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ANXIOLYTIC PROPERTIES OF ETHANOL LEAF EXTRACT AND RESIDUAL AQUEOUS FRACTION OF *ROTHMANNIA LONGIFLORA* FOLLOWING CHRONIC CONSTRICTION INJURY-INDUCED NEUROPATHIC PAIN IN RATS

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ABSTRACT

Rothmannia longiflora leaf is used in the management of pain and inflammation in African traditional medicine. Previous studies demonstrated its analgesic and anti-inflammatory properties but there are no documented reports on the anxiolytic properties of *Rothmannia longiflora.* The aim of this study is to investigate the anxiolytic properties of the ethanol leaf extract of *Rothmannia longiflora* and its aqueous fraction following chronic constriction injury-induced neuropathic pain in rats. The anxiolytic potentials of ethanol leaf extract of *Rothmannia longiflora* and its residual aqueous fraction at 500 and 1000 mg/kg (*p.o*) were investigated using anxiety open field model (OF) and elevated plus maze (EPM) test. The negative control received Distilled water 1 mL/kg while the positive control received Minocycline 30 mg/kg. The ethanol extract of *Rothmannia longiflora* (EERL) significantly (p <0.05) increased the time spent by the rats in central square and decreased the number of stretch attend posture significantly (p <0.05) compared to the negative control. Similarly, the rats that received residual aqueous fraction and the minocycline showed reduced number of stretch attend posture significantly (p <0.05) compared to the negative control in OF model. The EERL significantly (p <0.05) increased the number of entries of the rats into the open arm, the time spent in open arm, and the time spent in central square compared to the negative control in EPM model. EERL and residual aqueous fraction were found to exhibit anxiolytic properties following chronic constriction injury-induced neuropathic pain in rats.

Keywords: anxiolytic, minocycline, neuropathic, open field, *Rothmannia longiflora*

INTRODUCTION

An anxiety disorder is a type of mental health condition which makes a victim respond to certain things and situations with fear and dread (American Psychiatric Association, 2023). Several types of anxiety disorders include: Agoraphobia is a type of anxiety disorder in which an individual fear and often avoid places or situations that might cause panic and make an individual feel trapped, helpless or embarrassed. Anxiety disorder due to a medical condition includes symptoms of intense anxiety or panic that are directly caused by a physical health problem. Generalized anxiety disorder includes persistent and excessive anxiety and worry about activities or events

— even ordinary, routine issues (American Psychiatric Association, 2023). The worry is out of proportion to the actual circumstance, is difficult to control and affects how one feels physically. It often occurs along with other anxiety disorders or depression.

Panic disorder involves repeated episodes of sudden feelings of intense anxiety and fear or terror that reach a peak within minutes (panic attacks). It comes with feelings of impending doom, shortness of breath, chest pain, or a rapid, fluttering or pounding heart (heart palpitations). These panic attacks may lead to worrying about them happening again or avoiding situations in which they've occurred. Selective mutism is a consistent failure of children to speak in certain situations, such as school, even when they can speak in other situations, such as at home with close family members. This can interfere with school, work and social functioning. Separation anxiety disorder is a childhood disorder characterized by anxiety that's excessive for the child's developmental level and related to separation from parents or others who have parental roles. Social anxiety disorder (social phobia) involves high levels of anxiety, fear and avoidance of social situations due to feelings of embarrassment, self-consciousness and concern about being judged or viewed negatively by others (Chand and Marwaha, 2023). Specific phobias are characterized by major anxiety when exposed to a specific object or situation and a desire to avoid it. Substance-induced anxiety disorder is characterized by symptoms of intense anxiety or panic that are a direct result of misusing drugs, taking medications, being exposed to a toxic substance or withdrawal from drugs. Many of the impacts of anxiety (such as physical tension, nervous system hyperactivity or harmful use of alcohol) are also known risk factors for diseases such as cardiovascular disease (Chand and Marwaha, 2023). In turn, people with these diseases may also find themselves experiencing anxiety disorders due to the difficulties associated with managing their conditions.

An estimated 4% of the global population currently experience an anxiety disorder (GBD Results Tool, 2019). In 2019, 301 million people in the world had an anxiety disorder, making anxiety disorders the most common of all mental disorders. Drugs like Benzodiazepines, and Antidepressants (such as selective serotonin reuptake inhibitors (SSRIs), are used in treating patients with anxiety disorders but these drugs are associated with serious side effects with high potential for dependence as well as their limited long-term effectiveness and are often more costly (Alonso *et al*., 2018). On account of these adverse effects and limited effectiveness, there is need to seek for alternative drugs from the wealth of herbal plants that are more safe, effective and cheaper in the management of anxiety disorders. There also appears to be an elevated prevalence of anxiety disorders among individuals with a variety of medical conditions, including arthritis (Bystritsky, 2023). Studies have suggested that people with chronic pain may have a pre-existing anxiety disorder (Cosio and Meshreki, 2021).

More severe chronic pain has been associated with more severe anxiety symptoms (De La Rosa *et al*., 2024). The presence of anxiety also has been shown to lead to more frequent reports of pain (Cosio and Meshreki, 2021). Anxiety disorders cause distress in more than 30 million Americans in their lifetimes. The direct and indirect costs of anxiety disorders are estimated to be about $42 billion per year in the United States (Ruthanne *et al*., 2023). Relative to depression, anxiety disorders have received less attention in the chronic pain literature. Interestingly, the aggregated association of anxiety disorders with chronic pain may be stronger than the association with depression, while both anxiety and depression are strongly linked to chronic pain, some evidence suggests the aggregated association of anxiety disorders with chronic pain may be stronger than the association with depression (Cosio and Meshreki, 2021). This finding, supported by a major 2025 meta-analysis, indicates that anxiety can be both a cause and consequence of chronic pain through shared biological mechanisms, cognitive processes, and fear-avoidance behaviors, leading to intensified pain and reduced function (Aaron *et al*., 2025).

Rothmannia longiflora is found in Gambia, Sudan, Kenya, Tanzania, Angola and is also found in Nigeria, Ghana, Sierra Leone, Democratic Republic of Congo, Ivory Coast, Uganda, Liberia and Cameroon. *Rothmannia longiflora* is from the family Rubiaceae. In Nigeria, it is called 'Katambiri,' in Hausa, 'Alankita uku' in Igbo and 'Kere buje' in Yoruba. In Nigeria the roots are used to treat bowel complaints (Dalziel, 1937). In Democratic Republic of Congo, a root infusion is applied as a treatment for throat abscesses, toothache and leprosy. In some West Africa like Ghana and Cameroon, leaf pulp is used as an enema against kidney pain and diarrhoea with blood, and drinking the leaf juice is said to help during labour and childbirth. In Sierra Leone leaves are used to treat itchy skin diseases and the fruit pulp is said to be an emetic (Dalziel, 1937). Previous studies demonstrated its analgesic and anti-inflammatory properties (Mallam *et al*., 2016) but there are no documented reports on the anxiolytic properties of *Rothmannia longiflora.* The aim of this study is to investigate the anxiolytic properties of the ethanol leaf extract of *Rothmannia longiflora* and its aqueous fraction following chronic constriction injury-induced neuropathic pain in rats.

MATERIALS AND METHODS

Drugs, Chemicals, and Equipment

Stop watch, elevated plus maze, open field chamber, dissecting kits, hand gloves, cotton wool, oral cannula, Animal cages, Chromic catgut 3/0, Nylon silk 3/0, Dettol, Razor blades, Minocycline (Actavis Barnstaple, U.K, Lot HI49), Ketamine HCl (Rotex Medica, Germany), Amoxicillin (Hebe New Century Pharmaceuticals Co Ltd, China).

Laboratory Animals Care and Ethical Issues

Wistar Rats (120–180g) of either sex were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The animals were maintained at ambient temperature and were fed with laboratory feed and water *ad libitum.* The experiments were carried out after securing approval from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) with approval number: ABUCAUC/2021/067.

Plant Collection and Identification

The plant sample comprising of the stem, fruits and the leaves were collected from Bagauda forest along Kaduna-Kano Road in October, 2019 and were identified by a taxonomist, Mal. Namadi Sanusi in the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University Zaria by comparing with the existing Specimen (Voucher Number: ABU037195) previously deposited in the herbarium.

Extraction and Fractionation of Plant Material

The extraction and fractionation were carried out according to the method described by Abubakar and Haque (2020). The leaves of *Rothmannia longiflora* were air-dried at room temperature for several days until constant weight was obtained. The dried material was size-reduced into powder with the aid of an electrical blender. The powdered material (2 kg) was extracted with 70% (2.8 L) ethanol and 30% water (1.2 L) by cold maceration for 48 hours with occasional shaking. Ethanol solvent was recovered through rotary evaporator. The Ethanol extract was then stored in a bottle in a fridge prior to use, solution of the extract was freshly prepared with distilled water for each study. The percentage yield of the extract was calculated using the formula below:

$$\text{Percentage yield} = \frac{\text{Weight of the extract}}{\text{Weight of powdered material}} \text{ X } 100$$

One hundred grams (100 g) of the ethanol extract was weighed and fractionated by suspending in water (500 mL / 5 times) and partitioned with Hexane (500 mL /5 times) to obtain Hexane fraction. The aqueous layer was further partitioned with Dichloromethane (500 mL /5 times) to obtain Dichloromethane fraction, Ethyl acetate (500 mL /5 times) to obtain Ethyl acetate fraction. The aqueous layer was further partitioned with n-Butanol (500 mL /5 times) to obtain n-Butanol fraction and the Residual aqueous was concentrated to obtain Residual aqueous fraction. The n-Butanol and

residual aqueous fractions were concentrated on water bath set at 40°C while the Hexane, Dichloromethane and Ethyl-acetate fraction were air dried. The concentrated and dried fractions were also stored in separate desiccators in the fridge ready for use. Solutions of the fractions were prepared freshly with distilled water for each study. The percentage yield of the fractions was also calculated using the formula above.

Confirmation of Phytochemicals

The method described by Kowalska and Sajewicz, (2022) was employed. The thin layer chromatography plates were prepared and the solution of the extract and the fractions (DF, EAF, n-BUTF and RAQF) were spotted on the bottom of the plates with the aid of capillary tubes and were dipped in different mobile phases in the chromatogram tank for separation of the phytochemicals. The best reagents for specific phytochemicals were subsequently sprayed.

Median Lethal Dose (LD_{50}) Determination

Acute oral toxicity was investigated using the Organization for Economic Cooperation and Development 425 guidelines (up and down procedure) in rats. OECD 425 guideline was used in five non-pregnant female rats which were fasted prior to dosing (Food but not water was withheld overnight for the rats). The fasted body weight was determined for each animal and the dose was calculated according to the body weight. Food was then further withheld for 3-4 hours in rats after the extract had been administered using an oral cannula; a limit dose of 5,000 mg/kg was administered to one rat and was observed for 48 hours, then two rats were further dosed with the same dose of 5,000 mg/kg and were observed for another 48 hours and for 14 days, observations included changes in skin and fur, eyes and mucous membranes, and behavioral pattern, autonomic and central nervous systems etc. Animals were also observed for tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The same procedure was repeated for all the fractions. At the end of the test, the animals were sacrificed using Diethyl ether.

Neuropathic Pain Model

The chronic constriction injury (CCI) model of neuropathic pain was performed under ketamine anaesthesia (15 mg/kg) according to Bennett and Xie (1988). Wistar rats were divided randomly into seven groups (n = 5). The left sciatic nerve of each rat was exposed surgically and three ligations were loosely tight round the nerve with 1 mm gap between the adjacent ligatures. Group 1 with CCI received distilled water (I ml/kg *p.o*). Group 2 without CCI (sham/naïve) received distilled water (1 ml/kg *p.o*). Group 3 with CCI received Minocycline 30 (mg/kg *p.o*) (standard drug). Group 4 with CCI received 500 (mg/kg *p.o*) RAQF. Group 5 with CCI received 1000 (mg/kg *p.o*) RAQF. Group 6 with CCI received 500 (mg/kg *p.o*) EERL and Group 7 with CCI received 1000 (mg/kg *p.o*) EERL. The administration of the above started a day before surgery and was continued for 28 days post-surgery.

The Open Field Test

This study was conducted on the day 10 after CCI surgery as described by Souza *et al.* (2013). Each rat was placed on the centre of the Open Field (90 ×90 cm) and was allowed to explore for 5 minutes. The following parameters were observed and recorded: Number of peripheral line crossing (NPLC), number of central square entries (NCSE), time spent in central square (TSCS), number of rearing (NOR), time spent grooming (TSG), number of urination (NU), number of faecal boli (NFB), and number of stretch attend posture (NSAP).

Elevated Plus Maze

This study was conducted on day 11 as described by Souza *et al.* (2013). Each rat was placed in the central platform facing an open or closed arms and allowed to explore for 5 minutes. The following parameters were observed and recorded: Number of entries into the open arms, number of entries into the closed arms, time spent in open arms, time spent in closed arms and time spent in centre squares.

Statistical Analysis

Data were analysed and expressed as Mean ±Standard Error of Mean (Mean ± SEM) and were presented as tables. Data were analyzed

using One Way Analysis of Variance (ANOVA), SPSS version 20 followed by Bonferroni's post-hoc test where appropriate. Values of $p \leq 0.05$ were considered significant.

RESULTS

Oral Median Lethal Doses

In the acute toxicity test, the oral median lethal doses (LD_{50}) of EERL and all its fractions Dichloromethane, Ethyl acetate, n-Butanol and Residual aqueous fractions (DFRL, EAFRL, n-BUTFRL and RAQFRL) were estimated to be greater than 5,000 mg/kg in rats according to OECD 425 guidelines.

Effect of Ethanol Leaf Extract of *Rothmannia longiflora* and its Residual Aqueous Fraction on Open Field Parameter following CCI-induced Neuropathic Pain in Rats

The EERL significantly ($p<0.01$) increased the time spent in central square compared to the control and decreased the number of stretch attend posture significantly ($p<0.05$) at 1000 mg/kg compared to the control. Similarly, the residual aqueous fraction and the minocycline reduced the number of stretch attend posture significantly ($p<0.05$) at 500 and 1000 mg/kg respectively compared to the control. The number of rearing, urinating, and number of fecal boli decreased but not significantly compared to the negative control (CCI+D/W 1mL/kg) (Table 2).

Effect of Ethanol Leaf Extract of *Rothmannia longiflora* and its Residual Aqueous Fraction on Elevated Plus Maze Parameter following CCI-induced Neuropathic Pain in Rats

The EERL significantly ($p<0.01$) increased the number of entries into the open arm and the time spent in the open arm at 500 and 1000 mg/kg compared to the control. Similarly, EERL significantly ($p<0.05$) increased the time spent in central square at 1000 mg/kg compared to the control (CCI+D/W 1 mL/kg). The RAQF increased the number of entries into the open arm and the time spent in the open arm but not significantly at 500 and 1000 mg/kg compared to the control (Table 3).

Table 1. Phytochemical Constituents of Ethanol Leaf Extract of *Rothmannia longiflora* and its fractions

Chemical constituents	Ethanol Extract	Dichloromethane Fraction	Ethyl acetate Fraction	n-Butanol Fraction	Residual Aqueous Fraction
Alkaloids	+	+	+	+	+
Saponins	+	+	+	+	+
Flavonoids	+	+	+	+	+
Tannins	+	+	+	+	+
Carbohydrate	+	+	+	+	+
Cardiac glycosides	+	-	-	-	+
Anthraquinones	+	+	+	+	+
Steroids	+	+	+	+	+
Triterpenes	+	+	+	+	+

+ = Present - = Absent

Table 2. Effect of Ethanol Leaf Extract of *Rothmannia longiflora* and its Residual Aqueous Fraction on Open Field Parameter following Chronic Constriction Injury-induced Neuropathic Pain in Rats

Treatment (mg/kg)	NPLC	NCSE	TSCS (Sec.)	NOR	TSG (Sec.)	NU	NFB	NSAP
CCI+D/W (1 mL/kg)	56.50±23.89	3.50±0.00	3.0±0.00	8.00±2.83	19.00±4.92	1.67±0.67	4.60±1.17	4.00±0.00
D/W 1 (mL l/kg)	42.83±11.54	3.00±0.00	1.50±0.50	8.60±2.69	32.17±9.14	1.00±0.00	4.33±2.85	3.13±0.00
CCI+MCN 30	49.33±29.09	1.00±1.50	6.50±1.50*	5.33±2.04	15.00±091	1.33±0.33	3.00±0.58	1.67±0.33*
CCI+RAQFRL 500	44.83±21.34	2.50±0.00	1.33±0.33	6.40±1.93	23.33±6.85	1.00±0.00	3.40±0.51	1.33±0.33*
CCI+RAQFRL 1000	36.83±11.67	1.00±2.00	2.00±0.00	7.17±2.24	24.75±12.10	1.00±0.00	4.00±1.20	1.00±0.00*
CCI+EERL 500	48.33±9.61	2.00±0.00	1.33±0.33	7.67±0.71	46.40±6.76	1.00±0.00	3.50±0.50	-
CCI+EERL 1000	37.20±7.02	1.00±0.00	7.00±1.00**	6.17±2.60	33.00±7.20	1.50±0.50	3.20±1.39	1.00±0.00*

Values are expressed as Mean± S.E.M, Data were analyzed using One Way ANOVA followed by Bonferroni's post hoc test, * = $p<0.05$, ** = $p<0.01$ compared to CCI+D/W and *= $p<0.05$ compared to D/W. n = 5, NPLC = Number of peripheral line crossing, NCSE = Number of central square entry, TSCS = Time spent in central square, NOR = Number of rearing, TSG = Time spent grooming, NU = Number of urinating, NFB = Number of fecal boli, NSAP = Number of stretched attend posture , D/W = Distilled water, CCI = Chronic constriction injury, MCN = Minocycline, RAQFRL = Residual Aqueous Fraction of *Rothmannia longiflora*, EERL = Ethanol Extract of *Rothmannia longiflora*, S.E.M = Standard Errors of Mean.

Table 3. Effect of Ethanol Leaf Extract of *Rothmannia longiflora* and its Residual Aqueous Fraction against Elevated Plus Maze Parameter following Chronic Constriction Injury-induced Neuropathic Pain in Rats

Treatment (mg/kg)	**No. of entries into open arm**	**No. of entries into closed arm**	**Time spent in open arm (Sec.)**	**Time spent in closed arm (Sec.)**	**Time spent in central square (Sec.)**
CCI+D/W (1 mL/kg)	2.67±0.67	4.50±0.85	35.33±6.01	204.67±20.07	39.40±10.73
D/W 1 (mL/kg)	3.33±0.33	6.00±1.21	32.67±13.86	224.33±12.61	26.00±5.97
CCI + MCN 30	6.83±0.48**	4.83±0.75	58.67±1.75**	213.50±20.37	56.17±2.17**
CCI +RAQFRL 500	3.50±0.62	3.83±1.49	30.83±1.30	234.67±10.14	34.33±4.31
CCI +RAQRL 1000	4.83±0.48	4.50±1.12	40.00±3.54	243.00±0.97	35.50±4.73
CCI + EERL 500	6.50±0.43**	3.17±1.25	60.00±2.03**	232.67±9.74	39.67±3.64
CCI + EERL 1000	7.33±0.67**	4.67±1.48	62.50±3.53**	224.83±12.60	47.67±2.29*

Values are expressed as Mean± S.E.M, Data were analyzed using One Way ANOVA followed by Bonferroni's post hoc test, *= $p<0.05$, **= $p<0.01$ as compared to CCI+D/W and D/W, n = 5, D/W = Distilled water, CCI = Chronic Constriction Injury, MCN = Minocycline, RAQFRL =Residual Aqueous Fraction of *Rothmannia longiflora*, EERL = Ethanol Extract of *Rothmannia longiflora*

DISCUSSION

The oral administration of EERL and its residual aqueous fraction at doses up to 5,000 mg/kg did not show any sign of toxicity or death in rats and mice. This suggests that the EERL and its residual aqueous fraction is relatively safe when administered orally. Acute lethal dose (LD_{50}) study is carried out to determine the range of doses that could be toxic to the animal and also used to estimate the therapeutic index of the drugs (Maleki *et al.,* 2016). All the doses selected and subsequently used in this experiment were 250, 500 and 1000 mg/kg which is below 30% of the estimated LD_{50} (above 5000 mg/kg) as suggested by Vongtau (Vongtau *et al.,* 2004). Open field test (OFT) model is used to evaluate spontaneous locomotor, exploratory activities and emotional behaviours, with the most established indicators of number of defecation and ambulation (Wycliffe *et al.,* 2019).

The EER significantly increased the time spent in central square and EERL and RAQF significantly reduced the number of stretch attend posture leading to decreased in both the ambulatory activities of the rats and the number of stretch attend posture indicating calmness and suggests the manifestation of sedation and or anxiolytic effects. The non-significant decrease in the number of fecal boli, number of urine and the number of central square entries also suggest the reduction in the level of anxiety in rats. Similarly, elevated plus maze (EPM) is a model used to measure level of anxiety and to screen anxiolytic drugs (Camargos *et al*., 2020). Avoidance of open arm was due to fear as open arms are more aversive for rodents than closed arms (Camargos *et al*., 2020). Indicators of fear are increasing mobility, freezing and defecation in open arms than in closed arms (Schrader *et al*., 2018). (Camargos *et al*., 2020).

The EERL significantly increased the number of entries into the open arm, increased time spent in the open arm and the time spent in the central square, which also suggests that the EERL possesses anxiolytic effects. The EERL and RAQF revealed the presence of phytochemicals such as alkaloids, flavonoids, saponins, etc, these may contribute in reducing the levels of anxiety. In rats, alkaloids, flavonoids, and saponins reduce anxiety through distinct yet sometimes overlapping mechanisms that primarily involve modulating neurotransmitters, protecting against neuronal damage, and regulating stress hormones. Research suggests these compounds may offer safer alternatives to conventional anxiety medication, which can have significant side effects.

Many alkaloids interact with key neurotransmitter systems in the central nervous system. Some, like the β-carboline alkaloids, act as inhibitors of monoamine oxidase (MAO), an enzyme that breaks down neurotransmitters like serotonin and dopamine. By inhibiting MAO, alkaloids help increase the levels of these calming neurotransmitters in the brain (Balkrishna *et al.,* 2025). Alkaloids can bind to and activate gamma-aminobutyric acid (GABA) receptors, specifically the GABA-A subtype, which are the same receptors targeted by benzodiazepine drugs like diazepam. This action promotes "phasic" inhibition of neurons, reducing neuronal excitability and causing a sedative and anxiolytic effect. Similar to alkaloids, some flavonoids are known to act as ligands for the benzodiazepine site on GABA-A receptors, producing anxiolytic and sedative effects (Bruni *et al.,* 2021). Flavonoids can modulate brain monoaminergic neurotransmitters, particularly serotonin (5-HT). Studies on rats exposed to chronic stress have shown that flavonoids can reverse the stress-induced reduction of serotonin levels in the hippocampus, a brain region crucial for anxiety and mood regulation. Saponins contribute to reducing neuroinflammation and oxidative stress. By inhibiting pathways like NF-κB, they decrease inflammatory mediators such as TNF-α and nitric oxide, providing neuroprotective effects that reduce anxiety (Riya *et al*., 2025).

CONCLUSION

The findings of the study suggest that the ethanol leaf extract and the residual aqueous fraction of *Rothmannia longiflora* possess anxiolytic properties following chronic

constriction injury-induced neuropathic pain in rats.

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Conflict Of Interest

All authors declare no conflict of interest.

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