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In silico and *in vivo* anti-stress potential of *Stachytarpheta cayennensis* (Verbenaceae) in mice

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ABSTRACT

Background: *Stachytarpheta cayennensis* is locally used as an antistress agent but no scientific rationale for its use. Hence, this study evaluated the antistress potential of ethanol leaf extract of *Stachytarpheta cayennensis* (ELSC) in mice.
Methods: The stress induced anxiety was assessed using elevated plus maze following acute restraint stress in mice. The *in silico* studies of previously reported compounds from *Stachytarpheta cayennensis* (*S. cayennensis*) were also carried out.
Results and discussion: There was significant ($p<0.001$) increase in anxiety status of stressed control mice compared to the unstressed control suggesting stress induction. However, ELSC stressed at 125 and 250 mg/kg and diazepam stressed control significantly ($p<0.001$) reduced the anxiety indices of mice compared to the stressed control group suggesting the reversal of stress-induced anxiety in mice. The results of *in silico* studies revealed betulinic acid was the most favourable compound in terms of pharmacokinetics, toxicity profile and binding affinity. The post docking analysis showed that betulinic acid and the positive antistress drug (diazepam) interacted with amino acid residues of the target receptor protein active pockets. Moreso, betulinic acid has a stronger binding affinity (-7.8 Kcal/Mol) compared to diazepam (-6.9 Kcal/Mol), suggesting that betulinic acid may be a more effective antistress agent than diazepam.
Conclusion: This study therefore, concluded that betulinic acid may at least in part be responsible for the observed antistress effect of *Stachytarpheta cayennensis.* However, further studies may be carried out on the antistress potential of betulinic acid.

Introduction

Stress is defined as a state of threatened or perceived threatened homeostasis caused by intrinsic or extrinsic stressors[1]. This threatened state of homeostasis is counteracted by the body complex neuroendocrine system known as 'stress system' which is made up of hypothalamic-pituitary-adrenal axis and the locus caeruleus/norepinephrine autonomic nervous systems[2]. The 'stress system' allows the body to deal effectively with the stressor and re-establish homeostasis[2]. Abberations in proper stress response system may lead to several pathological conditions[2].

The present-day life styles of high physical and psychological demands have made man prone to stress-related disorders such as anxiety, depression, insomnia[3,4], cognitive impairment[5,6], cardiovascular disorders[7] and psychosis[8]. Lack of gainful employment, divorce, child delivery, environmental pollution including wars constitute

common stressful events in life[9,10]. The consequence of body responding to stress includes hormonal imbalances, immune disorders and an increased incidence of cardiovascular disease[11].

Several synthetic drugs such as benzodiazepine anxiolytics have been developed to manage stress but these drugs have failed in its utilisation against stress induced negative impacts on cogntion, immunity, hypertension and peptic ulcer as well as its teratogenic effects on the unborn babies and deleterious effects on suckling infants[12,13]. Hence, the needs for new effective anti-stress agents to mitigate stress-induced disorders particularly from medicinal plants becomes pertinent and imperative[14].

For many centuries ago, people of the ancient days have used plants to alleviate symptoms of illnesses and promote healing[15]. Plant-based medicines still play a pivotal role in many traditional and modern medical practices[15]. To this effect, many medicinal plants have been evaluated for their antistress potentials[16,17]. However, *Stachytarpheta cayennensis* leaf has not been evaluated for its antistress potential despite its use for combating stress in traditional settings (verbal communication)

The plant *Stachytarpheta cayennensis* (Rich.) Vahl, popularly known as verbena, belongs to the family Verbenaceae [18]. This species is perennial herb native to America and distributed in tropical and subtropical parts of America, Brazil, Asia, and Australia and also found in other countries of the world such as Africa, Mexico and many more[19,20]. It is an erect, perennial, branching, somewhat angular, fibrous subshrub that is very resistant to traction[21].The plant is traditionally employed to treat inflammation, pain, fever, cough, arthritis, malaria, gastric, liver and mental disorders, to induce sleep and for its diuretic and laxative potentials[22-26].

Experimental findings have shown that *Stachytarpheta cayennensis* has anthelmintic[27], anti-inflammatory[28], antinociceptive[29], antioxidant[21] anti-ulcerogenic[30], antidiabetic[31], antimalarial[32], anti-diarrhoea activities[33], sedative[34] and anxiolytic effects[35]. The presence of therapeutic phytocompounds such as alkaloids, tannins, steroids, terpenoids, saponins, phenols and flavonoids, quinones, glycosides, phenolic compounds and gluconic acid have been reported[23,36].

The goal of this study is to provide scientific basis for the traditional use of *Stachytarpheta cayennensis* leaves in the treatment of stress.

Plate 1: Picture of *Stachytarpheta cayennensis* in its natural habitat
Source: Obtained from the Obafemi Awolowo University campus in Ile-Ife, Osun State.

Materials and methods

Collection of Plant Materials

Stachytarpheta cayennensis leaves were identified and collected from the wild on the campus of Obafemi Awolowo University (OAU) Ile-Ife, Nigeria during the month of August 2023. Herbarium voucher (FHI-106491) for *S. cayennensis* has been deposited with the National Herbarium, Forestry Research Institute of Nigeria, Ibadan.

Preparation and extraction of plant materials

The plant leaves were subjected to fourteen (14) days of air drying in the laboratory at room temperature. The dried leaves were pulverized and 122 g of the powdered leaves was extracted by maceration with 1.5 litres of seventy percent (70%) ethanol solution for 72 hours. The marc was re-extracted twice. The extract was concentrated in a water bath at a set temperature of 40°C and subsequently freeze-dried to yield 13.22 g (10.84%) crude ethanol extract (ELSC). The crude extract was freshly prepared by dissolving in normal saline on each day of experiment.

Animals

The animals used for the experiment were adult swiss albino mice of both sexes (21 ± 1 g). The animals were bred having free access to drinking water and standard commercial diet (Guinea feeds brand, Bendel Feeds, Nigeria), housed in the well aerated and lit animal house situated at the Central Animal House, Igbinedion University, Okada, Edo State. The mice were maintained under natural daylight and night condition. The studies were carried out between the hours of 9:00 am to 3:00 pm.

Drug

Diazepam (Roche, Basel, Switzerland) and normal saline (Unique Pharmaceutical Limited, Lagos, Nigeria). Drugs and ELSC were dissolved and made up to the required concentration with normal saline on each day of the experiment.

Acute toxicity test

The guideline described by the Organization for Economic Co-operation and Development (OECD) Annex 2 Test Guideline 42537 was used to determine the oral acute toxicity using a limit test at 2000 mg/kg p.o. as single dose for the extract. Mouse were kept without food for 3–4 h prior to dosing but had free access to water. A dose of 2000 mg/kg b.w was orally administered to one female mouse and closely observed for 30 mins and then 4 hr. Upon the survival of the mouse, 4 additional female mice were orally administered the same dose of the drug and observed as done for the first mouse. The same procedure was followed for control group of 5 mice which were administered normal saline. All the mice were thereafter monitored in the morning and evening of each day for 14 consecutive days.

Experimental Design

The adult mice were divided into 6 experimental groups (n=5). Group-1 mice (control unstressed) received normal saline (10 mL/kg p.o.) without stress, Group-2 mice (control stressed) received normal saline (10 mL/kg p.o.) 1 hr prior to acute restraint stress (ARS), Group-3 mice (standard drug-treated unstressed) received diazepam (2 mg/kg p.o.) without stress, Group-4 mice (standard drug-treated stressed) received diazepam (2 mg/kg p.o.) 1hr prior to ARS, Group-5 mice (Dose 1 stressed) were treated with ELSC (125 mg/kg p.o.) 1hr prior to ARS, Group-6 (Dose 2 stressed) received ELSC (250 mg/kg p.o.) 1hr prior to ARS.

Acute restraint stress (ARS) procedure

The acute restraint stress model employed in this investigation was modified from previous research[17]. The animals were divided into six groups as mentioned above. Stressed groups were administered normal saline or ELSC and subjected to stress 1 hour after. Diazepam (2 mg/kg p.o.) was administered 30 min post treatment; the mice were then subjected to stress. In the 1 h for control and ELSC or 30 minutes for diazepam period passed between the stress procedure and treatment groups, the animals were inserted inside the plexiglas mouse restrainers for 1 hour applying immobilization. This restrained all physical movement causing no pain in animal (Plate 2). The animals were deprived of food and water during the entire restraint period. After 1 hour, the animals were removed and immediately subjected to behavioral tests.

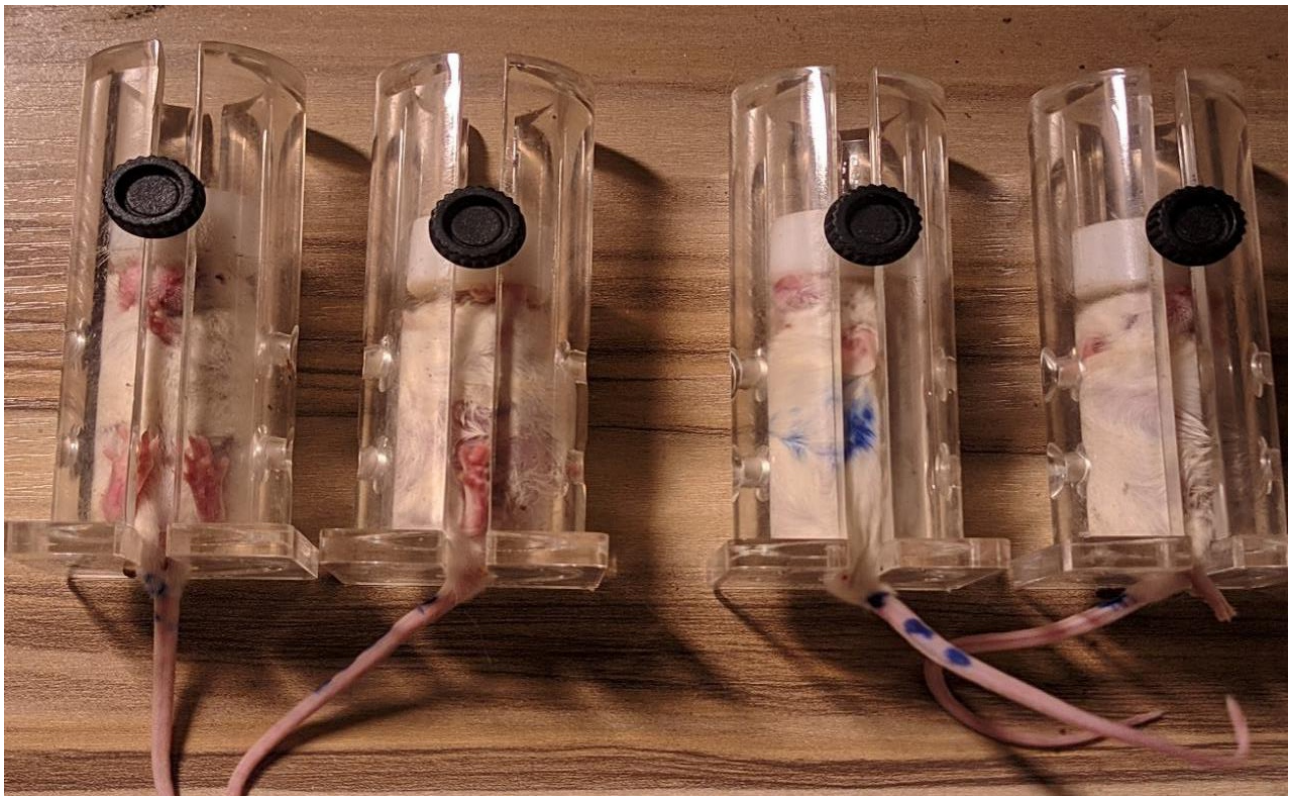


Plate 2: Mice being subjected to acute restraint stress (ARS)

Assessment of novelty-induced rearing and locomotion in open field test

The open field test model is used to observe the general motor activity and exploratory behavior of animal (novelty induced behavior)[38]. This model was adapted from previous work[39] to assess the stress-related behavior in mice on the basis of changes in the exploration, general locomotor activity and spontaneous activity. Each mouse was exposed to the open field test for 10 min in a dimly light room with the mice placed at the center of the open field and the number of rearing (frequency with which the mouse stood on its hind legs) and line crossings (locomotion which is the frequency with which the mouse crosses one of the grid lines with all four paws) noted.

Elevated Plus Maze (EPM)

This test was used to assess the behavioral changes was as earlier reported[40] based on the rodent's innate fear of heights and open areas. The plus maze comprises of two open arms (30 x 5 x 0.25 cm) and two enclosed arms (30 x 5 x 15 cm) extending from a common central platform (5 x 5 cm) with identical arms opposite each other. Between each examination, the maze was cleaned with 70% alcohol to remove any remaining smell cues. Each trial was recorded for 6 minutes with the parameters of open arm and close arm entries and time spent in open arm and close arm of EPM recorded. The anxiety index of each mouse was calculated using the percentage number of open arm entries (%OE) and percentage open arm duration (%OT) as previously done[41].

Retrieval of previously reported compounds from *S. cayennensis*

Sixty eight compounds from *S. cayennensis* were retrieved online from previously published data[32,42-47].

Preparation of target protein

The receptor protein used in this study to evaluate the in silico antistress potentials of the bioactive components of *S. cayennensis* was gotten from previous study[48] and downloaded from protein data bank (PDB) (https://www.rcsb.org/). The downloaded target protein (Crystal structure of a human gamma-aminobutyric acid receptor, the GABA (A) R-beta3 homopentamer: PDB ID 4COF)[48] was prepared using Chimera 1.10.2 software. The water molecule and all non-standard residues were removed from the protein. Thereafter, the protein was optimised for docking using dockprep tools. Polar hydrogen atom and Gasteiger charges were added to the protein and saved in pdb format.

Ligand preparation

The bioactive compounds retrieved from *S. cayennensis* were downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) with their PubChem CID numbers and saved in SDF format. Diazepam, a positive antistress agent was also downloaded from PubChem database. These ligands were prepared using Open Babel integrated in PyRx49 to minimize their energy and translated to pdbqt format in readiness for molecular docking.

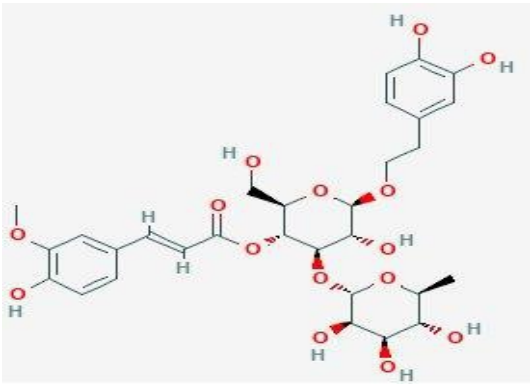
Molecular docking of protein and ligands

Molecular docking simulations were carried out using Vina Wizard integrated in PyRx. Prepared protein and ligands were loaded into PyRx and converted to pdbqt. The compounds were docked into the binding sites/pockets of the target protein using Vina Wizard of Pyrex software[50].

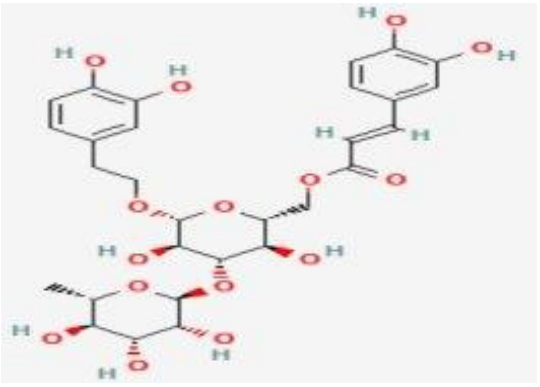
Ligands for *in silico* pharmacokinetics and toxicity predictions

From the result of molecular binding, compounds with stronger binding affinity than the positive antistress drug (diazepam) were used for the *in silico* pharmacokinetics and toxicity predictions.

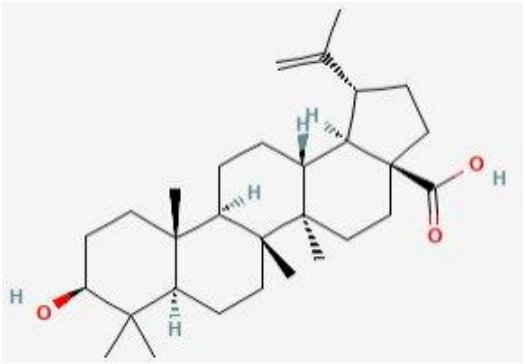
The in silico pharmacokinetics studies were carried out using SwissADME (https://www.swissadme.ch) and ADMETlab (admetmesh.scbdd.com) online servers[51]. The drug-likeness of these compounds were predicted using the Lipinski's rule of five[52]. The 2D structures of Leucosceptoside A (PubChem ID: 10394343), Isoacetoside (PubChem ID: 6476333), Betulinic acid (PubChem ID: 64971), Jionoside D (PubChem ID: 9895632), Verbascoside (PubChem ID: 5281800), Martinoside (PubChem ID: 13989933) and Martynoside (PubChem ID: 5319292) were downloaded from PubChem database in structured data file (SDF) format. Their respective SMILES were used through swissADME (http://www.swiss.adme.ch/) and ADMETlab online servers (https://admetlab3.scbdd.com/) to evaluate physicochemical and pharmacokinetics properties as well as the drug likeness of these compounds[51,53].



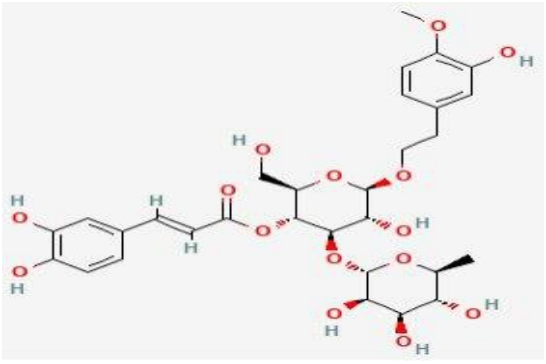
Leucosceptoside A



Isoacteoside (Isoverbascoside)



Betulinic acid



Jionoside D

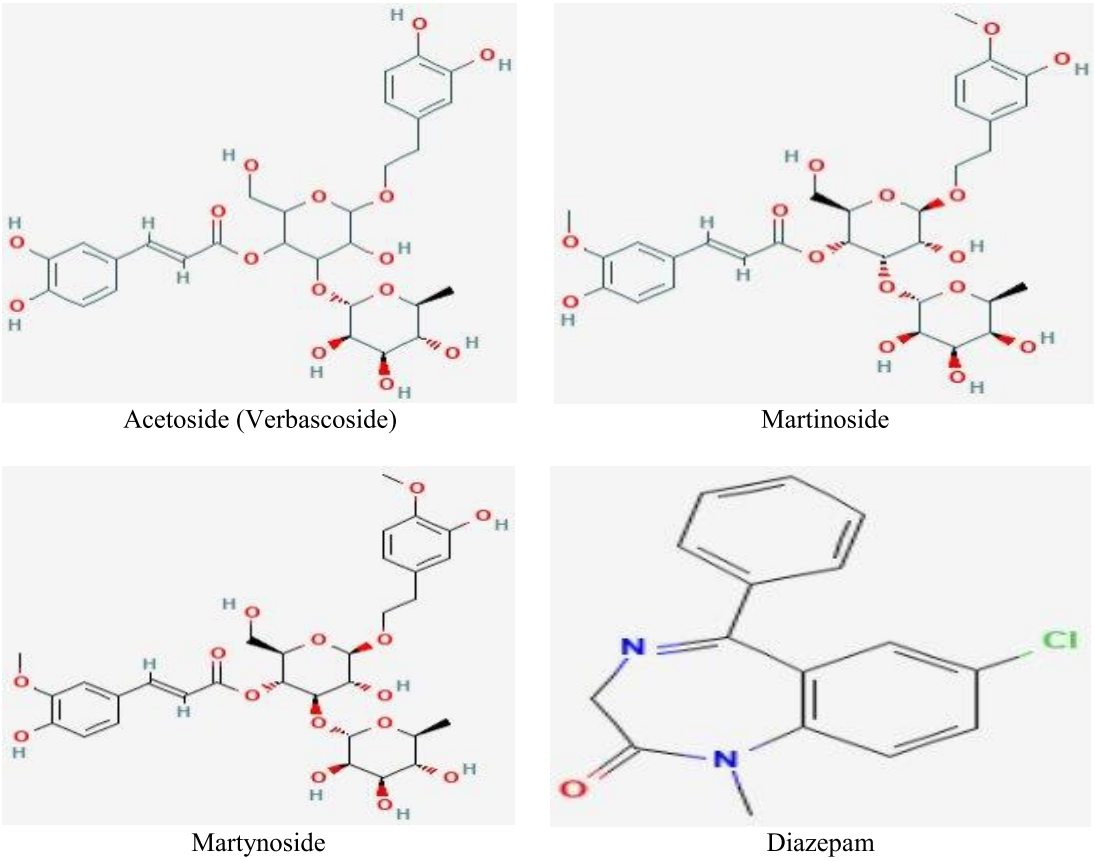


Plate 3: Chemical structure of some compounds from *S. cayennensis* plant and diazepam. Source: Downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov/)

***In silico* toxicity predictions**

The toxicity prediction was carried out using ProTox II web server (http://tox.charite.de/protox3/). The 2D chemical structures of the compounds were converted to their respective SMILES and inputted into the online server for toxicity prediction. The oral acute toxicity (LD_{50}) of the compounds was predicted. The organ toxicity (hepatotoxicity, nephrotoxicity and neurotoxicity) and toxicological endpoints (carcinogenicity, immunotoxicity, cytotoxicity, and mutagenicity) were also predicted[54,55].

Protein-ligand interactions analysis

From the results of *in silico* pharmacokinetics prediction, compound (s) that did not violate the drug-likeness test **(**Lipinski's rule of five)[52] has/have their receptor-ligand-interactions analyzed using LigPlot+ software[56].

Statistical Analysis

The results obtained from the experiments were expressed as mean ± SEM and statistically analyzed using one way ANOVA (Analysis of Variance) followed by Dunnett's post hoc analysis. GraphPad InStat® Biostatistics software (GraphPad Software, Inc., La Jolla, USA) was used as statistical tool. All tests were carried out with the significance level set at $p<0.05$, $p<0.01$ and $p<0.001$ compared to the control or stress control.

Results

Acute toxicity studies

Acute oral toxicity studies showed that all the treated mice survived beyond 14 days hence, no mortality was observed for up to the dose of 2000 mg/kg of ESC.

Effect of ethanol leaf extract of *Stachytarphetata cayennesins* on novelty-induced rearing in mice

The stress control significantly ($p<0.01$) increased novelty-induced rearing compared to the control. However, DZP stress and ELSC at 125 and 250 mg/kg significantly ($p<0.001$) reversed the stress induced rearing compared to the stress control mice (Figure 1A).

Effect of ethanol leaf extract of *Stachytarphetata cayennesins* on novelty-induced locomotion in mice

The stress control significantly ($p<0.01$) increased novelty-

induced locomotion compared to the control. However, DZP stress and ELSC at 125 mg/kg significantly ($p<0.001$) reversed the stress induced locomotion compared to stress control. Similarly, ELSC at 250 mg/kg significantly ($p<0.05$) reversed the stress induced increase in locomotion compared to the stress control mice (Figure 1B).

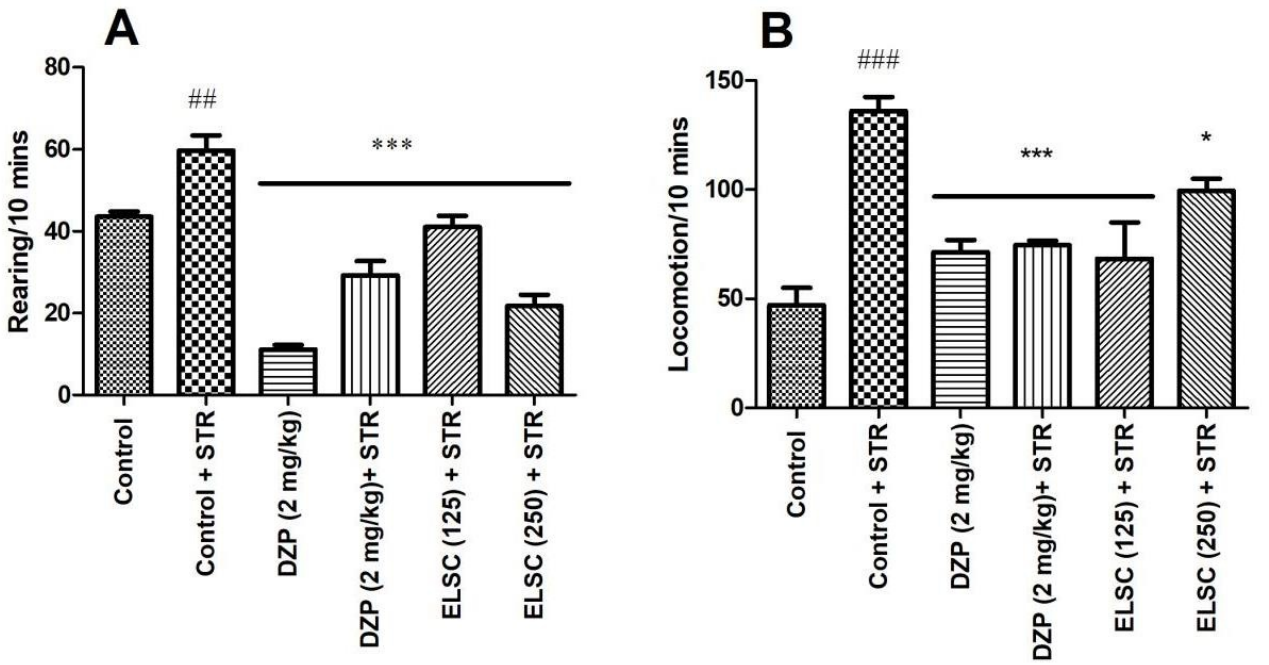


Figure 1: Effect of ethanol leaf extract of *Stachytarphetata cayennesins* on rearing (A) and Locomotion (B) in mice.

Control; Normal saline (10 mL/kg, p.o.), ELSC (mg/kg); ethanol leaf extract of *Stachytarpheta cayennensis,* DZP; diazepam, STR; stress. Each bar represents Mean ± SEM, n=5 (ANOVA; Dunnett's post hoc), $^{###}p<0.001$ compared to control and $^{*}p<0.05$ and $^{***}p<0.001$ compared to stress control.

Effect of ethanol leaf extract of *Stachytarphetata cayennesins* on percentage number of open arm entry in mice

There was significant ($p<0.001$) reduction in the percentage number of open arm entry in stress control compared to control group. However, DZP stress and ELSC (250 mg/kg) significantly ($p<0.01$) reversed the stress induced reduction in the percentage number of open arm entry compared to control. Similarly, ELSC (125 mg/kg) significantly ($p<0.05$) reversed the stress induced reduction in the percentage number of open arm entry compared to control (Figure 2A).

Effect of ethanol leaf extract of *Stachytarphetata cayennesins* on percentage number of open arm duration in mice.

There was significant ($p<0.001$) reduction in the percentage number of open arm duration in stress control compared to control group. However, ELSC (125 mg/kg) and ELSC (250 mg/kg) significantly ($p<0.001$) and ($p<0.05$) reversed the stress induced reduction in the percentage number of open arm duration compared to control respectively. Similarly, DZP stress significantly ($p<0.05$) reversed the stress induced reduction in the percentage number of open arm duration compared to control (Figure 2B).

Effect of ethanol leaf extract of *Stachytarphetata cayennesins* on percentage number of open arm duration in mice.

The stress control significantly ($p<0.001$) increased the anxiety indices of mice compared to the control. However, DZP stress, ELSC at 125 mg/kg and 250 mg/kg significantly ($p<0.001$) ameliorated the increased anxiety indices induced by stress control in mice (Figure 2C).

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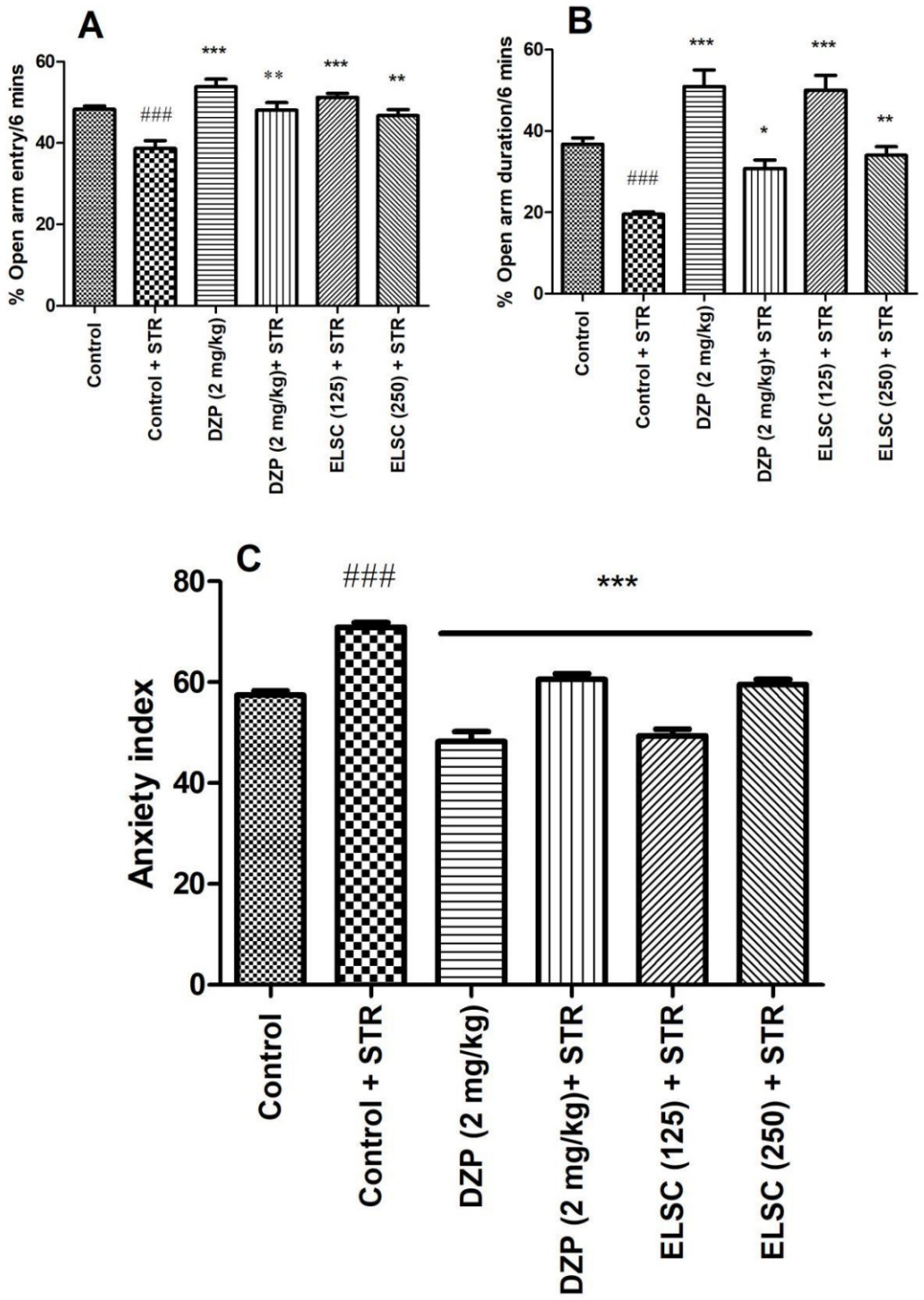


Figure 2: Effect of ethanol leaf extract of *Stachytarphetata cayennesins* on percentage number of open arm entry (A), percentage number of open arm duration and antianxiety index (C) in mice.

Control; Normal saline (10 mL/kg, p.o.), ELSC (mg/kg); ethanol leaf extract of *Stachytarpheta cayennensis,* DZP; diazepam, STR; stress. Each bar represents Mean ± SEM, n=5 (ANOVA; Dunnett's post hoc), $^{###}p<0.001$ compared to control and $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to stress control.

Results of molecular docking

Of all the 68 compounds docked, Leucosceptoside A (-7.9 Kcal/Mol), Iso-acteoside (-7.8 Kcal/Mol), Betulinic acid (-7.8 Kcal/Mol), Jionoside (-7.5 Kcal/Mol), Verbascoside (-7.3 Kcal/Mol), Martinoside (-7.1 Kcal/Mol), Martynoside (-7.1 Kcal/Mol) have higher docking scores compared to the positive control drug diazepam (6.9 Kcal/Mol) on $GABA_A$ receptor site [Table 1].

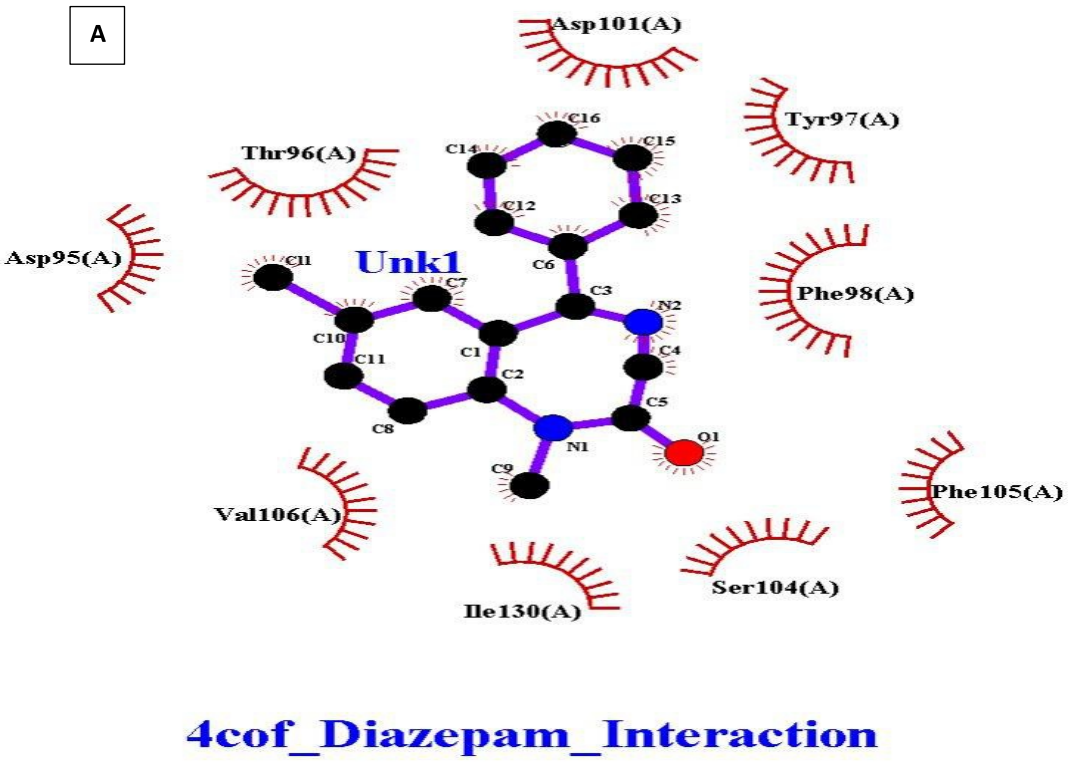
The post docking analysis using LIGPLOT showed that diazepam and betulinic acid interacted with GABAA receptor protein at 8 common position that is TYR97, TYR157, PHE105, PHE98, ILE130, ASP101, VAL106, SER104, and THR106 amino acids residues [Figure 3].

Table 1: Binding affinity of retrieved compounds from *C. cayenensis* and diazepam at $GABA_A$ receptor site

			Docking scores (Kcal/Mol)
	Compound	PubChem CID	4COF
1.	Leucosceptoside A	10394343	-7.9
2.	Isoacteoside	6476333	-7.8
3.	Betulinic acid	64971	-7.8
4.	Jionoside D	9895632	-7.5
5.	Verbascoside	5281800	-7.3
6.	Martinoside	13989933	-7.2
7.	Martynoside	5319292	-7.1
8.	Diazepam	3016	-6.9

Results of pharmacokinetics predictions of compounds from *S. cayennensis* using Swiss ADME

All the evaluated compounds (Leucosceptoside A, Isoacetoside, Betulinic acid, Jionoside, Verbascoside Martinoside and Martynoside) have low gastrointestinal (GIT) absorption values. All except betulinic acid (0.85) have bioavailability values of 0.17. Similarly, all except betulinic acid violated the Lipinski's rule of five for drug-likeness [Table 2].



4cof_Diazepam_Interaction

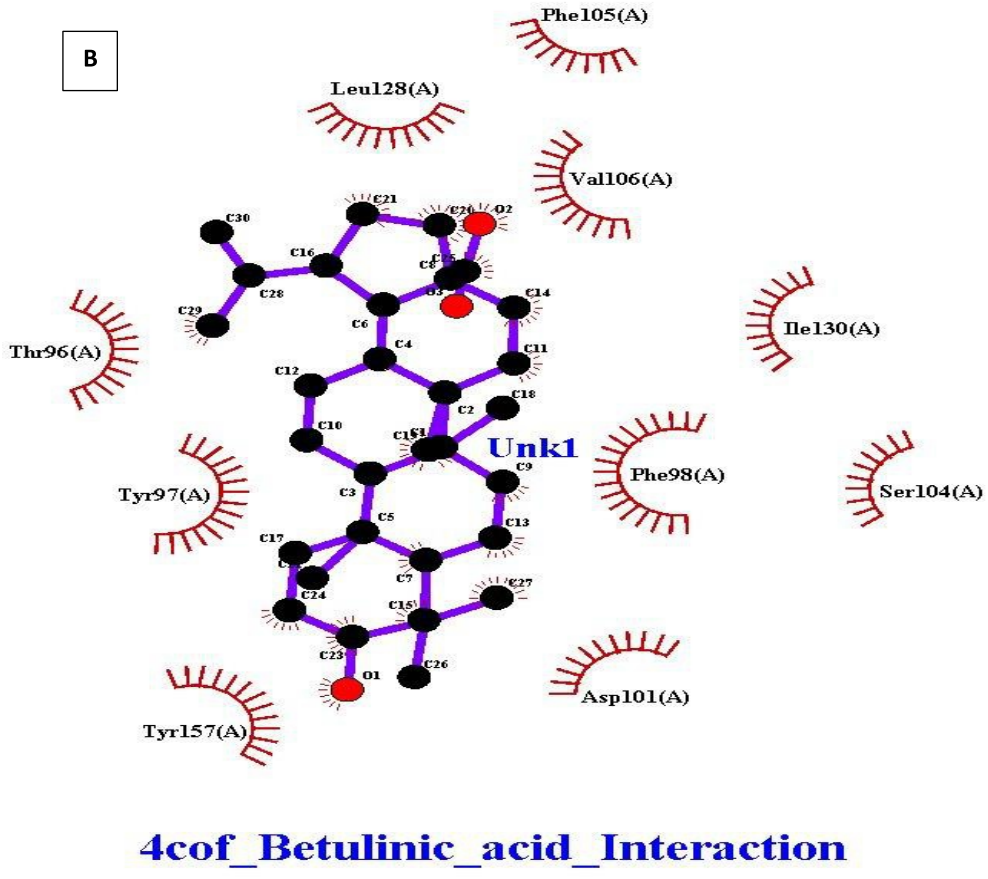


Figure 3: 2D representation of Diazepam (A) and Betulinic acid (B) showing interactions with different amino acid residues of 4COF receptor.

Table 2: *In-silico* pharmacokinetics predictions of previously isolated compounds from *S. cayenensis*

Compounds	MW	GIT absorption	Bioavailability	T1/2	CLp	Drug -likeness	
A	638.61	Low		0.17	3.39	2.85	No
B	624.59	Low		0.17	3.11	4.51	No
C	456.7	Low		0.85	0.79	6.35	Yes
D	638.6	Low		0.17	3.55	2.63	No
E	624.6	Low		0.17	4.31	3.18	No
F	652.6	Low		0.17	3.83	2.86	No
G	652.6	Low		0.17	3.29	2.12	No

MW = Molecular weight in g/mol; T1/2 = Half life; CLp = plasma clearance; A = Leucosceptoside A; B = Isoacetoside; C = Betulinic acid; D = Jionoside D; Verbascoside; Martinoside; F = Martynoside

Results of toxicity predictions of compounds from S. stachytarpheta using ProTox II

All the compounds evaluated except Jinoside showed to be inactive in hepatotoxicity, neurotoxicity except Jinoside in *in silico* toxicity predictions. However, 4 compounds (57.1%) were active for nephrotoxicity (Leucosceptoside A, Isoacetoside, Verbascoside, Martinoside and Martynoside), 1 compound (14.3%) was positive for carcinogenicity (Betulinic acid). All compounds were inactive for mutagenicity and cytotoxicity and all are active for immunotoxicity. All the evaluated compounds belong to toxicity class 5 except Jionoside that belongs to class 4 [Table 3].

Table 3: *In silico* toxicity prediction of compounds previously isolated from *Starchytarpheta cayenensis*

Compounds			Organ toxicity						End point toxicity							
	LD_{50}	T-class	Hepatotoxicity		Neurotoxicity		Nephrotoxicity		Carcinogenicity		Immunogenicity		Mutagenicity		Cytotoxicity	
			Toxicity	Prob	Toxicity	Prob	Toxicity	Prob	Toxicity	Prob	Toxicity	Prob	Toxicity	Prob	Toxicity	Prob
A	5000	5	Inactive	0.82	Inactive	0.86	Active	0.74	Inactive	0.82	Active	0.99	Inactive	0.88	Inactive	0.75
B	5000	5	Inactive	0.79	Inactive	0.89	Active	0.75	Inactive	0.82	Active	0.99	Inactive	0.86	Inactive	0.75
C	2610	5	Inactive	0.54	Inactive	0.79	Inactive	0.60	Active	0.53	Active	0.74	Inactive	0.74	Inactive	0.97
D	1190	4	Active	0.69	Active	0.87	Inactive	0.90	Inactive	0.62	Active	0.96	Inactive	0.97	Inactive	0.93
E	5000	5	Inactive	0.81	Inactive	0.87	Active	0.75	Inactive	0.81	Active	0.99	Inactive	0.87	Inactive	0.77
F	5000	5	Inactive	0.82	Inactive	0.86	Active	0.73	Inactive	0.81	Active	0.99	Inactive	0.86	Inactive	0.73
G	5000	5	Inactive	0.82	Inactive	0.86	Active	0.73	Inactive	0.81	Active	0.99	Inactive	0.86	Inactive	0.73

LD_{50} = median lethal dose; T-class = toxicity class; Prob = probability; A = Leucosceptoside A; B = Isoacetoside; C = Betulinic acid; D = Jionoside; Verbascoside; Martinoside; F = Martynoside

Discussion and conclusion

This study investigated the in vivo antistress potential of ethanol leaf extract of *Stachytarpheta cayennensis* (ELSC) using acute restraint stress (ARS) in mice. It went further to carry out *in silico* analyses (molecular docking, pharmacokinetics and toxicity predictions) using $GABA_A$ receptor site[48] and retrieved ligands previously reported to be present in *Stachytarpheta cayennensis*[31,42-47].

In the oral acute toxicity test, no death that was observed up to 2000 mg/kg suggest that the extract may be safety margin of up to 2000 mg/kg in mice. Hence the extract may be considered to belong to a lower class of toxicity57. This in vivo acute toxicity finding may also be supported at least in part by the *in silico* toxicity prediction, which predicted toxicity class 5 (may be harmful if swallowed) for six out of the seven compounds and class 4 (harmful if swallowed) for the seventh compound from *S. cayennensis* subjected to *in silico* toxicity predictions in this study[58]. Based on the acute toxicity finding, lower doses of 125 and 250 mg/kg were used in this study.

The novelty-induced behaviours have been extensively used to study the gross effects of agents on the central nervous system (CNS)[59]. Agents that stimulates the CNS increase novelty-induced rearing, grooming and locomotion, while agents that decrease these parameters depress the CNS[59]. Therefore, the increase in rearing and locomotion by stress control suggest that stress induces CNS excitation. However, the reversal of the induced stress by ELSC suggested that the extract may possess anti-stress potential in mice. This assertion can further be corroborated by the reduction in these parameters by a standard anti-stress drug diazepam used as positive control drug in this experiment.

The acute restraint stress (ARS) is a broadly used behavioral model in studying the molecular basis of stress-related issues[60] such as anxiety and depression. Earlier report has also shown that quantification of anxiety states post ARS induction made the mice to experience a higher level of anxiety on elevated plus maze[16,17,61]. In this study, ARS induced anxiety-like behavior as observed by the reduction in the percentage number of open arm entries and percentage time spent on the open arms as well as increased anxiety index as recorded from the index of open arm avoidance. The reversal of anxiety induced by ARS by the ELSC suggests that the extract may have mitigating effect on stress induced anxiety in mice. This assertion is in conformity with earlier experimental findings that ARS induced anxiety-like behavior which could be ameliorated by therapeutic agents [16,17,62]. Although the mechanism of ani-stress effect of ESC on anxiety-like behavior on EPM was not delineated in the study but it could be suggested that ESC might be acting like an agonist as does diazepam through $GABA_A$-benzodiazepine receptor-Cl-channel complex in the elicitation of its antistress effect. This is in consonance with the earlier suggested agonistic action of *Euphorbia hirta* involved in the depletion of stress-induced anxiety-like behavior via $GABA_A$- benzodiazepine receptor-Cl2 channel complex[63].

Molecular docking (MD) is an essential procedure in drug discovery and rational drug design[64]. This is because MD predicts he binding orientation of small molecule drug candidates into their protein targets to predict the affinity and activity of small molecules64,65. The stronger binding affinities of leucosceptoside A, isoacetoside, betulinic acid, jionoside D, verbascoside, martinoside, martynoside with respect to the binding affinity of diazepam [Table 1] on GABAA receptor site suggest that these compounds may be

the promising compounds in *S. cayennensis* that is at least in part responsible for the antistress potentials of the plant in this study. However, pharmacokinetics study revealed betulinic acid as the only drug candidate of all the compounds screened using Linpiski's rule of five[52]. Likewise, betulinic acid has the most favourable toxicity profile compared to other compounds in *in silico* toxicity predictions [Table 3]. This finding of the safety profile of betulinic acid from our *in silico* toxicity predictions can be corroborated by the findings of Pisha *et al*.[66] who reported the safety of betulinic acid in the treatment athymic mice carrying human melanomas. However, of concern in our toxicity prediction in this study, is the carcinogenicity and immunotoxicity associated with betulinic acid. Hence, further study may be warranted to clear this contradictions. The favourable *in silico* pharmacokinetics properties of betulinic acid can also be supported by the report of Udeani *et al.*[67]. Since betulinic acid was the most favourable compound in terms of binding affinity, pharmacokinetics and toxicity profile, this compound was further analyzed for its ligand-protein interaction using LIGPLOT[56,68].

The ligand-receptor interaction for diazepam and betulinic acid showed that betulinic acid occupied the same active pockets like diazepam in 4COF receptor as also depicted in PDBSUM. Moreover, betulinic acid has a stronger binding affinity than diazepam suggesting that betulinic acid may be a more promising antistress candidate than diazeapm (a positive control drug).

Betulinic acid is one of the naturally occurring pentacyclic lupane-type triterpenoid which is usually isolated from birch trees but also found widely distributed in other medicinal plants[69,70]. The observed antistress potential of betulinic acid in this study, along with its neuroprotective effect in other brain disorders involving the GABAA receptor, such as epilepsy[71], anxiety[72], and insomnia[73], may be at least partially explained by its previously reported anti-oxidant and anti-inflammatory[74,75] effect as well as its high ability to cross the blood-brain barrier from earlier in silico studies[76]. It will therefore be plausible to suggest that betulinic acid in *S. cayennensis* may at least in part be responsible for the earlier reported anxiolytic[35], sedative-hypnotic[34,35] and anticonvulsant[77] effects of the plant.

Conclusion

In conclusion, the ethanol leaf extract of *Stachytarpheta cayennensis* may have anti-stress effects against stress-induced anxiety in mice. The study further concluded that betulinic acid may be acting in additive or synergy with other phytocompounds present in the extract to elicit the observed antistress potential and GABAergic system may be involved in the antistress effect.

CONFLICT OF INTEREST

None declared

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