

SEDATIVE AND ANXIOLYTIC EFFECTS OF THE EXTRACTS OF THE LEAVES OF *STACHYTARPHETA CAYENNENSIS* IN MICE.

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*E-mail: gbolayiw@oauife.edu.ng**Abstract**

The leaves are used ethnomedicinally in Nigeria and other parts of the world for insomnia and anxiety among other uses. The investigations sought scientific evidence for the ethnomedicinal use of the leaves for the management of insomnia and anxiety as well as the neural mechanisms for the activities. The sedative and anxiolytic effects of the extracts of the leaves of *Stachytarpheta cayennensis* were examined in this study. The methanolic extract (5-50 mg/kg, i.p.) as well as the ethylacetate (10-50 mg/kg, i.p.), butanol and aqueous fractions (5-50 mg/kg, i.p.) of the extract were examined. Sedation was assessed as reduced novelty-induced rearing (NIR), reduced spontaneous locomotor activity (SLA) and increased pentobarbitone-induced sleeping time (PIST) in mice. The anti-anxiety effect (methanol 2.5-5.0; butanol 5.0; aqueous 20.0; ethylacetate 25.0 mg/kg, i.p.) was assessed using an elevated plus maze. LD₅₀ was calculated for the extract and the fractions after the intraperitoneal route of administration using the Locke method. The methanolic extract, the butanol and the aqueous fractions inhibited rearing and spontaneous locomotion but prolonged pentobarbitone induced sleep. The ethylacetate fraction however increased both rearing and locomotion and decreased pentobarbitone sleeping time. The butanol and aqueous fractions, but not the methanol extract showed indices of open arm avoidance consistent with anti-anxiety effect. Naltrexone (2.5 mg/kg, i.p.) reversed the inhibition of rearing, locomotion and prolongation of pentobarbitone sleep due to the aqueous fraction of the extract. Flumazenil (2mg/kg, i.p.) abolished the effects of both methanolic extract and the butanol fraction on rearing, locomotion, pentobarbitone sleep and anxiety model. The methanolic extract, the butanol and aqueous fractions possess sedative activity while the ethylacetate fraction possesses stimulant property. The anxiolytic effect was found in both the aqueous fraction and the butanol fraction but not in the main methanol extract and also not in the ethylacetate fraction. Flumazenil, blocked the effect of the leaves of *Stachytarpheta cayennensis* on rearing, locomotion and elevated plus maze suggesting that GABA receptors are involved in the observed sedative and anxiolytic activities. This study also found opioid receptors involved in the sedative activity of the leaves of *Stachytarpheta cayennensis*. The rationale for the ethnomedicinal use of the leaves for the management of insomnia and anxiety were confirmed scientifically in this study.

Keywords: *Stachytarpheta cayennensis*; sedative; anxiolytic; opioid receptors; GABA receptors.

List of abbreviations: Aq. -Aqueous fraction; BuOH -Butanol fraction; CPZ-Chlorpromazine; DZP-Diazepam; EtOAc -Ethylacetate fraction; FMZ-Flumazenil; MeOH-Methanol Extract; NIR-Novely-induced rearing; SLA-Spontaneous locomotor activity; PIST-Pentobarbitone-induced sleeping time

Introduction

Stachytarpheta cayennensis (L.C.Rich) Vahl. Family verbanaceae is found in several regions of the world including the Bahamas, Brazil, Ghana, Haiti, India, Jamaica, Cameroon, Malaysia, Mexico, West Indies and Nigeria as weeds (Schwontkowski, 1993). The extracts of the leaves are used ethnomedicinally in many tropical countries including Nigeria as sedative, anxiolytic and antipsychotic remedies (Burkill, 1966; Akanmu et al, 2005). Also in Ghana, a West African nation like Nigeria, the extracts of the leaves of *Stachytarpheta cayennensis* (*S.cayennensis*) are employed in their traditional medicine for the management of mental illness (Burkill, 1966). It is an annual and sometimes perennial herb growing to the height of 60 to 150cm (Haselwood and Motter, 1966).

Neurological conditions such that can be precipitated by insomnia are normally treated with sedatives (Heller and Benington, 1995). Insomnia and other sleep disorders are worldwide medical problems and attempts to find new remedies, especially with herbs are steps in the right direction. Currently there are no suitable drugs for the treatment of chronic insomnia. People rapidly develop tolerance to existing sleeping medications, leading them to take higher doses and to mix medications. This can result in bad side effects and even worse insomnia when they try to reduce the medications (Morrissette and Heller 1998). The extracts of the leaves of *S. cayennensis* were then investigated pharmacologically to look at the possibility of confirming the use of the leaves to resolve insomnia. It is also possible that some of the shortcomings of synthetic drugs for insomnia, particularly drowsiness, dependence and addiction as we have with the benzodiazepines may be absent in the extracts of the leaves of *S. cayennensis*. Medicinal herbs may also be cheap and accessible, especially in resource limited regions of Africa, hence the need to screen potential herbal remedies for safety and potency.

Anxiety disorders afflict approximately 10% of the world population in one or many of its forms such as panic attacks, social phobias or generalized anxiety disorders (Ballenger et al, 2001). Anxiety is characterized by excessive or irrational fear associated with a real or anticipated stimulus. Anxiety is often accompanied by phobic avoidance and a constellation of somatic symptoms. Phobic avoidance may be seen as an adaptive mechanism that enables the individual to minimize exposure to situations that may be anxiety provoking. But such avoidance can become maladaptive when it leads to significant behavioral changes, including social isolation and agoraphobia (Connor and Blazer 2007). Common somatic manifestations of anxiety include cardiovascular (palpitations, non-cardiac chest pain), respiratory (dyspnea), neurological (dizziness, headache, tremulousness), laryngeal (lump in the throat), and gastrointestinal (diarrhea, abdominal cramps) symptoms. The somatic complaints are often the impetus for the individual to seek treatment, usually with a primary care provider, and can result in extensive medical workups that fail to find an underlying medical etiology for the symptom (Ballenger et al, 2001; Connor and Blazer 2007). This investigation into the potential of the extracts of the leaves of *S. cayennensis* as an anxiolytic remedy is aimed at confirming the ethnomedicinal use as well as advice on its safe use.

Previous work on the leaves of *S. cayennensis* reported its anthelmintic (Alice et al, 1991), anti-inflammatory (Vela et al, 1997), anti-nociceptive (Perido et al, 2006), anti-ulcerogenic (Almeida et al, 1995), antimalarial (Okonkon et al, 2008) and anti-diarrhoea effects (Kvist et al, 2006). Our previous study reported the sleep modulation and toxicity of the leaves of this plant with a projection that the mechanism of its

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sedative activity and other effects would be investigated (Akanmu et al, 2005). The present study reports the sedative and anxiolytic effects as well as the neural mechanism of the alterations of the extracts of the leaves of *S. cayennensis* as a sedative and an anxiolytic agent. The receptors involved in the observed sedation and anxiolytic effects of the plant are also elucidated in this study.

Materials and Methods

Plant Materials

S. cayennensis leaves were collected from the wild on the campus of Obafemi Awolowo University, Ile-Ife, Nigeria during the months of August – September. The staff of the Department of Botany (Professor O. Olorode and Dr. H. C. Illoh), Faculty of Science of the Obafemi Awolowo University identified the plants as *S. cayennensis*. Herbarium voucher (FHI-106491) for *S. cayennensis* has been deposited with the National Herbarium, Forestry Research Institute of Nigeria, Ibadan.

Preparation of Plant Extracts for biological activities

The leaves of the plants were air-dried for 48 hours in the laboratory at a room temperature of about $27 \pm 1^\circ\text{C}$. The dried leaves were pulverized and 500 g of the powdered leaves of the plant was soaked in 3 litres of 1:1 methanol: water solution for 48 hours. The marc was re-extracted twice and the combined extracts were concentrated *in vacuo* to yield 88.52 g (17.7%) methanolic extracts. The extract obtained was concentrated to a waxy cake on dryness. The methanolic extract was successively partitioned into ethylacetate, n-butanol and water. The fractions were concentrated to give ethylacetate fraction (19.20 g, 21.69%), butanol fraction (35.52 g, 40.13%), and aqueous fraction (28.82 g, 32.56%). 20ml stock solution of each extract and fraction was prepared by dissolving 2g of the extract in 20ml 0.9% saline to give a 100 mg/ml solution for some of the tests. Equal amount of the ethylacetate fraction was dissolved in DMSO (3%) in 0.9% saline. The partitioning of the methanolic extract into butanol and ethylacetate was to locate the fraction with the active principle, particularly to see if the polar medium (butanol) or the non-polar medium (ethylacetate) contains the active pharmacological principle.

Animals

The experiments on total activity and anxiety were carried out with MNRI male mice (32-40 g) purchased from Charles River Inc. (Germany). The mice were housed in an air-conditioned room with temperature set at 22°C , 80% relative humidity, light on at 8 a.m-6 p.m and maintained on a standard laboratory pellet diet (Altromin, Lage/Lippe, Germany).

For the experiments on novelty induced rearing, male Swiss mice (20 – 27g) were used. The animals were bred and housed in a well-lit and aerated room (temperature $26 \pm 1^\circ\text{C}$, relative humidity 66%) in the Animal House, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria. The animals had free access to drinking water and standard commercial diet (Guinea Feeds Brand, Bendel Feeds and Flour Mills Ltd, Nigeria). The cages were cleaned once a week and five mice were housed in a standard cage. The animal experiments were carried out according to the protocol approved by the committee on animal use and care of the Obafemi Awolowo University, Ile-Ife, Nigeria.

Chemicals and Drugs

The drugs used for the experiments in this report are: atropine sulphate (BDH Chemicals Ltd England), pentobarbitone sodium (Saggittal, M&B, England), diazepam (Roche, Basle, Switzerland), chlorpromazine (Sigma Chemical Co, St Louis, USA), flumazenil (Sigma Chemical Co, St Louis, USA), naltrexone (Sigma Chemical Co, St Louis, USA), yohimbine HCl (Sigma Chemical Co, St Louis, USA). All drugs were administered dissolved in 0.9% saline on each day of the experiment.

Acute Toxicity Tests

The method described by Hays (1989) was used in determining LD_{50} , which is an index of acute toxicity. The experiment was done with graded doses of the methanolic extract, butanol, aqueous and ethylacetate fractions (20-140 mg/kg, i.p.) of the leaves of *S. cayennensis* in mice ($n=10$ for each dose level) to see the effect of the extract on rearing for the 30 minutes duration of the test for each mouse. Observations of both the activities of the mice during the time of the experiment and the behavioural pattern at all doses employed were noted. The time of death and the pattern of death were noted immediately and over 24 hours. The control group received saline. The LD_{50} was calculated using the arithmetic method of Spearman Karber (Hays, 1989) and the results were validated by Probit Analysis Program for the Determination of LC/EC values, Version 1.5. The ED_{50} was also calculated by plotting the percentage inhibition against the log dose for the methanolic, butanolic and the aqueous fractions and reading from the plot, the ED_{50} for each of the extract/fractions. These values were used to determine the Therapeutic Indices for the extracts.

Behavioural Assays

Assessment Of Novelty-Induced Rearing (NIR)

Rearing was assessed as part of novelty-induced behaviour, and was evaluated by the method previously described (Ajayi and Ukponmwan, 1994). The animals were placed directly from home cages into an opaque Plexiglas observation cage (45 x 25 x 25 cm) with only one side transparent for observation. All animals were observed and assessed singly in the Plexiglas cage, after the administration of the saline, positive control, extracts and fractions. Each animal was used only once, and the Plexiglas cleaned with 70% alcohol after each assessment to remove olfactory cue from one animal to the other (Blanchard et al, 2001; Bolivar et al, 2000; Brown et al, 1999). The time of the experiments was kept constant (10a.m – 2p.m. daily) to avoid influence of changes in biological rhythm (Siqueira et al, 1998). The laboratory was brightly lit, with an ambient temperature of $26 \pm 1^\circ\text{C}$.

The frequency of the episodic rearing was quantified by using a counter and a timer for 30 minutes. Methanolic extract (5 – 50 mg/kg), butanol fraction (5-50 mg/kg), aqueous fraction (5-50 mg/kg) and ethyl acetate fraction (10-100 mg/kg) were injected intraperitoneally to mice and the effect on rearing was compared to the saline (10ml/kg) control group. Animals were divided into eleven groups of five mice per each dose level and rearing was recorded as frequency over a period of 30 minutes.

Table 1: Treatment groups for the behavioural study

Group	Treatment
1	Saline (10 ml/kg, i.p.)
2	Diazepam (2 mg/kg, i.p.)
3	Methanolic extract (5-50 mg/kg, i.p.)
4	Atropine (0.5 mg/kg, i.p.)
5	Atropine (0.5 mg/kg, i.p.) 15 minutes prior to methanolic extract (50 mg/kg, i.p.)
6	Flumazenil (2.0 mg/kg, i.p.)
7	Flumazenil (2.0 mg/kg, i.p.) 15 minutes prior to methanolic extract (50 mg/kg, i.p.)
8	Naltrexone (2.5 mg/kg, i.p.)
9	Naltrexone 15 minutes prior to methanolic extract (50 mg/kg, i.p.)
10	Yohimbine (1.0 mg/kg, i.p.)
11	Yohimbine 15 minutes prior to methanolic extract (50 mg/kg, i.p.)

The protocol was repeated for the butanol, aqueous and ethylacetate fractions in mice, using the intraperitoneal routes of drug administration.

Assessment of the extract and fractions of *S. cayennensis* on spontaneous locomotor activity in mice

The spontaneous locomotor activity of the mice was assessed using an automated and computerized system. In this experiment, the total activity was recorded as the summation of all individual activities of the animal. These include rearing, grooming, stretching, face wash, paw licking, and all manners of movements by the animal. Methanolic extract of the leaves of *S. cayennensis*, and the fractions (10-40 mg/kg), diazepam (2 mg/kg, i.p. as positive control) or vehicle (0.9 % saline, as negative control) was administered to the mice. The cage contained an infrared sensor system using a multi-Fresnel lens that was sensitive to any motion within it (Sugiura et al, 1997). The cages were connected to a computer loaded with dBase software programmed for the scoring of activity by the animals. Mice were placed individually in each cage (5 cages in all), 5 minutes after receiving intraperitoneally, methanol extract (10-40 mg/kg, i.p.), butanol (10-40 mg/kg, i.p.), aqueous (10-40 mg/kg, i.p.), ethylacetate (10-40 mg/kg, i.p.) fractions, diazepam (2 mg/kg, i.p.) or saline and the effects observed on the total activity (Xiao-Ming et al, 2002). The total number of light beam interruptions was recorded every 5 minutes for 30 minutes. Five mice were used in each group, and in each session, control mice which received saline and diazepam were also tested under the same conditions with those animals receiving *S. cayennensis* treatment. The change in locomotion was expressed as a percentage using the following ratio:

$$\% \text{ Change} = 100 - (\text{total counts of treated mice} / \text{total counts of control mice}) \times 100$$

Assessment of the extract and fractions of *S. cayennensis* on Pentobarbitone Sleeping Time.

The method described by Dandiya and Collumbin (1959) was used. Mice were used for the experiments. The sleep latency is defined as the time in minutes after treatment with pentobarbitone (40 mg/kg) that the animal presents with loss of righting reflex whilst the time in minutes between loss and regain of righting reflexes was taken as duration of hypnosis or sleeping time (Dandiya and Collumbin, 1959; Grases et al, 1994; Vogel, 2002). The following treatment protocol was used to assess the effect of the extract and fractions of *S. cayennensis* on pentobarbitone induced sleep. Mice were divided into dosage groups of at least 5 mice each.

Table 2: Treatment groups for pentobarbitone induced sleep experiment.

Group	Treatment
1	saline (10 ml/kg, i.p.),
2	methanolic extract 5-50 mg/kg, i.p., 30 minutes before pentobarbitone (40 mg/kg, i.p.),
3	diazepam (2.0 mg/kg, i.p.), 15 minutes before pentobarbitone (40 mg/kg, i.p.)
4	flumazenil (2.0 mg/kg, i.p.),
5	flumazenil (2.0 mg/kg, i.p.) 15 minutes before pentobarbitone (40 mg/kg, i.p.)

The protocols were repeated for all the fractions (butanol, aqueous, and ethylacetate fractions) to see their effect on pentobarbitone-induced sleep.

Assessment of the anti-anxiety effect of the extract and fractions of the leaves of *S. cayennensis* in mice using the elevated plus maze.

The method of Lister (1990) was used in this assessment. The method utilizes an elevated plus maze model previously described (Lister, 1987, 1990.) which is based on the natural aversion for open spaces and heights by rodents. The plus-maze is made of plexiglas and consists of two open arms 30 X 5 cm. and two enclosed arms 30 X 5 X 15 cm. The arms extend from a central platform 5 X 5 cm. The open arms, the central platform and the floor of the closed arms are painted black. The apparatus is mounted on a wooden base, raising it 38.5 cm. above the floor. The open arms contain a slight ledge 4 mm. high, to prevent the mice from slipping and falling off the edge. To eliminate any lingering olfactory cues, the apparatus is cleaned between each examination using 70% ethyl alcohol. Each trial was recorded for 5 minutes with the following behaviours scored:

- (a) open arm entries
- (b) closed arm entries
- (c) time spent in the open arm
- (d) time spent in the closed arm

The index of open arm avoidance (Trullas and Skolnick, 1993), interpreted as level of anti-anxiety is calculated as:

$$[100 - (\% \text{ time spent on open arm} + \% \text{ entries into open arms}) / 2]$$

The effects of the extract and fractions of *S. cayennensis* on open arm entries and duration were compared with the effect of the anxiolytic dose (1.0 mg/kg, i.p.) of diazepam (Reddy and Kulkarni, 1997). Doses of the extract and fractions which did not affect motor coordination were used

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in the experiments. In another group of mice, flumazenil (2.0 mg/kg, i.p.) was administered 15 minutes prior to administration of the extract and fractions of *S. cayennensis* to establish the neuronal pathway of the anxiolytic effect observed in the fractions.

Statistical Analysis of Results

Results of the experiments and observations are expressed as mean \pm standard error of mean (s.e.m) in the text, tables and graphs in this report. The significance of differences between groups was determined using one-way analysis of variance (ANOVA), followed by post hoc analysis using the Student-Newman-Keuls (SNK) test. In the determination of median lethal dose (LD₅₀), the Spearman-Kärber arithmetic method was used. The results were validated by a computer programme developed by the United States Environmental Protection Agency (USEPA)-Probit Analysis Program for the Determination of LC/EC values, Version 1.5. In all the observations, statistical significance was accepted at p values less than or equal to 0.05. In all these statistical determinations, a computer programme-the Primer of Biostatistics (Version 3.01) was used (Glantz, 1992).

Results

Results of Toxicity Testing

The mice in the treatment groups (methanol extract, butanol, aqueous and ethylacetate fractions 20, 40, 60, mg/kg, i.p.) all survived beyond 14 days. The median lethal dose (LD₅₀) was calculated by the arithmetic method of Karber (Hays 1989) as follows: methanol extract 90 mg/kg, i.p; butanol fraction 90 mg/kg, i.p; aqueous fraction 102 mg/kg, i.p; ethylacetate fraction 88 mg/kg, i.p. The median effective dose (ED₅₀) from the dose-response curve for methanol is 15 mg/kg, i.p; for butanol, 10 mg/kg, i.p and for aqueous fraction 10 mg/kg, i.p. (Fig.1.). The Therapeutic Index (LD₅₀/ED₅₀) for methanolic extract is 6, for butanol fraction it is 9 and for aqueous fraction it is 10.2. (Figure 1.)

Place figure 1 here

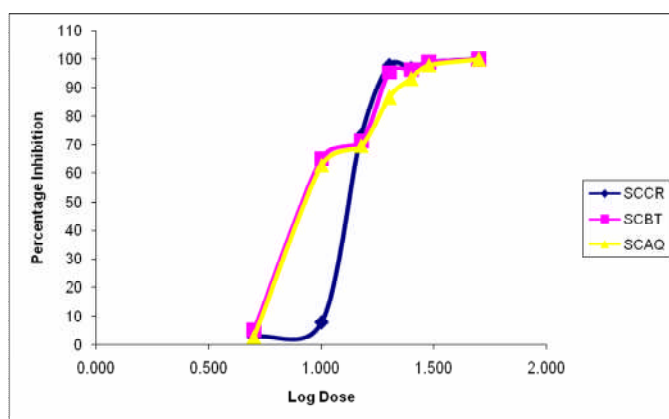


Figure 1: Percent inhibition of rearing versus log dose to calculate ED₅₀ and therapeutic index (TI) for MeOH, BuOH, Aq and EtOAc in mice.

Effect of the extract and fractions of *S. cayennensis* on Novelty Induced Rearing (NIR) in mice

There were significant differences between mice that received saline (10 ml/kg, i.p.) and the mice that were treated with the extract and fractions (10-50 mg/kg, i.p.) as well as those that received diazepam (2 mg/kg, i.p.) (Fig.2.). Maximal inhibition of NIR was observed at 20 mg/kg, i.p. for the methanolic extract and this was 96% lower than saline control. Diazepam was only able to elicit 91.8% inhibition of NIR. There were no significant differences in the inhibitory effects of the methanolic extract given at doses between 25 and 50 mg/kg, i.p. (Fig.1; $p=0.025$). The inhibition of rearing induced by the butanol fraction (5-50 mg/kg, i.p.) was significant and dose-dependent as the inhibition offered by each dose level was significantly different ($p<0.05$) from one another. Maximum inhibition of NIR was observed at 50 mg/kg, i.p. and it was 98.5% lower than control. The aqueous fraction (5-50 mg/kg i.p.) significantly inhibited NIR in mice compared to the 0.9% saline control ($p<0.05$, Fig.2.). The inhibition of rearing due to the aqueous fraction was dose dependent ($p<0.05$). Maximal inhibition of NIR was observed at the dose of 50 mg/kg for the aqueous fraction and this was 96% reduction of NIR compared with control. The ethyl acetate fraction (10-50 mg/kg) produced significant increase ($p<0.05$) in NIR. The increase in NIR due to the ethylacetate fraction was dose-dependent. Only at the dose of 10 mg/kg for the extract and the aqueous and butanol fractions was a difference in the inhibitory activity on NIR observed, with the butanol and aqueous fractions showing more inhibitory activity (64 and 60.5% respectively) at this dose compared to the methanolic extract (8%) (Figure 2).

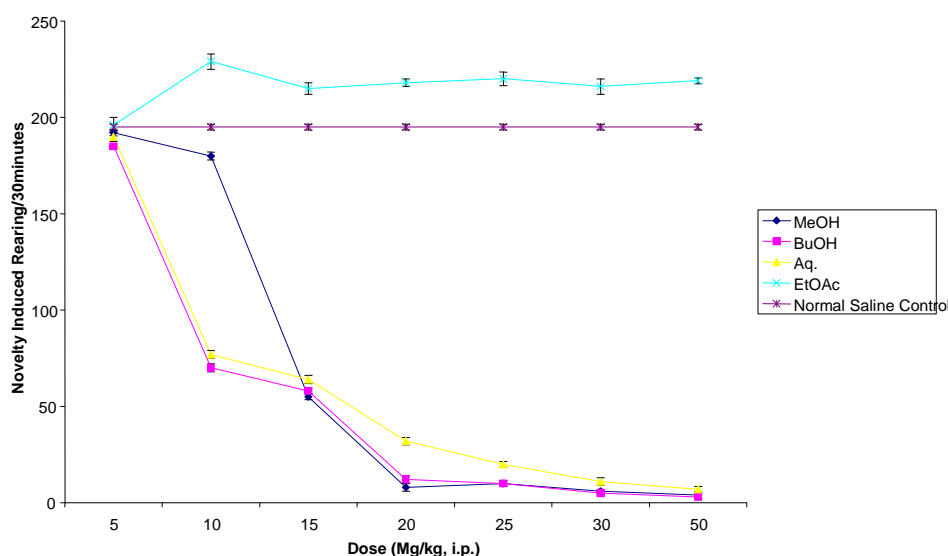


Figure 2: Effects of methanolic extract, butanolic, aqueous and ethylacetate fractions of *S. cayennensis* leaves on novelty induced rearing in mice. Each point is expressed as mean \pm S.E.M. ; Number of mice per extract treatment is 5.; There is no difference in the degree of inhibition produced by MeOH, BuOH and Aq particularly from 15 mg/kg, i.p. EtOAc induced stimulation of NIR.; *indicates significant difference from saline control.

Effect of flumazenil (2.0 mg/kg, i.p.) on the NIR inhibitory action of the methanol extract, butanol and aqueous fractions of *S. cayennensis* in mice.

The inhibition of NIR induced by the extract and the butanol fraction was blocked by flumazenil, a GABA_A and benzodiazepine specific antagonist. The reversal of methanolic extract-induced inhibition of NIR by flumazenil was significant ($p < 0.05$) so is the effect of flumazenil on the butanol fraction ($p < 0.05$). A 37-fold more rearing or 97% increase in NIR was observed for the methanolic extract while a 49-fold increase was observed for the butanol fraction. However, flumazenil did not reverse the inhibition of NIR induced by the aqueous fraction ($p = 0.399$, Figure 3).

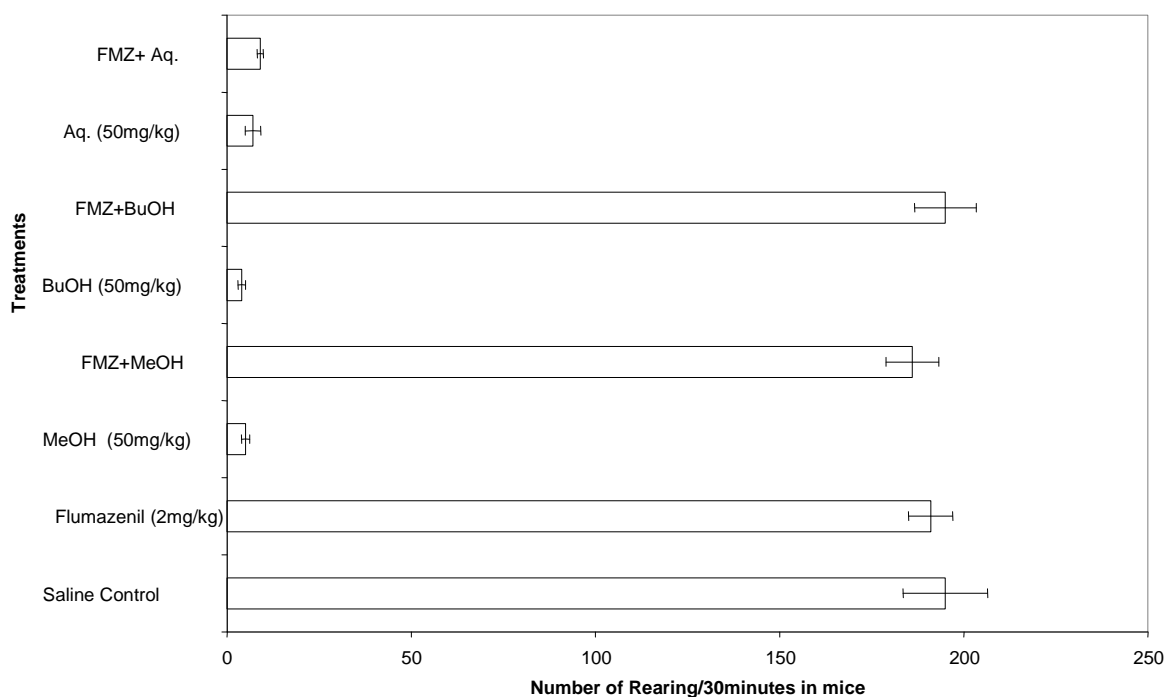


Figure 3: Effects of flumazenil (2mg/kg) on inhibition of rearing induced by methanolic extract, butanolic, aqueous and ethylacetate fractions of *S. cayennensis* leaves in mice. One-way ANOVA revealed that there is significant difference between the various treatment groups. ; Each bar is expressed as mean \pm S.E.M. Number of mice per treatment is 5.; *indicates significant difference from saline control $p < 0.05$ (SNK test)

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Effect of atropine (0.5 mg/kg, i.p.) on the NIR inhibitory action of methanol extract, butanol and aqueous fractions (50 mg/kg, i.p.) of *S. cayennensis* in mice.

Atropine did not have any effect on the inhibition of NIR induced by the methanolic extract ($p=0.52$), butanol fraction ($p=0.50$) and aqueous fraction ($p=0.173$). However, atropine administered alone increased NIR significantly compared with 0.9% saline ($p<0.05$) in mice (Data not shown).

Effect of naltrexone (2.5 mg/kg, i.p.) on the NIR inhibition action of methanol extract, butanol and aqueous fractions (50 mg/kg, i.p.) of *S. cayennensis* in mice.

Administration of naltrexone significantly ($p<0.05$) reversed the inhibition of rearing induced by the aqueous fraction of the leaves of *S. cayennensis*. The increase in rearing episode was 18-fold compared to the control. Naltrexone also decreased the inhibition of NIR induced by the methanol extract ($p<0.05$) thereby increasing NIR by 12-fold. However, the inhibition of NIR induced by the butanol fraction was affected to a lesser extent (9.5-fold) by naltrexone (Figure 4).

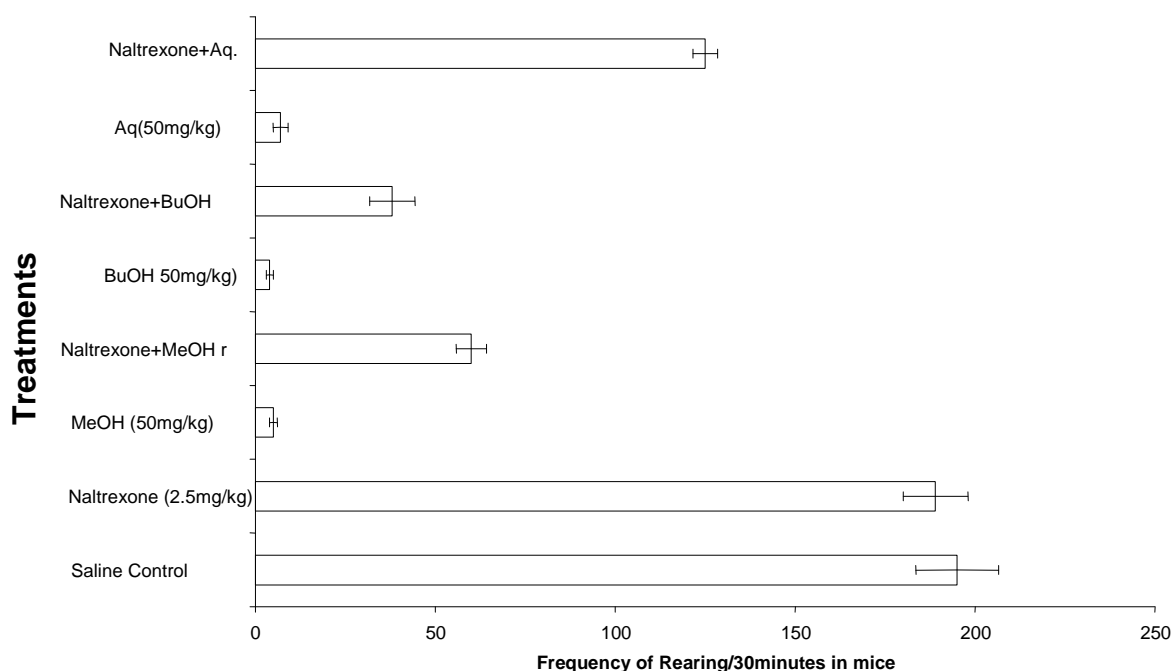


Figure 4: Effects of naltrexone (2.5 mg/kg) on inhibition of rearing induced by methanolic extract, butanolic and aqueous fractions of *S. cayennensis* leaves in mice

One-way ANOVA revealed that there is significant difference between various treatment groups. ; Each bar is expressed as mean \pm S.E.M. Number of mice per treatment group is 5. ; *indicates significant difference compared with saline controls $p<0.05$ (SNK test).

Effect of yohimbine (1.0 mg/kg, i.p.) on NIR inhibition action of methanol extract, butanol and aqueous fractions (50 mg/kg, i.p.) of *S. cayennensis* in mice.

Administration of yohimbine alone reduced NIR episode in mice. However, administration of yohimbine 15 minutes prior to the administration of the extract and fractions of the leaves of *S. cayennensis* did not alter the inhibitory effect of the extract ($p=0.56$), the butanol fraction ($p=0.53$) and the aqueous fraction ($p=0.569$) on NIR (Figure 5).

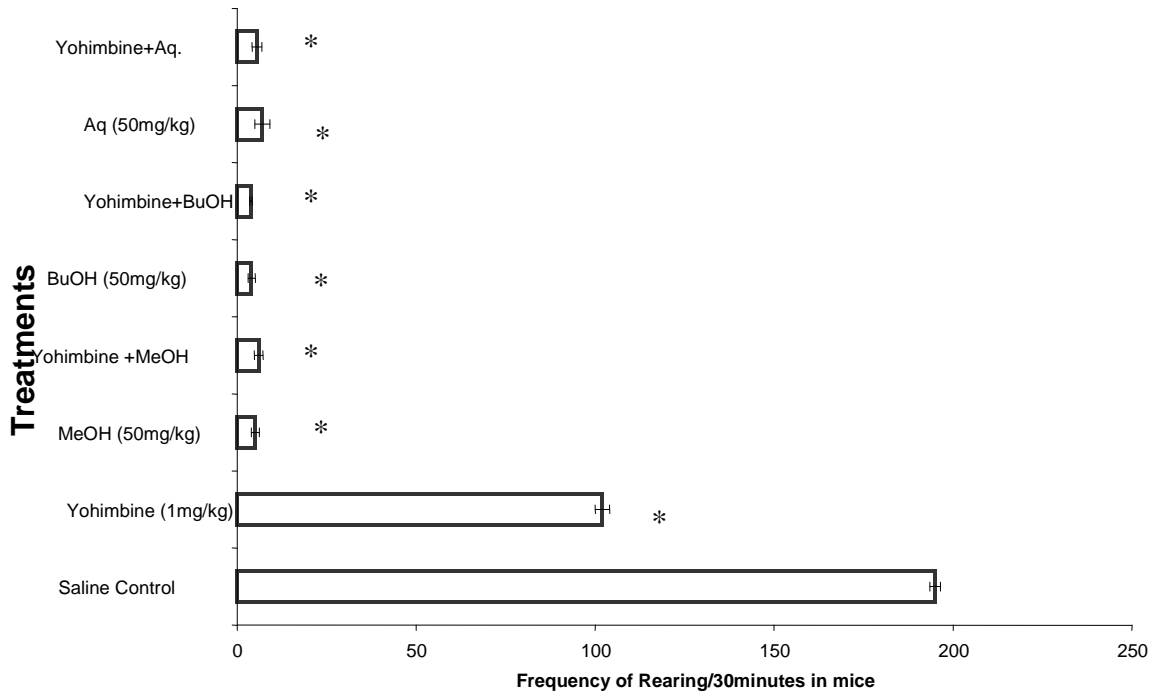


Figure 5: Effects of yohimbine (1mg/kg) on inhibition of rearing induced by methanolic extract, butanolic and aqueous fractions of *S. cayennensis* leaves in mice
 One-way ANOVA revealed that there is significant difference between various treatment groups. $F(12, 64) = 441.26; p < 0.001$. ; Results are expressed as mean \pm SEM. ; Number of mice per extract treatment is 5. ; *indicates level of significance relative to saline control $p < 0.05$. SNK test.

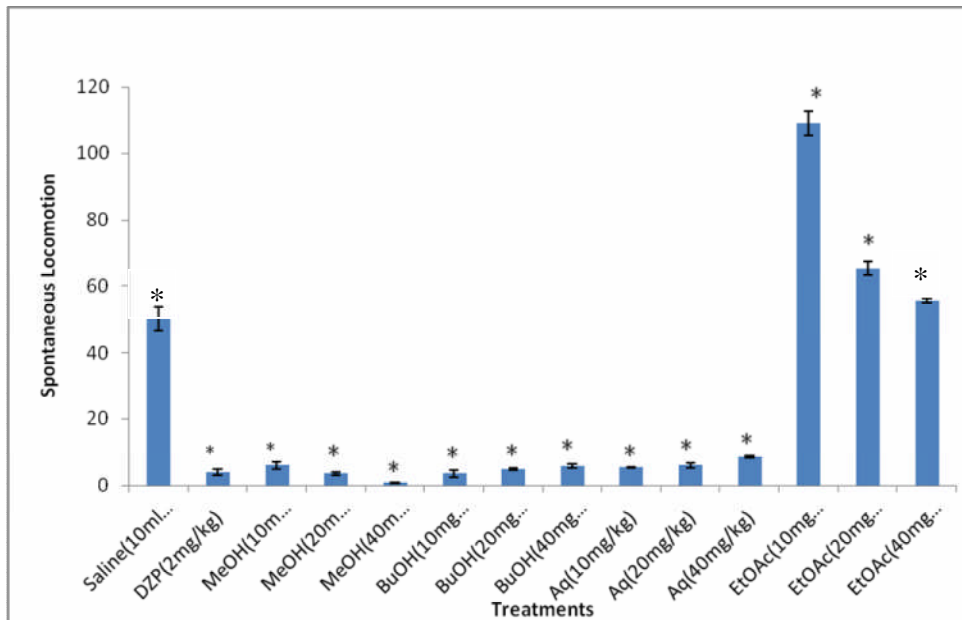


Figure 6: Effects of methanolic extract, butanolic, aqueous and ethylacetate fractions of *S. cayennensis* leaves on spontaneous locomotor activity in mice
 One-way ANOVA revealed that there is significant difference between various treatment groups. $F(12, 64) = 441.26; p < 0.001$. ; Results are expressed as mean \pm SEM. ; Number of mice per extract treatment is 5. ; *indicates level of significance relative to control experiment $p < 0.05$. SNK test.; ** indicates significant stimulation relative to other inhibition of locomotion.

Effect of the Extract and fractions of *S. cayennensis* on spontaneous locomotor activity (SLA) in mice.

Effect of the methanol extract, the butanol, aqueous and ethylacetate fractions (10-40 mg/kg, i.p.) on SLA was observed in mice. The results showed that the methanolic extract significantly and dose-dependently inhibited the SLA in mice compared to the saline control ($p < 0.05$) (Figure 6). Administration of the butanol fraction (10 mg/kg, i.p.) induced inhibition of SLA in mice that is greater than that induced by 20 mg/kg (92.8% versus 90.0%), which in turn induced inhibition greater than that produced by 40 mg/kg (88.2%). Unlike the butanol fraction, the aqueous fraction exhibited significant inhibition of SLA, with the higher doses having more NIR inhibitory activity than lower doses. Ethylacetate fraction did not induce inhibition of SLA in mice in the fashion of the methanol extract, butanol or the aqueous fractions. Administration of ethylacetate fraction (10 – 40 mg/kg, i.p.) caused significant and dose-dependent increase in SLA (117% for 10mg/kg; 29.8% for 20 mg/kg and 10.8% for 40 mg/kg) compared to the saline control ($p < 0.05$). (Figure 6).

Effect of the Extract and fractions of *S. cayennensis* on pentobarbitone-induced sleeping time (PIST) and sleep latency in mice.

Doses of methanolic extract, butanolic, aqueous and ethylacetate fractions (5-50 mg/kg i.p) were administered to mice, alone and in the presence of flumazenil (2.0 mg/kg i.p) prior to administration of pentobarbitone (40 mg/kg, i.p.). Methanol extract (10-50 mg/kg, i.p.) significantly reduced sleep latency in mice ($p < 0.05$), but the starting dose of 5.0 mg/kg; i.p. did not alter sleep latency in mice compared with saline control. The sleep latency modulation by the methanolic extract is however not dose-dependent ($p = 0.07$) (Table.3.). The sleep latency reduction induced by the methanolic extract (20-50 mg/kg, i.p.) in mice is comparable to that by diazepam (2.0 mg/kg, i.p.). Administration of Flumazenil (2.0 mg/kg, i.p.) reversed the reduction of sleep latency induced by the extract in mice (Table 3.). Administration of the extract (10-50 mg/kg, i.p.) significantly and dose-dependently potentiated pentobarbitone (40 mg/kg, i.p.)-induced sleeping time in mice ($p < 0.05$). Maximum potentiation of sleep was observed with the extract (20 mg/kg, i.p.) and this effect was comparable to that produced by diazepam (2.0 mg/kg, i.p.) and chlorpromazine (10 mg/kg, i.p.) ($p = 0.825$). Flumazenil (2 mg/kg, i.p.) reversed or abolished the sleep potentiation by the methanol extract (20 mg/kg, i.p.) in mice (Figure 7.).

Butanol fraction (5-40 mg/kg, i.p.) significantly reduced sleep latency in pentobarbitone (40 mg/kg, i.p.)-induced sleep in mice compared with saline control ($p < 0.05$), but the effect is not dose-dependent. Butanol fraction (10 mg/kg, i.p.) induced maximum reduction of sleep latency and this effect was reversed by flumazenil (2.0 mg/kg, i.p.). Administration of butanol fraction (5-40 mg/kg, i.p.) induced a dose-dependent potentiation of pentobarbitone induced sleep in mice ($p < 0.05$). Maximum effect was observed at 10 mg/kg, i.p. and this effect was reversed by flumazenil (2.0 mg/kg, i.p.) (Fig.7). This observed maximal potentiation is comparable to the effect due to diazepam and chlorpromazine ($p = 0.725$) and it is 3.5-fold more than the control. Administration of the aqueous fraction (10-80 mg/kg, i.p.) significantly reduced sleep latency in mice ($p < 0.05$). The sleep latency reduction induced by doses 10-80 mg/kg, i.p. is comparable with that induced by diazepam ($p = 0.053$). Administration of flumazenil (2.0 mg/kg, i.p.) did not alter significantly ($p = 0.396$), the effect of the aqueous fraction. In the same experiment, aqueous fraction (10-80 mg/kg, i.p.) induced a significant and dose-dependent potentiation of pentobarbitone-induced sleep in mice ($p < 0.05$). This potentiating effect of the aqueous fraction was not altered by flumazenil in mice (Fig.7). Administration of the ethylacetate fraction (10-40 mg/kg, i.p.) in mice did not potentiate pentobarbitone-induced sleep in the manner of the methanolic extract, butanolic or the aqueous fractions. The ethylacetate fraction (10-40 mg/kg, i.p.) did not alter pentobarbitone-induced sleep in mice compared with the saline control (Figure 7.).

Table 3: Effect of methanolic extract (MeOH), butanol fraction (BuOH), aqueous fraction (Aq.) and ethylacetate fraction (EtOAc) of the leaves extract of *Stachytarpheta cayennensis* on sleep latency in mice.

Pentobarbitone (40mg/kg) in the presence of:	Sleep latency (min) in mice	Pentobarbitone (40mg/kg) in the presence of:	Sleep latency (min) in mice	Pentobarbitone (40mg/kg) in the presence of:	Sleep latency (min) in mice	Pentobarbitone (40mg/kg) in the presence of:	Sleep latency (min) in mice
Saline (control)	3.8±0.1						
3% DMSO Control	3.6±0.3						
Diazepam 2 mg/kg, i.p	2.8 ±0.2*						
MeOH 5mg/kg,i.p	3.4±0.1	BuOH 5mg/kg,i.p	2.8±0.2*	Aq. 5mg/kg,i.p.	3.7±0.1	EtOAc 5mg/kg, i.p.	3.6±0.2
MeOH 10mg/kg:i.p	3.0±0.1*	BuOH 10mg/kg	2.1±0.1*	Aq. 10mg/kg	3.1±0.1*	EtOAc 10mg/kg	4.3±0.4
MeOH 20mg/kg i.p	2.5±0.2*	BuOH 20mg/kg	2.6±0.2*	Aq. 20mg/kg	2.7±0.2*	EtOAc 20mg/kg	4.6±0.1*
MeOH 25mg/kg i.p	2.6±0.1*	BuOH 40mg/kg	2.9±0.2*	Aq. 40mg/kg	2.5±0.1*	EtOAc 40mg/kg	4.2±0.2
MeOH 50mg/kg i.p	2.5±0.3*			Aq. 80mg/kg	2.5±0.1*		
Flumazenil 2mg/kg i.p	No Sleep Observed						
Flumazenil(2mg/kg, i.p) + MeOH 50 mg/kg i.p.	4.2±0.2*	Flumazenil + BuOH 10mg/kg i.p.	3.5±0.2	Flumazenil + Aq. 25mg/kg,i.p.	2.7±0.2*	Flumazenil + EtOAc 10mg/kg	No Sleep

^aOne-way ANOVA revealed that there is significant difference between various treatment groups.

^bResults are expressed as mean ± S.E.M.

^cNumber of mice per treatment group is 5.

^d*indicates significant difference from saline control. $P < 0.05$ (SNK test).

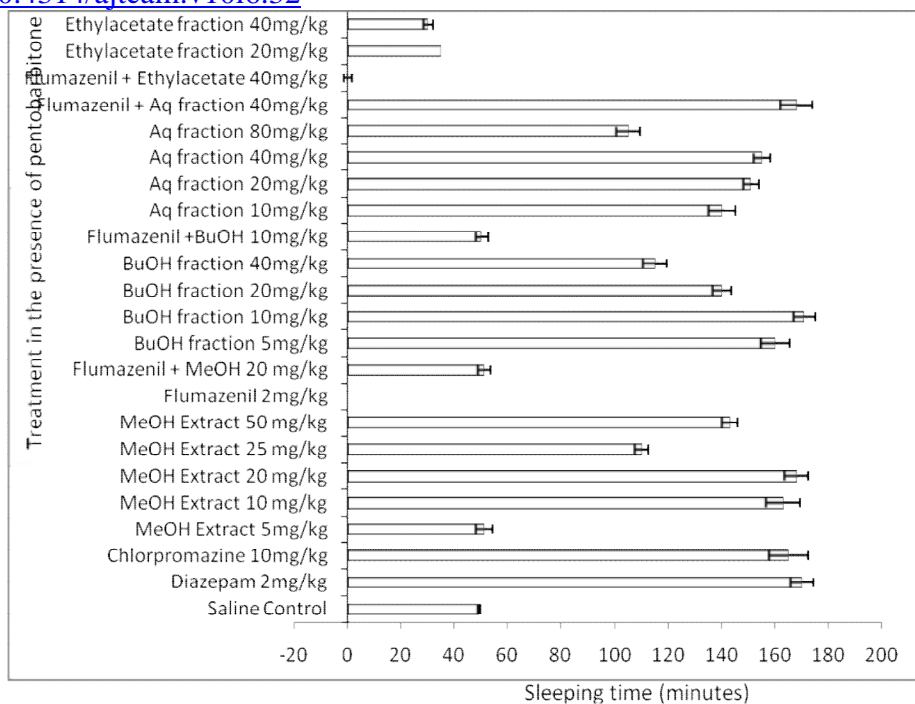


Figure 7: Effects of methanolic extract, butanolic, aqueous and ethylacetate fractions of *S. cayennensis* leaves on pentobarbitone (40mg/kg) induced sleeping time in mice

One-way ANOVA revealed that there is significant difference between various treatment groups. $F(12, 64) = 441.26; p < 0.001$. Results are expressed as mean \pm SEM. ; Number of mice per extract treatment is 5. ; *indicates level of significance relative to control experiment $p < 0.05$. SNK test.

Effect of the extract and fractions of *S. cayennensis*, diazepam and flumazenil on anxiety in mice

The butanol fraction (5.0 mg/kg, i.p.) and aqueous fraction (20 mg/kg, i.p.) induced significant increase in frequency of open arm entries and duration in the open arms compared with saline control (Table 4). The methanol extract (2.5-5.0 mg/kg, i.p.), the butanol fraction (2.5 mg/kg, i.p.) and the aqueous fraction (10-40 mg/kg, i.p.), all representing sub-sedative doses, did not alter the numbers of entries into the open arm compared with controls. The ethylacetate fraction induced increase in frequency of entries into the closed arms and index of open arm avoidance in a dose-dependent manner compared with saline control.

Pre-treatment with flumazenil (2.0mg/kg, i.p.) 15 minutes prior to administration of the butanol (5.0 mg/kg, i.p.), aqueous (20mg/kg, i.p.) and diazepam (1.0 mg/kg, i.p.) reversed the effect on the frequency of open and closed arms entries and the effect on index of open arm avoidance in both butanol and diazepam but not on aqueous fraction.(Table 4).

Table 4: Effect of Methanolic extract (MeOH), butanol (BuOH), aqueous (AqOH), ethyl acetate (EtOAc) fractions of *Stachytarpheta cayennensis* leaves and Diazepam on anxiety in mice.

Treatment	Number of Entries Into Arms		Time Spent in Each Arm (secs)		%Time Spent In open arm	Index of Open Arm Avoidance
	Open	Close	Open	Close		
0.9% Saline 10 ml/kg, i.p.	4 \pm 1.0	11.7 \pm 1.8	70 \pm 3.2	202 \pm 6.3	25.7	74.4
MeOH (5mg/kg, i.p.)	4.3 \pm 1.0	11.7 \pm 1	85.7 \pm 4.1	189.7 \pm 5.1	31.1	71
BuOH (5mg/kg, i.p.)	16.7 \pm 3.*	8.0 \pm 1.6	181.7 \pm 5.*	73.0 \pm 3.9	71.3	30.6*
AqOH (20mg/kg, I,p)	7.0 \pm 2.1*	3.7 \pm 1.1	144.7 \pm 5.*	98.3 \pm 4.3	59.5	37.6*
EtOAc(25mg/kg, i.p.)	2.7 \pm 1.2*	7.3 \pm 2.3	57.7 \pm 4.2	202.3 \pm 5.8	22.2	75.4
Diazepam(1mg/kg, i.p)	13.0 \pm 2.1*	3.0 \pm 1.1*	164.3 \pm 6.0*	114.0 \pm 7.7*	59.0	36.3*

^aOne-way ANOVA revealed that there is significant difference between various treatment groups. $F(5, 29) = 115.33; p < 0.001$.

^bResults are expressed as mean \pm S.E.M.

^cNumber of mice per treatment group is 5.

^dThe anxiolytic effect of BuOH and AqOH measured as index of open arm avoidance is comparable to that of Diazepam 1 mg/kg, i.p (positive control).

^eSub-sedative doses of the extract, fractions and diazepam were used to elicit anxiolytic effect.

^fAdministration of BuOH (5 mg/kg, i.p.) and AqOH (20 mg/kg, i.p.) elicited significant anxiolytic effect in mice, while MeOH (5 mg/kg, i.p) has an effect comparable to 0.9% saline control.

^g*Indicates significant difference from the 0.9% saline control $p < 0.05$, SNK test.

^h. Total test time is 300 seconds

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Discussion

The sedative property and the mechanism of the sedative activity of the methanolic extract and the n-butanol, aqueous as well as the ethylacetate fractions of the leaves of *S. cayennensis* have been investigated in this study. The sedative parameters examined include novelty-induced rearing (NIR), spontaneous locomotor activity and pentobarbitone-induced hypnosis. These investigations were carried out in mice, and the tests also established the safety/toxic limit for the extract and fractions in the test animals by the determination of LD₅₀, ED₅₀ and the calculation of the Therapeutic Index (T.I). (Hays, 1989; Glantz, 1992; Subhan et al, 2010).

The LD₅₀ determined for the methanolic extract, the butanol, aqueous and ethylacetate fractions of *S. cayennensis* via the intraperitoneal route is 90 mg/kg, 90 mg/kg, 102 mg/kg, and 88 mg/kg, i.p respectively. The corresponding T.I. calculated is 6, 9 and 10.2 for the methanolic, butanol and aqueous fractions respectively. It is important to note that all the pharmacological effects of the extracts of the leaves in this study occurred well below the LD₅₀ values for both extract and fractions of the leaves. T.I. in this range calls for caution in the use of the extract as it reveals a small therapeutic window. Previous study on the aqueous extract of the same leaves by the oral route showed a very high LD₅₀ (Akanmu et al, 2005). Additional insight is gained into the characteristic of the leaves of *S. cayennensis* on account of the route of its administration. Novelty induced rearing is a behaviour of rodents in novel environments. Rearing is part of the exploratory behaviour employed by rodents as one of the survival strategies in assessing the environment for food, sex, protection and possibly escapes (Abdel-Barry and Al-Hakeim, 2000; Golani et al, 1993). Measurement of the frequency of rearing in rodents and the modification of same can therefore be employed in assessing test drugs and extracts for both sedative property and central nervous system stimulation (Vogel, 2002; Wishaw et al, 1994). In this study, while the methanol extract, the butanol and aqueous fractions have principles that inhibit NIR in mice, the ethylacetate fraction has principle that stimulates the animals; leading to increase in novelty induced rearing. The methanolic extract, the butanol and the aqueous fractions are suggested to possess sedative activity while the ethylacetate fraction is essentially stimulant in nature.

Some of the receptor antagonists (atropine for muscarinic receptors, yohimbine for adrenergic receptors, and naltrexone for opioid receptors and flumazenil for GABA receptors) were administered to the test animals to establish the receptors involved in the observed modulation of NIR by the extract and fractions (Jordan, 1991; Lai and Siegel, 1991; Watling, 1998; Zedkova et al, 2001). In this study, flumazenil (2.0 mg/kg, i.p.) blocked the NIR inhibition induced by the methanol extract and the butanol fraction, while that induced by the aqueous fraction was abolished by naltrexone. This may show that while the extract and the butanol fraction may be exhibiting their NIR inhibitory activity via GABA, the aqueous fraction may be acting via an opioid receptor (Fig.3.). GABA is known to participate in locomotor activity in rodents as one of the inhibitory neurotransmitters. The effect of GABA is partly that of inhibiting locomotor activity (Anguilio and McEwen, 1994). Sedation ensues when there is stimulation of the inhibitory GABA receptors (Sinnamon and Benaour, 1997). The influence of flumazenil in this experiment suggests that the mechanism of action that may explain the sedative action of the extracts of *S. cayennensis* in mice is gabaergic neural system.

Naltrexone (2.5mg/kg, i.p.) an opioid receptor antagonist inhibited the rearing effect due to both the extract and fractions of *S. cayennensis*, blocking some of the opioid receptors to influence the rearing episode observed in mice. In the experiment with the methanolic extract, the dose administered (50mg/kg, i.p.) was the maximal dose that produced the maximal response (Fig. 4). In the presence of naltrexone, a 12-fold increase in the number of rearing was observed, indicating that the inhibition of rearing observed with the extract was not due to a single receptor type. The explanation may be that while naltrexone blocked the opioid receptors, some receptor types were still left and this was responsible for some residual inhibition ascribable to the methanolic extract. It is convenient to suggest that opioid receptors may also be involved in the inhibitory activity of the extract on rearing and therefore on sedation as rearing inhibition was used as an index of sedation. The fact that there was significant frequency of rearing after naltrexone blockade shows that GABA receptors alone could not have been responsible for the observed inhibition. The participation of the noradrenergic and cholinergic systems in the inhibition of NIR by the extract and fractions of *S. cayennensis* could not be established in this study.

The results of the test of the extract and fractions for their effect on spontaneous locomotor activity follow the pattern obtained for NIR study. The NIR-spontaneous locomotor behaviour phenomenon is regulated by multiple neurotransmitter systems (Karczmar, 1993). Such transmitters include acetylcholine (ACh), dopamine, serotonin (5-HT), gamma-amino butyric acid (GABA), opioid and noradrenaline (Garrett et al, 2003; Jordan, 1998; Karczmar 1993). This study was able to establish that the inhibitory activity of the extract and the butanol fractions of the plant on NIR and spontaneous locomotor activity (SLA) were mediated via the GABA and (to a lesser extent) opioid receptors. The study also showed that the methanolic extract at a dose of 20 mg/kg body weight and 10 mg/kg of the butanol fraction are empirically equipotent to 2 mg/kg body weight of diazepam for the sedative effect in mice. This study therefore concludes that the inhibition of novelty induced rearing and spontaneous locomotor activity; both measures of the sedative property of the extract are effected via the dual receptor complex of GABA and opioid receptors.

The experiments with pentobarbitone-induced hypnosis showed that the methanolic extract, the butanol as well as the aqueous fractions reduced sleep latency in the test animals while prolonging sleeping time induced by pentobarbitone in the animals. The reduction of sleep latency and the prolongation of sleeping time induced by the extract and fractions of *S. cayennensis* are both significant (compared with the saline control), empirically comparable with standard hypnotic drugs (Diazepam and Chlorpromazine) (Table 3 and Figure 7) and this prolongation was reversed by flumazenil, a GABA antagonist. It is interesting to note that flumazenil, a GABA/benzodiazepine antagonist (Costall et al, 1981; Watling 1998), reversed the potentiating effect of methanol extract and the butanol fraction of *S. cayennensis* on pentobarbitone-induced sleeping time in mice but not the sleeping time due to the aqueous fraction. (Fig.7). This shows that while GABA receptors appear to be involved in the prolongation of sleeping time induced by pentobarbitone in methanolic extract and butanolic fraction, it appears that GABA is not involved in the events that are modulated by the aqueous fraction. It is known that increased GABAergic transmission produced profound sedation in mice and rats (Alifimof and Miller, 1993; Gottesmann, 2002). Pentobarbitone-induced hypnosis is achieved by the activation of the GABA inhibitory system, and also by decreasing brain nitric oxide concentration (Trevor and Way, 1997). It should be noted that prolongation of pentobarbitone-induced sleep is an indication of sedative activity of drugs under investigation (Stahle and Ungerttedt, 1987).

The novelty induced rearing, spontaneous locomotor activity and sleep prolongation are all mediated by serotonin (5HT), dopamine, GABA, opioid and GABA-benzodiazepine receptors complex (Ajayi and Ukponmwan, 1994; Haefely, 1984; Hellion-Ibarrola et al, 1990). Additional studies will be necessary to ascertain if other neurotransmitters and their receptors (5HT and dopamine,) are also involved in the observed inhibitory effects of the extract of the leaves of *S. cayennensis*.

The methanolic extract did not show anxiolytic effects at the dose employed in this study. Doses were selected such that they were lower than the sedative doses to avoid false positive or false negative results in this study (Trullas and Skolnick, 1993); the butanol fraction caused significant increase in open arm entries as well as time spent in the open arm of the elevated plus maze (Table 4). The induction of the low index of open arm avoidance is interpreted as anxiolytic effect of the test substance as found in this study and in perfect agreement with the position of Trullas and Skolnick, 1993. The aqueous fraction of the extract also caused significant increase in open arm entries and time spent in the open arm but this effect was less than that caused by the butanol fraction. Diazepam employed as the standard anxiolytic agent in this study caused less open arm entries than the butanol fraction of the leaves of *S. cayennensis*. This may suggest that the anxiolytic effect of the butanol fraction is

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empirically superior to that of diazepam. It is interesting that the main extract did not demonstrate anxiolytic effect probably because the constituents in the whole extract before separation, particularly the constituents of the ethylacetate fraction were balancing the effect of the anxiolytic principles revealed by fractionation. Flumazenil, a competitive antagonist of GABA and diazepam blocked the anxiolytic effect due to butanol fraction and diazepam Table 4.

In conclusion, this study showed that the leaves extract of *S. cayennensis* possess sedative effect and this effect resides in the main methanolic extract, the aqueous as well as the butanol fractions of the extract. The neural mechanisms by which the observed sedation was induced by the leaves extracts are suggested to be both gabaergic and opioid. The anxiolytic effect of the extracts of the leaves was expressed by the aqueous and butanol fractions of the main methanol extract while the methanol extract itself did not exhibit any anxiolytic property from this study. The low LD₅₀ via the intraperitoneal route is instructive of potential for toxicity but in a previous study, the oral route which is the conventional route for the administration of the leaves extract ethnomedically was found to be safe (Akanmu et al, 2005).

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