Niger. J. Physiol. Sci. 36 (December 2021): 173– 180
www.njps.physiologicalsociety.com
https://doi.org/10.54548/njps.v36i2.5

Niger. J. Physiol. Sci.

Full-Length Research Article

Effects of Ruzu, a Polyherbal Mixture, on Neurobehaviour and Expression of Serotonin and Dopamine Transporters in Rats

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Summary: There is an increased possibility that combined herbal constituents may interact to increase toxicity and lower efficacy. Ruzu herbal bitters (RHB) is a blend of extracts from *Curculigo pilosa, Uvaria chamae*, and *Citrullus colocynthis*, each of which has been shown to possess important bio-effects. There is anecdotal evidence for efficacy of RHB in neurological disorders; however, there are no data on possible neurotoxic effects of RHB. Using behavioural, biochemical and molecular indices as surrogates of neurotoxicity, this study therefore evaluated the nervous system effects of RHB. Twenty male Wistar rats were grouped into two – a control group and RHB group (n=10). RHB (0.5ml/kg) was administered to the RHB group twice daily while control group took water (0.5ml/kg). Treatments lasted 6 weeks after which behavioural tests were carried out. Animals were subsequently sacrificed and the expression of serotonin transporter (SERT) and dopamine transporter (DAT) was determined in the striatum by immunofluorescence while specific activities of catalase, alkaline phosphatase and gamma glutamyltransferase were determined. In the elevated plus maze and light and dark box tests which are models of anxiety, animals treated with RHB showed significant anxiety compared to control. They also showed impaired locomotor activity in the open field and wire hang tests. The activity of catalase was significantly increased in the brain of the RHB treated rats while an increase in the expression of both DAT and SERT was observed in the striatum.

Keywords: *Ruzu, Polyherbalism, serotonin, dopamine, behaviour*

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Manuscript received- October 2021; Accepted- November, 2021

INTRODUCTION

Polyherbalism started as a result of the inadequacy of the active phytochemical constituents of individual plants to provide attractive pharmacological action and attain the desirable therapeutic effects (Showande and Amokeodo, 2014). The concept of polyherbalism which involves the combination of several medicinal herbs in meticulous ratios has been proven to achieve greater therapeutic effectiveness (Karole *et al*. 2019). According to the World Health Organization, 80% of the world's population still rely majorly on the different species of plants for their health care (Parasuraman *et al*; 2014). Owning to cost effectiveness, perception of wonderful healing, curative, and preventiveness coupled with the notions for fewer side effects, the use of polyherbal formulations such as *Ruzu* herbal bitter is strongly encouraged in Nigeria (Oreagba *et al*. 2011).

Ruzu herbal bitters (RHB) is a poly-herbal mixture widely used as an anti-malaria, anti-typhoid, and anti-obesity concoction in Nigeria (Ogunlana *et al*. 2018). It has been claimed to be useful in the management of sexually transmitted diseases. It is also claimed to detoxify the kidneys, tone the liver, shrink fibroid tumor cells, lower high blood pressure and relieve waist, back and joint pains. As an aqueous preparation, RHB contains 40% *Curculigo pilosa* (Squirrel groundut) root, 20% *Uvaria chamae* (Bush Banana) stem, and 40% *Citrullus colocynthis* (Bitter Apple or Desert Gourd) bark.

The phytochemical investigation of *Curculigo pilosa*, which is used in the traditional treatment of arthritis, impotence, gastrointestinal and heart diseases, revealed that the plant has antioxidant, hepatoprotective, and neuroprotective activity (Nie *et al*. 2013). *Uvaria chamae*, has been reported to be effective in the treatment of sickle cell anemia, severe abdominal pains, cough, urinary tract and cerebral infections, and diarrhea (Oluremi *et al*. 2010). *Citrullus colocynthis* plant is widely known for its efficacy in the management of various diseases including diabetes, cough, asthma, jaundice, mastitis, cancer, leprosy, common cold, toothache, wound, joint pain, and gastrointestinal (Hussain *et al*. 2014).

The safety and efficacy of polyherbal mixtures has become a public health concern. The perception that "natural" equates to "safe" blinds users from the adverse effects of polyherbal mixture (Vidushi 2013). Although each of the RHB active constituents has been scientifically investigated and documented to be safe, the synergism in polyherbal mixtures such as RHB predisposes them to herb-herb interactions capable of affecting their pharmacological

or toxicological profile. Serious psychiatric and neurological effects have been associated with the use of herbal medicines (Ernst 2003). Furthermore, there are limited scientific data to support the claimed safety and therapeutic efficacy of polyherbal formulations like RHB. Hence, this study was designed to evaluate the neurobehavioural effects of *Ruzu* Herbal Bitters. This is important because the nervous system is a common site of natural products' toxicities (Harris & Blain 2004) and this toxicity is often manifested as both downstream (behaviour) and upstream (brain) disruptions. We evaluated the effects of RHB on anxiety, memory, and motor functions. It was also of interest to examine the expression of the serotonin and dopamine neurotransmitter transporters to have an insight into the molecular basis of any behavioural deviations caused by sub-chronic RHB.

MATERIALS AND METHODS

Animals: 20 Male Wistar rats (140g-160 g) were utilized for the study. The animals were acquired and housed at the animal house of the Department of Physiology, Ladoke Akintola University of Technology, Ogbomoso Oyo State, Nigeria. The animals were kept under standard laboratory conditions and housed in well ventilated plastic cages at room temperature and relative humidity with light and dark cycles (12hr/12hr). The animals were acclimatized for 2 weeks and were provided standard rat pellet and water *ad libitum* throughout the experiment. Animal management and experimental procedures were performed in accordance with the requirements of the university's guide for the use of laboratory Animals.

Materials: *Ruzu* herbal bitters (RHB) (NAFDAC Registration Number A7-1102L) was obtained from A2W Global Ltd., Lagos, Nigeria. Rat-reactive anti-SERT and anti-DAT rabbit polyclonal antibodies were purchased from Elabscience Biotechnology (USA). Anti-rabbit secondary antibody was purchased from R&D Systems (MN, USA). Every other reagents were of pure analytical grades from British Drug House (UK) and Sigma-Aldrich (USA).

Experimental design: After acclimatization, the animals were randomly grouped into two with each group made up of 10 animals. The first group (control) were given 0.5ml/kg of distilled water orally while the second group (RHB) received 0.5 ml/kg of RHB orally. This dose was extrapolated from the manufacturer-recommended adult daily dose. Treatment was twice daily (7am and 7pm) for six consecutive weeks. After the treatment period, animals were subjected to a battery of behavioural tests before being euthanized for whole brain harvesting.

Phytochemical screening: The qualitative phytochemical screening of *Ruzu* Herbal Bitters was conducted by adopting the methods as described by Odebiyi and Sofowora (1979) and Onwuka, (2005). The bioactive substances determined were Tannin, Saponin, Flavonoid, Cardiac Glycoside, Steroid, Terpenoids, Anthraquinones, Phenols, and Alkaloid.

Proximate Analysis: Proximate analysis was conducted on *Ruzu* Herbal Bitters to determine the nutritional value of the herbal bitter. Following the standard methods of Association of Official Analytical Chemists (AOAC, 2005), the proximate composition of the samples with respect to moisture content, Ash content, carbohydrate, crude protein, crude fiber, and crude fat were determined . Caloric value was calculated.

Behavioural Studies

Elevated plus maze: The elevated plus maze (EPM) has been documented to be very effective in assessing anxiety-like behavior (Komada *et al*., 2008). The EPM apparatus consists of two opposing open arms (50 × 10 cm) and two opposing closed arms (50 × 10 × 40 cm), elevated 50 cm above floor level. The arms are connected by a common central platform (10 × 10 cm) where the rat is placed. The edges, 6 mm high, surround the open arms, reducing the chances of rats falling from the apparatus.

Each animal was individually placed in the common central platform of the maze, with the animal facing one of the enclosed arms. Each animal was allowed to explore the maze freely for 5 mins. The rat's behavior was recorded by an overhead digital camera for the duration of the test and then video-analyzed by an assistant blinded to the study. The number of open and enclosed arm entries and time spent in each arm were scored. An entry is recorded when all four paws enter the arm. After each rat, the entire maze was cleaned using 70% ethanol before the next rat was put on the maze.

The numbers of entries into the open arms (OAE) and time spent in the open arms (OAT) reflect the general behavior of the rat. Anxiety-like behavior was deduced based on the less common entrance of the rat into the open arms of the maze and the decreased amount of time spent in the open arms of the maze.

Light and dark box test: The light/dark box test is based on the innate aversion of rodents to brightly illuminated areas and the spontaneous exploratory behavior of rodents in response to mild stressors like novel environment and light (Crawley and Goodwin 1980). The test is used to assess unconditioned anxiety and exploratory behavior. The light and dark box used was made of Plexiglass and consisted of two compartments which are equally divided. One compartment was covered outside with black cardboard making it the dark compartment while the other compartment was left uncovered making it the light compartment. The experiment was carried out in an isolated room away from noise, scents and movement.

Each animal was left to freely move from the first compartment to the second one through an open door between the compartments for 5 minutes. A rat was placed into the light box facing the open door, and the time the animal spent in each compartment was recorded by a video camera set up high above the plexiglass. The box was cleaned using 70% ethanol between rats to remove external scents, fecal deposits and urine.

Anxiolytic-like potential was deduced from increase in the time spent in the light chamber.

Open field test: In a bid to evaluated the general locomotor activity of the animals, they were subjected to an open field (OF) test. The OF apparatus is made of a wooden square box

(60 × 60 × 35 cm) and divided into nine equal squares (20 × 20 cm). Each animal was placed at the centre of the wooden box and allowed to explore the area freely for 5 minutes. Animals were filmed by an overhead digital camera. The distance walked in cm, analyzed for the whole arena (total distance) was quantified as the horizontal locomotor activity. The apparatus was cleaned with 70% alcohol after each trial.

Wire Hang Task: The wire hang task was used to assess forelimb strength as previously described (Jansone *et al*., 2016). Each rat was placed midway on a stainless-steel wire (90 cm length, 3 mm in diameter), mounted between two platforms at 60 cm above the ground. The performance was observed for a maximum of 2 minutes. Each rat was made to grasp the central position of the wire with its forepaws. The latency (s) to fall from the wire to the flat soft pad was measured. The trial was conducted three times for each rat with the longest duration used for the evaluation. Rats were allowed to rest for at least 3 minutes between consecutive attempts.

Oxidative Stress Status: Catalase Inhibition Assay was done to evaluate the oxidative stress status of the rats' whole brains. Catalase activity was determined using a colorimetric assay described in detail by Goth (Góth, 1991). The method is based on the formation of a yellow complex with molybdate and H_2O_2. Briefly, 50 µL of the supernatant of brain homogenate was added to 50 µL reaction mixture containing 50 mMH_2O_2 in sodium-potassium phosphate buffer (0.2 M, pH 7.4) in a 96-well microtiter plate. This was incubated for 3 minutes at 37°C. 100 µL ammonium molybdate (64.8 mM in H_2SO_4) was used to stop the enzymatic reaction, with the absorbance measured in a microplate reader at 405 nm (Micro READ 1000, Belgium).

Determination of Brain Markers: Activities of brain enzymes, such as gamma glutamyl transferase (GGT) as well as alkaline phosphatase (ALP) were assayed on a biochemical analyzer (Olympus AU-600, Tokyo, Japan) based on the protocols of commercial kits.

Immunofluorescence: Brain tissues from transcardially perfused (phosphate-buffered paraformaldehyde 4%) animals were removed and cryo-preserved in sucrose solution. Cryostat sections revealing the striatum were blocked with 1% BSA solution in PBS at room temperature for 60 minutes. Thereafter, the sections were incubated for 1 hour 30 minutes with rabbit anti-SERT/anti-DAT primary antibody which was diluted in blocking buffer (1:300). Subsequently, slides were incubated with anti-rabbit secondary antibody (1:200) for 60 minutes in a humidified dark chamber. Then slides were counterstained with Hoechst nuclear stain before being mounted with N-propyl gallate-supplemented glycerol/PBS solution. Mounted slides are stored at 4°C and away from light. Using SP-98-FL inverted fluorescent microscope (Brunel Microscope Limited), the slides were visualized. The images were analyzed, background noise was removed, and the mean optical density of the fluorescence signal were quantified with ImageJ software (NIH) to remove background noise and quantify.

Data Analysis: Video and data analyses were carried out by someone blinded to the experimental interventions. All values are expressed as mean ± SEM. Data were analyzed with GraphPad Prism, version 7.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Inter-group differences were analyzed with unpaired t-test while p values ≤ 0.05 were considered statistically significant.

RESULTS

Phytochemical screening: The result of the preliminary qualitative phytochemical screening of RHB showed that saponins, terpenoids, and alkaloids were strongly present in the polyherbal mixture. Flavonoids was found to be present in trace amount while tannins, cardiac glycosides, anthraquinones, steroids, and phenols were found to be absent.

Table 1
Phytochemical components of *Ruzu* herbal bitters

Component	Content
Saponins	++
Tannins	-
Flavonoids	+
Cardiac glycosides	-
Steroids	-
Anthraquinones	-
Terpenoids	++
Phenol	-
Alkaloids	++

Proximate analysis: Table 2 shows the proximate composition of RHB.

Table 2
Proximate composition of Ruzu herbal bitters

	Sample 1	Sample 2
Moisture (%)	74.44853	74.11395
CHO (%)	22.10301	22.45589
Calorific value (kj/100g)	476.7266	489.3274
Crude protein %	6.376324	6.778022
Crude fibre (%)	0.070667	0.071333
Ash (%)	0.525073	0.491159
Crude lipid (%)	0.029753	0.029735

Elevated plus-maze: As shown in Figure 1, RHB-treated animals had fewer open arm entries (RHB: 0.80±0.25 vs Control: 2.40±0.65; $p<0.05$), spent less time (seconds) in open arms (RHB: 6.90±1.48 vs Control: 24.60±2.70: $p<0.05$), and spent more time (seconds) in the closed arms (RHB: 295.90±1.28, $p<0.05$).

Light and dark box test: Figure 2 shows that the time (seconds) spent by the RHB-treated rats in the light compartment was less than that of the control (RHB: 130.40±11.33 vs Control: 151.40±10.23, $p>0.05$) although this was not statistically significant. Similarly, the RHB animals spent more time in the dark compartment than the control (227.20±7.52 vs Control: 215.50±9.22; $p>0.05$) although this also was not statistically significant.

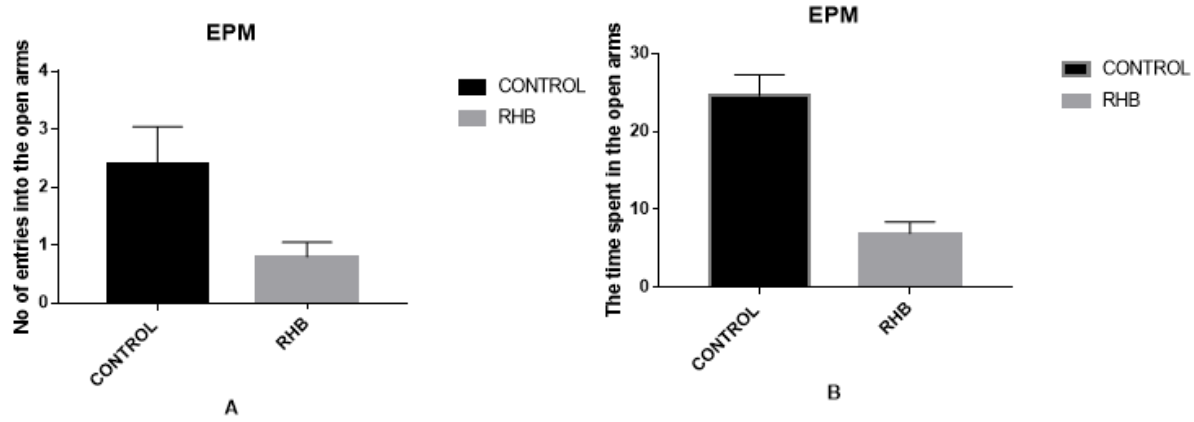

Figure 1
Elevated Plus Maze was used to asses anxiety. (A) The number of entries into the open arms during the test (B) The time spent in the open arms during the test. Histograms indicate the means±SD for the rats (n=10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters.

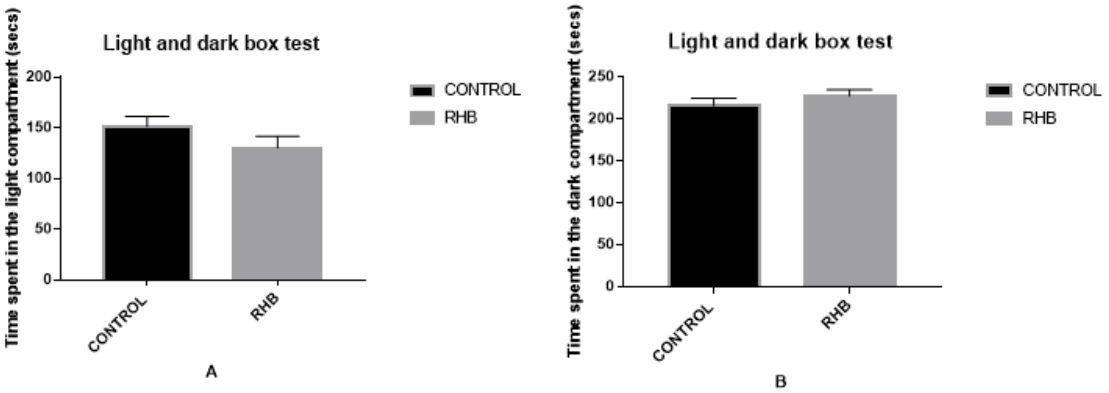

Figure 2
Light and Dark Box Test was used to assess anxiety. Histograms indicate the means±SD for the rats (n=10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters.

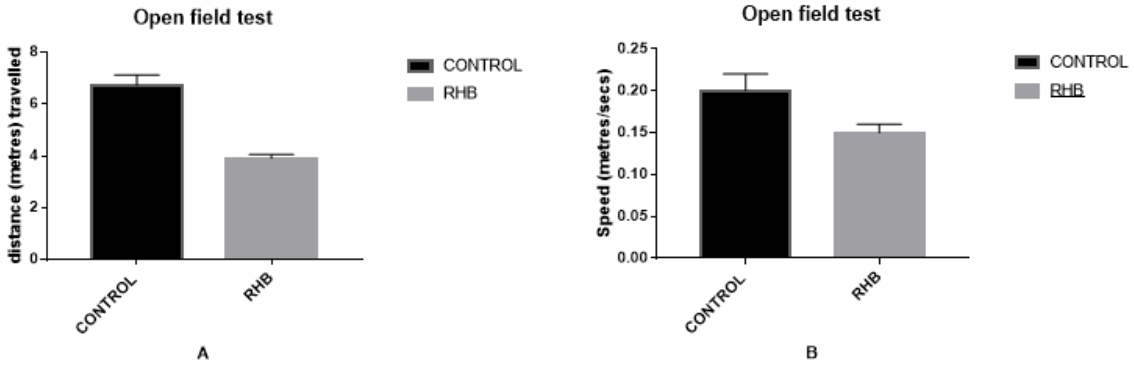

Figure 3
Open Field Test was used to assess general locomototry function. Histograms indicate the means ± SD for the rats. (n=10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters.

Open field test: The RHB animals displayed lower locomotive activity than the control as judged by the distance (metres) travelled (RHB: 3.90±0.15 vs Control: 6.73±0.40; p<0.05); they also moved at lower speed (RHB: 0.15±0.01 vs Control: 0.20±0.02, p<0.05) (Figure 3).

Wire hang test: The effect of Ruzu Herbal Bitters (RHB) on forelimb strength was assessed using the Wire hang task. As shown in figure 4, RHB animals displayed significantly lower wire-hang latency (seconds) than the control animals (RHB: 51.7±1.40 vs Control: 94.1 ±3.78, p<0.05).

Effects of RHB on oxidative stress markers in the brain: In the result of this study, the activity of Catalase in the brain of the RHB group (80.11±3.78 U/ mg protein) was significantly higher (p<0.05) compared to control group (41.2±5.172U/ mg protein) (figure 5). Alkaline phosphatase (ALP) was significantly lower RHB compared to control (RHB: 44.14±13.86 U/L vs Control: 63.34±8.654 U/L, p<0.05). The enzyme gamma glutamyltransferase (GGT) was also significantly lower in RHB than control group (RHB: 24.18±5.078 U/L vs Control: 29.3±5.86U/L, p<0.05) (Figure 6).

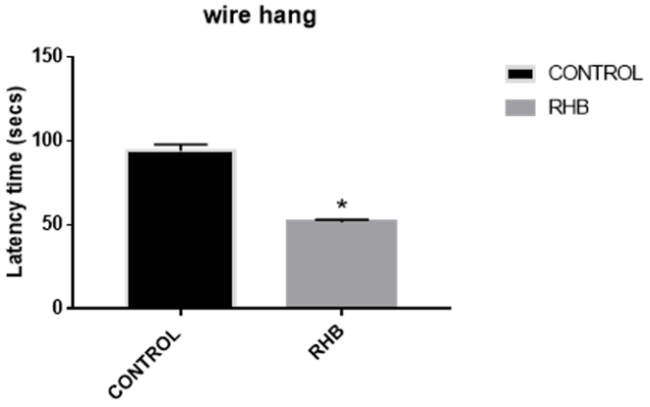

Figure 4
Wire Hang Task was used to assess forelimb strength. Histograms indicate the means ± SD for the rats (n = 10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters.

SERT and DAT expression: Immunofluorescence staining for DAT (Plate 1) and SERT (Plate 2) show that RHB animals had a statistically significant higher expression of DAT compared to control (RHB: 2.4±0.92 vs Control: 1.4±0.95, p<0.05) and a higher expression of SERT which was not statistically significant (RHB: 1.7±0.67 vs Control: 1.5±0.80, p>0.05) (Figure 7).

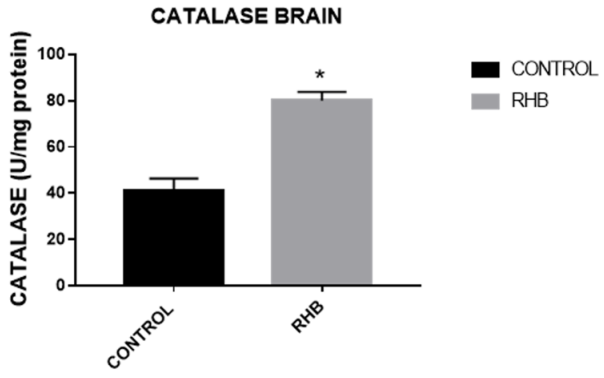

Figure 5
Effect of RHB on the activity of Catalase in the brain. Histograms indicate the means ± SD for the rats (n = 10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters

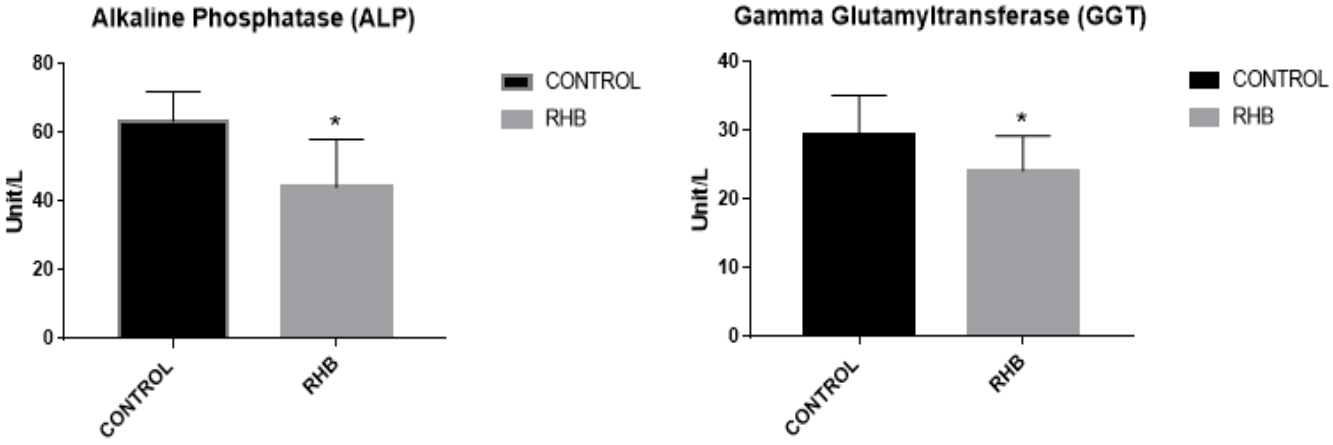

Figure 6
Effect of RHB on the activities of Alkaline Phosphatase and gamma Glutamyl transferase in the brain. Histograms indicate the means ± SEM for the rats (n = 10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters

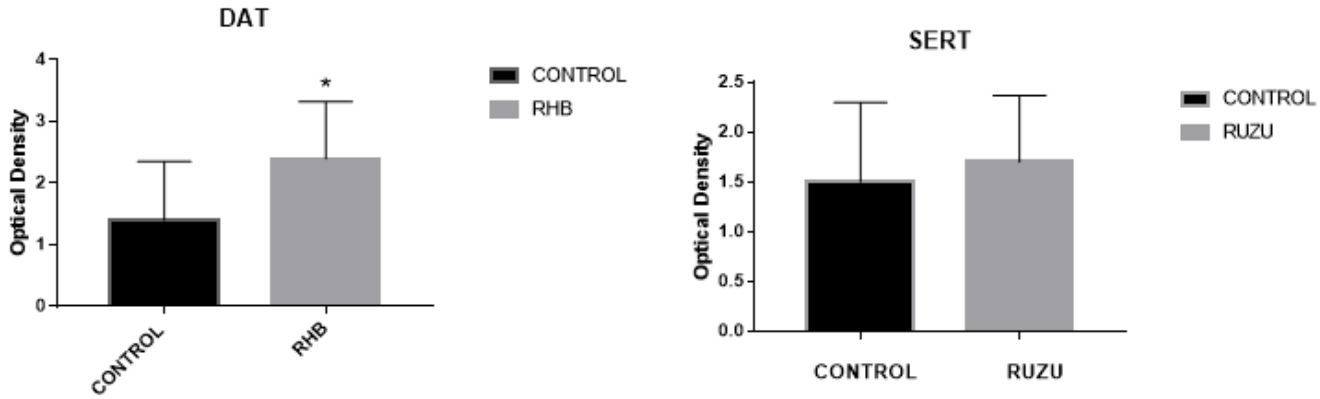

Figure 7
Effect of RHB on the expression of DAT and SERT in the brain. Histograms indicate the means ± SEM for the rats (n = 10/group). Data were analysed using unpaired t-test. *p <0.05, Control vs RHB. RHB = Ruzu Herbal Bitters

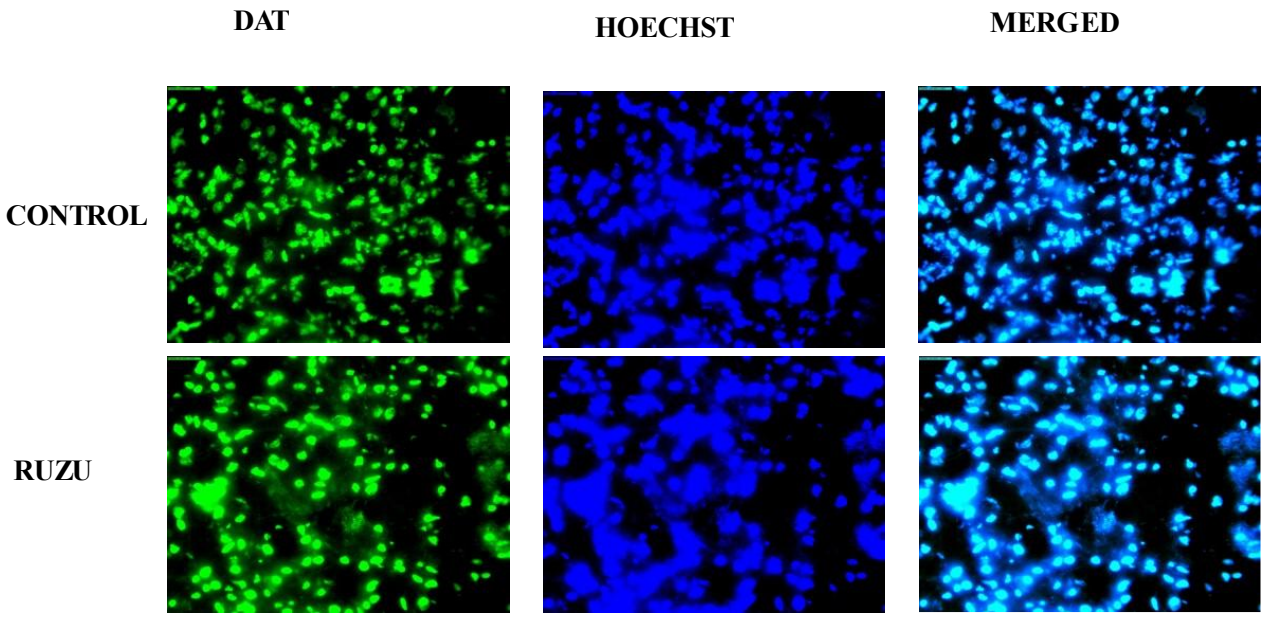

Plate 1
A photomicrograph of the brain of RHB treated animal compared with control animal showing DAT expression

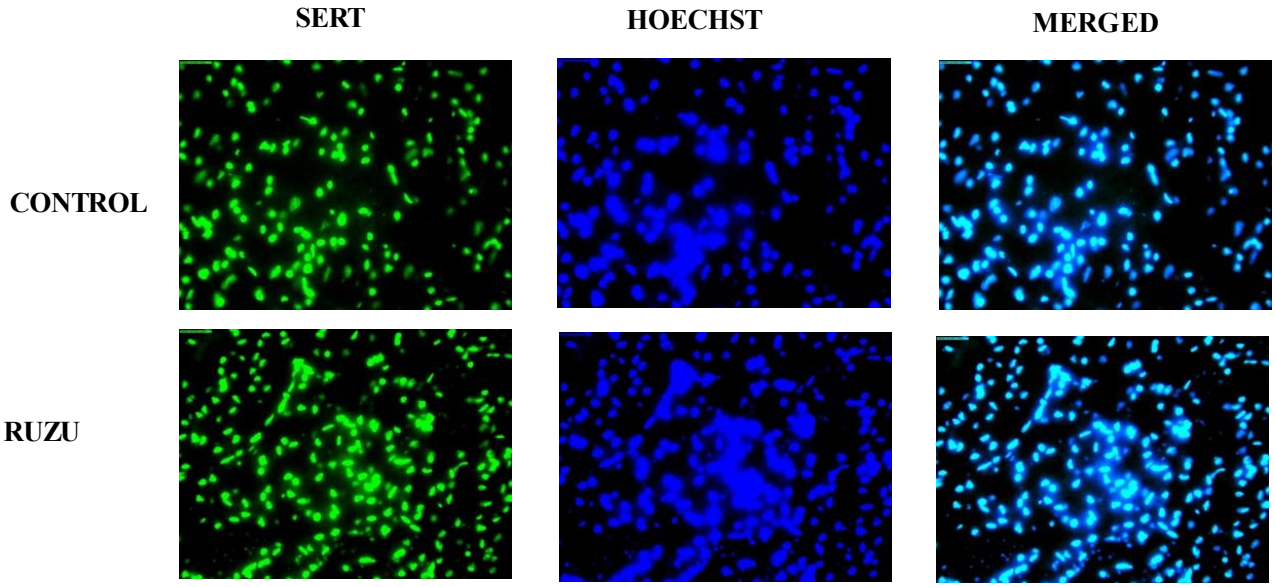

Plate 2
A photomicrograph of the brain of RHB treated animal compared with control animal showing SERT expression

DISCUSSION

The study evaluated possible neurotoxic effects of *Ruzu* Herbal Bitters (RHB) in rats by measuring animal behaviour, enzyme activities and neurotransporter expression as surrogates. Anxiogenic-like profile was observed in RHB treated rats; the observed anxiogenic-like profile is evidenced by the avoidance of the open arms by RHB-treated rats during the EPM. This behaviour is considered an anxiogenic behavior and a sign of anxiety (Belzung *et al*. 2001). In addition, animals treated with RHB spent lesser time and had fewer entries into the open arms, indicating an anxiogenic-like effect.

It is very likely that RHB contains extracts/active principles that could induce anxiety-like behaviour; some African herbal bitters have been similarly reported. For instance, the report of Woode *et al*., (2009), showed anxiogenic properties of a Ghanaian herbal extract with *Sphenocentrum jollyanum* as a major constituent. Aside compounded herbal bitters, individual herbal extracts such as *Agastache Mexicana* (Molina-Hernandez *et al*., 2000), *Visnea mocanera* (Hernandez-perez *et al*., 1995), *Myristica fragrans* (Sonavane *et al.,* 2002), and *Passiflora incarnata* (Elsas *et al.,* 2002) are known to elicit anxiogenic responses in animals. Interestingly, in some cases, these anxiogenic-like effects can occur side by side other positive effects such as anticonvulsant effects (Elsas *et al.,* 2002), and psychostimulant actions (Hernandez-perez *et al.,* 1995

The results of the Light and Dark Box Test (LDBT) showed that RHB-treated animals exhibited a preference for the dark compartment by spending less more time in the dark compartment and more time in the light compartment.

Although compared to the control animal the difference was not statistically significant, the data trend showed a clear leaning towards anxiety behaviour. Understandably, the LDBT suffers from high variability induced by age, weight, strain and genetics of animals (Bourin and Hascoet, 2003), but when combined with data from or physically with other tests such as EPM and open field, the data are a reliable predictor of anxiety-like states in rodents (Ramos *et al*., 2008). Therefore, put together with the EPM data, there is clear anxiogenic effects of RHB.

A decrease in locomotory and motor activity was observed in the rats treated with RHB; this was evidenced by the lower locomotive activity animals displayed during the open field test (OFT), an in vivo assay used in assessing the efficacy of therapeutic substances capable of improving locomotion and/or motor function (Malerba *et al*. 2011). The result of this study indicated that the rats treated with RHB covered shorter distances and also moved at lower speed. In addition, the rats displayed significantly lower wire-hang latency during the wire hang test (WHT), indicative of a decreased grip strength which can be attributed to a decrease in motor coordination and muscle tone (Carmela *et al*. 2012).

However, the data from OFT and WHT should be interpreted with a caveat: the possible sedative, depressant and muscle-relaxant activities of RHB were not independently studied. Depressant activity is physiologically linked to reduced motor activity (Bhosale *et al*., 2011) and this could account for reduced mobility and some muscular relaxation. This thinking is corroborated by the RHB phytochemical analysis data which show high presence of saponins, alkaloids, terpenoids and flavonoids. Several plants have been reported to have CNS depressant activity due to the presence of triterpenoids (Datta *et al*., 2004), saponins (Anandhan *et al*., 2010) and flavonoids (Datta *et al*., 2004; Anandhan *et al*., 2010). In addition, it has been hypothesized that triterpenoid saponins behave like benzodiazepines because of their agonistic/facilitatory activities at GABAA receptor complex (Chakraborty *et al*., 2010).

The activity of Catalase, a common enzyme which catalyzes the breakdown of hydrogen peroxide to oxygen and water, thus protecting the cell from oxidative damage caused by reactive oxygen species (ROS) (Chelikani *et al*. 2014) was significantly increased in the brain of the RHB treated rats, indicating that RHB has antioxidants capability. This correlates with the phytochemical investigation of one of the active components of RHB, *Curculigo Pilosa*, reported to have antioxidant activity (Nie *et al*. 2013).

Dopamine transporter (DAT) which controls the dynamics of dopamine (DA) neurotransmission by directing its extracellular reuptake has been implicated in the etiology of many diseases including depression and attention deficit (Vaughan & Foster 2013). RHB treated animals showed an increased expression of the protein DAT. This might be responsible for the observed behavioral decrease in locomotory and motor activity. This is in accordance with the study conducted by Vaughan & Foster (2013) which stated that over-expression of DAT causes a moderate increase in DAT function, leading to fine motor impairment. The serotonin transporter (SERT) which plays a central role in serotonin neurotransmission has been implicated in depression and anxiety (Ni *et al*. 2003). RHB treated animals showed a non-significant increase in the expression of the protein SERT. An increased expression of the protein SERT is most consistently associated with reduced anxiety-like behavior (Holmes *et al*. 2003; Murphy and Lesch 2008; Olivier *et al*. 2008). This contradicts the observed anxiogenic behaviour evidenced by the results of the EPM from this study. However, the motor impairment observed in RHB treated rats might be responsible for the observed anxiogenic behavior as changes in baseline locomotor activity is capable of presenting a difficulty when interpreting the results of anxiety tasks (Line *et al*. 2011). For instance, the reduced locomotive activity in RHB treated rats might be responsible for the reduction in open arm entries during the EPM and thus cause them to appear anxious.

We conclude that RHB produces anxiogenic-like behaviour in rats, and this might result from phytochemicals with depressant-like activities. This is however without prejudice to the clear antioxidant effects of RHB.

Acknowledgement:
The authors acknowledge the technical assistance of Dr. Awogbindin O. Ifeoluwa of Neuroimmunology group, Molecular Drug Metabolism & Toxicology Research Laboratory, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria

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