugs or test compounds that interact with the various neurotransmitter systems in the central nervous system. Drugs or test compounds with central depressant effects are known to reduce the frequency of these parameters in rodents [1]. The converse is also true, centrally acting stimulants increase these parameters. Gamma amino butyric acid (GABA) is an inhibitory transmitter that acts by reducing the rate of neuronal firing via the GABA$_A$ receptor in the central nervous system, hence reducing the overall activity of an animal [7]. GABA$_A$ is abundant in the cerebral cortex; it is a chloride ion channel that is activated by GABA. Activation of this channel by GABA results in an influx of chloride. This increased chloride flux serves to hyperpolarise the postsynaptic neurons and thus reduce the excitability of the neuron. The summation of this effect is observed as reduced overall activity. In this study, CAHLE significantly reduced the mean frequency of rearing compared to normal saline and diazepam treated groups. This suggests that CAHLE has a central depressant action that is more potent than a known standard diazepam. The decrease in activity may arise from central inhibitory influences of inhibitory neurotransmitters particularly GABA. In this study the CAHLE + flumazenil treated group showed significantly reduced mean frequency of rearing when compared with flumazenil and normal saline treated groups. This finding may suggest that CAHLE interacts with benzodiazepine receptor.

Benzodiazepine receptor is a component part of the $GABA_A$ macromolecular complex [7]. This site is blocked competitively by flumazenil, a known benzodiazepine receptor antagonist. The interaction of flumazenil with this site caused a significant increase in the mean frequency of rearing among mice groups treated with flumazenil compared to normal saline only treated mice. This rise in the mean frequency of rearing was reversed by the addition of CAHLE. The mean frequency of rearing reduced significantly among the mice group treated with flumazenil/CAHLE. This reversal of increased activity observed in flumazenil treated groups by the co-administration of CAHLE and flumazenil therefore suggest an interaction between CAHLE and the benzodiazepine receptor. This is further supported by the observation that gradually increasing the dose of CAHLE in the CAHLE/flumazenil combination (200 mg/kg-800 mg/kg) progressively reduced the mean frequency of rearing in a dose dependent fashion (Fig 3)**.** This may be explained by the fact that CAHLE through a gradually increasing dose overcame the antagonism at the benzodiazepine receptor (BZD) in a competitive way. The BZD and gamma amino butyric A ($GABA_A$) receptors form parts of the same macromolecular complex and although they constitute distinct binding sites, they are functionally coupled and regulate each other in an allosteric manner [7].In conclusion this study has shown that CAHLE may exert it central depressant effect by interacting with $GABA_A$ receptor, a major inhibitory transmitter receptor in the central nervous system through the benzodiazepine receptor site.

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