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ANTINOCICEPTIVE AND ANTIOXIDANT EFFECTS OF ETHANOL EXTRACT AND RESIDUAL FRACTION OF *Rothmannia longiflora* SALISB (RUBIACEAE) IN NEUROPATHIC PAIN INDUCED BY CHRONIC CONSTRICTION INJURY OF THE SCIATIC NERVE

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ABSTRACT

Rothmannia longiflora is a plant found in Nigeria and most African countries, which is used in the management of pain and inflammation. The anti-nociceptive properties of the ethanol leaf extract and residual aqueous fraction of *Rothmannia longiflora* (EERL and RAQF) in rats was studied after the qualitative screening and acute toxicity studies. The antinociceptive properties of EERL and RAQF (500 and 1000 mg/kg p.o) were evaluated using a neuropathic pain model in rats. The result of qualitative evaluation of EERL and RAQF revealed the presence of flavonoids, tannins, saponins, steroids, glycosides, anthraquinones and phenols. The acute oral toxicity (LD_{50}) of the EERL and RAQF in rats was found to be greater than 5,000 mg/kg body weight. The result of this work demonstrated the ability of EERL and RAQF to significantly increase the pain latency time, $p<0.01(9.40)$ on D7, $p<0.01(10.72)$ on D14, $p<0.05(11.72)$ and $p<0.05(8.73)$ on D7, $p<0.01(10.30)$ on D14, respectively, compared to the control. Similarly, the concentrations of SOD increased significantly $p<0.05(1.58)$, $p<0.01(1.60)$; CAT $p<0.01(1.80)$, $p<0.01(1.62)$; GSH $p<0.01(1.50)$, $p<0.01(1.36)$ compared to the control while the concentrations of MDA significantly decreased $p<0.001(111.88)$, $p<0.05(132.46)$ compared to control at 1000 mg/kg, thus validating the herbal medicinal activity of the plant for the management of neuropathic pain.

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INTRODUCTION

"The International Association for the Study of Pain defines neuropathic pain as pain initiated or caused by a primary lesion or dysfunction in the nervous system" [1]. Neuropathic pain can occur due to damage or injury of the nerves, especially the sciatic nerves; disease conditions such as diabetes, arthritis, tumours and any other medical condition associated with inflammation or neurological disorders and metabolic diseases [2]. The pharmacological properties of *Rothmannia longiflora* leaf extract have been shown to possess antipyretic, antinociceptive and anti-inflammatory

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effects when administered orally. *Rothmannia longiflora* has indicated the presence of alkaloids, flavonoids, saponins, tannins, anthraquinones and phenolic compounds.

The research on this plant seeks to validate the scientific bases of long-time use of *Rothmannia longiflora* in the management of pain and inflammation in herbal medicine, and this can lead to the discovery of new drugs with better efficacy, fewer side effects and less cost compared to the current orthodox analgesic and anti-inflammatory drugs used in the management of neuropathic pain. We investigated the anti-hyperalgesia and antioxidant activities of the ethanol extract and the residual aqueous fraction from *Rothmannia longiflora* leaves on chronic constriction injury (CCI) of the sciatic nerve (a rat model for neuropathic pain study). *Rothmannia longiflora* Salisb is from the Rubiaceae family and is found in most African countries, including Nigeria. In Nigeria, it is called 'Katambiri,' in Hausa, 'Alankita uku' in Igbo and 'Kere buje' in Yoruba**.** *Rothmannia longiflora* is considered to have febrifugal and analgesic properties, and a decoction of the leaves, twigs, bark and roots is applied internally or externally as lotions, washes and baths [3]. In Nigeria, the roots are used to treat bowel complaints [4]. The study on neuropathic pain using the ethanol leaf extract of *Rothmannia longiflora* has not been carried out before, hence the need for this study.

MATERIALS AND METHODS

Plant material

The animals used for this study were Wistar Rats (120–180g), which were purchased from the Animal House Facility of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The animals were housed at ambient temperature and were fed with laboratory feed (Chikun feed) and sufficient water. The study was performed after securing ethical approval from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) with approval number: ABUCAUC/2021/067.

Drugs and chemicals

Morphine (Biotuva, USA, CAS 57-17-2), Sodium hydroxide, Magnesium chips, Hydrochloric acid, Drangedorff's reagent, Wagner's reagent, Mayer's reagent, Glacial acetic acid, Ferric chloride, Sulphoric acid, Acetic anhydride, Chloroform, Ammonia, Molish reagent (Lobachemie India). Minocycline (Actavis Barnstaple, U.K., Lot HI49), Ketamine HCl (Rotex Medica, Germany), Amoxicillin (Hebe New Century Pharmaceuticals Co. Ltd, China).

Plant collection and identification

Rothmannia longiflora plant sample consisting of the stem, fruits and the leaves was collected from Bagauda forest along Kaduna-Kano Road in October 2019 and was 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 method described by Abubakar and Haque was employed [5]. The leaves of *Rothmannia longiflora* were dried at room temperature for several days. The dried material was size-reduced into powder with the help of an electrical blender. 2 kg of the powdered plant material 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 a rotary evaporator. The Ethanol extract was then stored in a bottle inside the fridge for use. The solution of the extract was freshly prepared with distilled water for each study. The formula below was used to calculate the percentage yield of the extract:

Percentage yield =

$$\frac{\text{weight of the extract}}{\text{weight of powdered material}} \text{ X } 100 \text{ .....Equation 1}$$

100 g of EERL was weighed and fractionated by suspending it in water (500 ml / 5 times) and partitioned with Hexane (500 ml /5 times) to obtain the Hexane fraction. This procedure was repeated with Dichloromethane (500 ml /5 times), Ethyl acetate (500 ml /5 times), n-Butanol (500 ml /5 times) to obtain: Hexane, Dichloromethane, Ethyl acetate, n-Butanol and the Residual aqueous fractions, respectively. The n-Butanol and residual aqueous fractions were concentrated on a water bath set at 40°C, while the Hexane, Dichloromethane and Ethyl-acetate fractions were air dried. The dried fractions were also stored in separate desiccators in the fridge, and the extracts were freshly prepared with distilled water for each study. ready for use.

Phytochemical screening

The Ethanol leaf extract of *Rothmannia longiflora* (EERL) and fractions (DF, EAF, n-Butanol and RAQF) underwent qualitative evaluation via the method described by Sofowora [6].

Determination of acute oral toxicity (LD_{50})

The OECD 420 guidelines were used to determine the lethal dose (LD_{50}) for both mice and rats [7].

Inducement of neuropathy via chronic constriction injury (CCI)

The method described by Bennett and Xie was employed for this procedure [8]. 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 tightened around the nerve with a 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 (most active fraction). 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.

Heat hyperalgesia (hot-plate test)

The day before surgery (day 0), the rats were subjected to heat sensitivity assessments, and on days 7, 14, 21 and 28, after surgery, pain latency time was measured by the method described by Eddy and Leimbach [9].

Sample preparation for quantification of oxidative and antioxidant markers

On day 28 of the neuropathic pain study, all the animals were humanely euthanized with ethyl ether and the spinal cord at the lumber (L4-5) segments was removed through a surgical procedure. Homogenate tissues were prepared with 0.9% saline using a glass homogenate and then centrifuged at 2500 RCF for 10 minutes. Supernatant (10% w/v) was used for the assessment of Anti-oxidative stress markers as described by Hasanvand [10].

Malondialdehyde (MDA) quantification

The method described by Atawodi was employed [11]; 2 cm^3 of 15 % trichloroacetic acid (TCA) was pipetted into a test tube, 2 ml of thiobabituric acid (TBA) and 100 µl of serum were added. The mixture was incubated at 80 ^{0}C for 30 minutes on a water bath and allowed to cool for 30 minutes, followed by centrifugation at 3,000 RCF for 10 minutes. The clear supernatant was collected, and the absorbance was measured at 535 nm using a spectrophotometer.

The concentrations of Thiobarbituric acid reactive substances (TBARS) were expressed in nmol/mg protein using the formula below:

Concentration (nmol/mg) protein =

$$\frac{\text{Absorbance of sample}}{1.5\text{x}10 - 5 \text{ x protein concentration (mg/ml)}} \quad \text{.....Equation 2}$$

The concentration of protein (mg/ml) was determined as described by Atawodi [11]; 5 ml of serum was mixed with 4 ml of biuret reagent and incubated at room temperature for 30 minutes, and the absorbance was measured at 540 nm. The determination of the concentration of protein was done from the calibration curve, which is a plot of the absorbance of egg albumin (standard) against reaction time.

Determination of the concentration of catalase (CAT)

The determination of the concentration of CAT was carried out using the method described by Abebi [12]. Serum concentration of 10 µl was added to a test tube containing 2.80 ml of potassium phosphate buffer (50 mM, pH 7.0). The reaction was initiated by adding 0.1 ml of freshly prepared 30 mM H_2O_2, and the decomposition rate of H_2O_2 was measured at 240 nm for 5 minutes using a spectrophotometer. A molar extinction coefficient (É) of 0.041 Mm^{-1}ml was used to calculate the catalase activity

$$\text{Catalase concentration} = \frac{\text{Absorbance of sample}}{\text{É}}$$

$$\text{Catalase activity} = \frac{\text{Catalase activity}}{\text{protein concentration (mg/ml)}}$$

Superoxide dismutase (SOD) enzyme determination

The determination of superoxide dismutase enzyme concentration was carried out by the method described by Fridovich [13]. This method demonstrates the ability of the superoxide dismutase enzyme to inhibit autooxidation of adrenaline 0.05 M carbonate buffer (pH 10.2) and Adrenaline (0.3 mM) solution, which were freshly prepared. Then, 0.2 ml of serum was added to 2.5 ml of 0.05 M carbonate buffer. The reaction was started with the addition of 0.3 ml of 0.3 mM adrenaline. The reference mixture contained 2.5 ml of 0.05 M carbonate buffer, 0.3 ml of 0.3mM adrenaline and 0.2 ml of distilled water. The absorbance was measured at 480 nm at 30 seconds as the initial and 180 seconds as the final absorbance, respectively.

$$\text{Absorbance per minute} = \frac{\text{A2}-\text{A1}}{2.5} \quad \text{.....Equation 3}$$

Where A1 = Initial absorbance, A2 = Final absorbance

$$\%\text{inhibition} = \frac{\text{increase in absorbance for sample}}{100-[(\text{ increase in absorbance of blank})x\ 100]} \quad \text{.....Equation 4}$$

One unit of SOD concentration is required to elicit 50% inhibition of the oxidation of adrenaline to adrenochrome in 1 minute.

Determination of GSH

The analysis was performed according to the method described by Rajagopalan [14]; 150 µl of serum was added to 10% TCA and centrifuged at 1,500 rpm for 5 minutes, and 1 ml of the supernatant was treated with 0.5 ml of Ellmans reagent and 3 ml of phosphate buffer (0.2 M,
pH 8.0). The absorbance was measured at 412 nm. The quality of GSH concentration was determined by the formula below:

GSH Concentration = $\frac{\text{Absorbance of sample}}{\text{É}}$ ……Equation 5

Where É = Molar extinction coefficient

Data Analysis

Data were analyzed using one-way and repeated-measure analysis of variance (ANOVA), SPSS version 20, followed by Bonferroni's post-hoc test. The results were presented as Mean ±Standard Error of Mean and as figures.

RESULTS

Qualitative analysis of phytochemicals and the LD_{50} result of EERL

The qualitative analysis of phytochemicals of EERL revealed the presence of alkaloids, cardiac glycosides, saponins, tannins, flavonoids, steroids/triterpenes, terpenoids, carbohydrates and anthraquinones. The LD_{50} of the EERL was estimated to be greater than 5000 mg/kg body weight.

The effect of ethanol leaf extract of *Rothmannia longiflora* and its residual aqueous fraction against the hot plate test following CCI-induced neuropathic pain in rats

The EERL and the residual aqueous fraction (RAQF) at a dose of 1000 mg/kg offered significant (p <0.05 and p <0.01) protection against the chronic constriction injury on day 7, 14 and 21 compared to the negative control (CCI+D/W 1 mL/kg). The Minocycline 30 mg/kg (positive control) also offered significant protection (p <0.05 and p <0.01) against the chronic constriction injury on day 7, 14, 21 and 28 compared to both the negative control (CCI+D/W 1 mL/kg) and the sham group (D/W 1 mL/kg) (Figure 1).

The effect of ethanol leaf extract of *Rothmannia longiflora* and its residual aqueous fraction on CCI-induced alteration of antioxidants in rats.

EERL and the residual aqueous fraction (1000 mg/kg) significantly (p <0.05, p <0.01 and p <0.001) increased the concentrations of SOD, GSH, and CAT and decreased the concentration of MDA, respectively. The standard drug, Minocycline 30 mg/kg, significantly (p<0.01 and p<0.001) increased the concentrations of SOD, GSH, and CAT and decreased the concentrations of MDA, respectively, compared to the control (Figures 2 - 5).

DISCUSSION

The antinociceptive and antioxidant properties of EERL were successfully investigated. The oral administration of EERL and its RAQF at 5000 mg/kg to the rats failed to show signs of toxicity, and no death was recorded. This confirmed the safety of the plant as demonstrated in the LD_{50} evaluation. The result of the acute toxicity (LD_{50}) study gives insights into the range of doses to select for in vivo experiments in animals or humans [15]. The doses used in this study were 20 % of 5000 mg/kg; this agrees with what was reported by Vongtau [16]. The hyperalgesia study is conducted to assess the central antinociceptive properties of drugs, medicinal plants and other substances. The paws of albino mice and Wistar rats are very sensitive to heat, hence were employed for this study. The manifestation of pain in this model is expressed by jumping of rats out of the hot plate, withdrawal from the effect of heat and chewing of the paws; the prolongation of the latency time for these responses to be seen after oral administration of the drugs or medicinal substances indicates the central analgesic activity [17]. The centrally acting drugs like morphine exert their mechanism of action in the spinal cord and the brain by binding to the opioid receptors (mu (μ) and kappa (κ)) thereby elevating the pain threshold level at the spinal cord and changing the brain perception to pain, hence contributing to antinociceptive action [18]. Therefore, the ability of EERL and RAQF to raise the pain threshold in the hot plate model applied to neuropathic rats indicates that they may possess central analgesic activity. This is in line with the study conducted by Germain and colleagues, which demonstrated that the roots decoction of *Nuclea latifolia* (Rubiaceae) possesses analgesic activity against the hot plate-induced pain model [19]. The CCI of the sciatic nerve is a valid model used to induce neuropathic pain (NP) [20]. The process where neuropathic pain is induced is moderately multifaceted, which comprises the central and peripheral mechanisms. It is well known that oxidative stress contributes to the formation and progression of neuropathic pain [21]. The free radicals and reactive oxygen species (ROS) occur because of normal cellular metabolism. Lipids, proteins, carbohydrates and other cell components are exposed to oxidation, which causes cell damage; this process is called lipid peroxidation [22]. Malondialdehyde acid (MDA) is a marker for oxidative stress and one of the products of lipid peroxidation. Antioxidant enzymes protect cells and tissues from the effects of oxidative stress. They serve as a body defence system, thereby offering protection against diseases caused by oxidative stress, for example, superoxide free radicals is transformed to H_2O_2 and H_2O by superoxide dismutase enzyme (SOD) [22]. Plants extract rich in antioxidants will mop up free radicals that constitute oxidative stress; therefore, the capacity of EERL and RAQF to increase the concentrations of SOD, catalase (CAT), and reduced-glutathione (GSH) and decrease the quantity of MDA suggests that they possess antioxidant effects and offer protection against neuropathic pain. The qualitative evaluation of medicinal herbs gives clues on the type of phytochemicals that are contained in these herbal extracts [23]. These phytochemicals have been proven to possess some pharmacological activities that are beneficial in the management of some disease conditions. For example, flavonoids, saponins, and triterpenes have been found to possess analgesic activity [24], [25].

CONCLUSION

Ethanol leaf extract and residual aqueous fraction of *Rothmannia longiflora* (EERL) demonstrated significant antinociceptive and antioxidant activities in the neuropathic pain model, which may be attributed to the occurrence of flavonoids, saponins, and triterpenes acting in combination with the antioxidant properties of plants that mop up the free radicals that constitute oxidative stress.

AUTHORS' CONTRIBUTION

Research conceptualization: **DM, NMD, MGM** and **BAC**

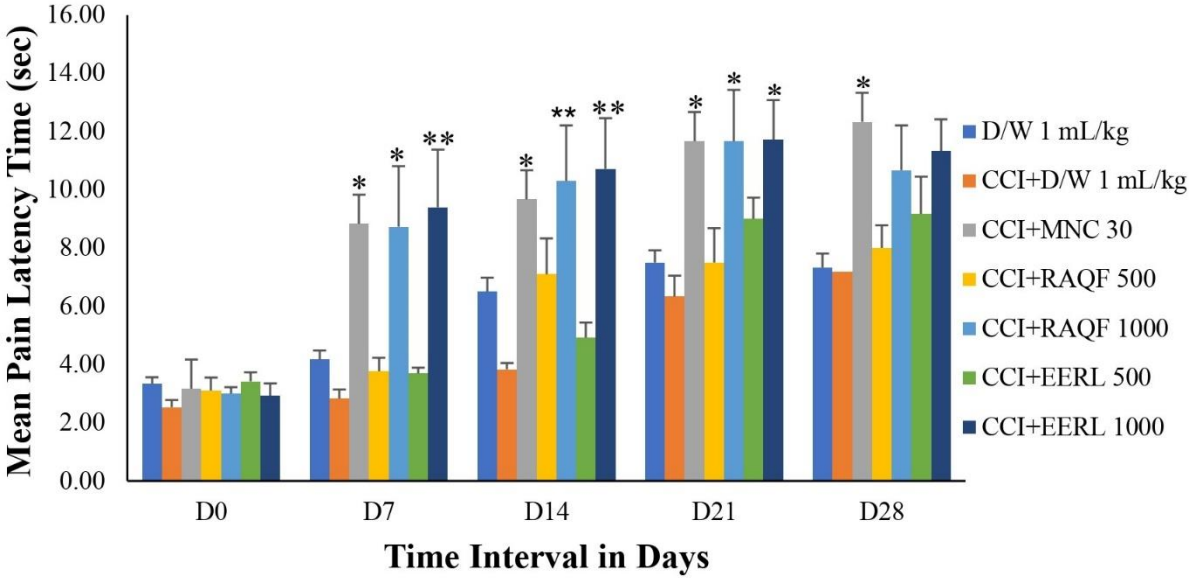

Figure 1: The effect of EERL and its RAQFRL against the hot plate test following CCI-induced neuropathic pain in rats. Values were presented as Mean± standard errors of the mean. Data were analyzed using repeated measure analysis of variance followed by Bonferroni's post hoc test, * = p <0.05, ** = p <0.01 compared to CCI+D/W, n = 5, D/W = distilled water, EERL = ethanol extract of *Rothmannia,* MNC = minocycline, CCI = chronic constriction injury.

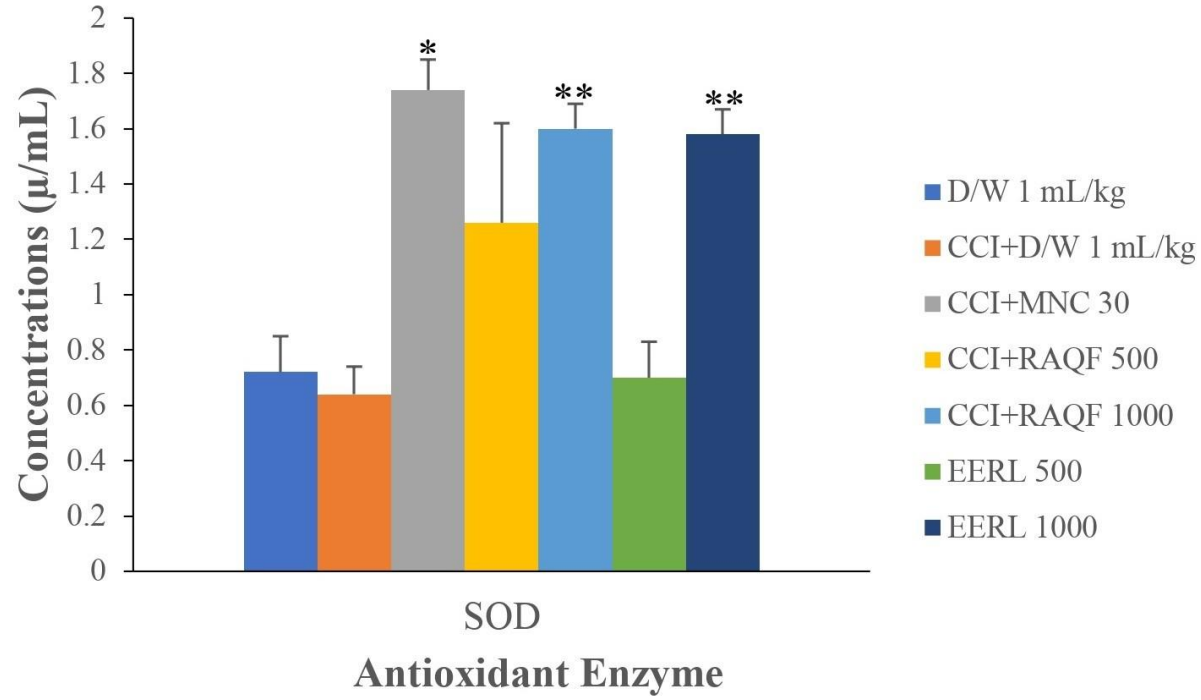

Figure 2: The effect of EERL and its RAQFRL on superoxide dismutase (SOD) following CCI-induced neuropathic pain in rats. Values are presented as Mean± S.E.M. Data were analysed using one-way analysis of variance followed by Bonferroni's post hoc test, * = p <0.05, ** = p <0.01, compared to CCI+D/W. n = 6, D/W = distilled water, EERL = ethanol leaf extract of *Rothmannia*

longiflora, RAQFRL = residual aqueous fraction of *Rothmannia longiflora* leaf extract, CCI = chronic constriction injury, S.E.M = standard errors of mean, SOD = superoxide dismutase.

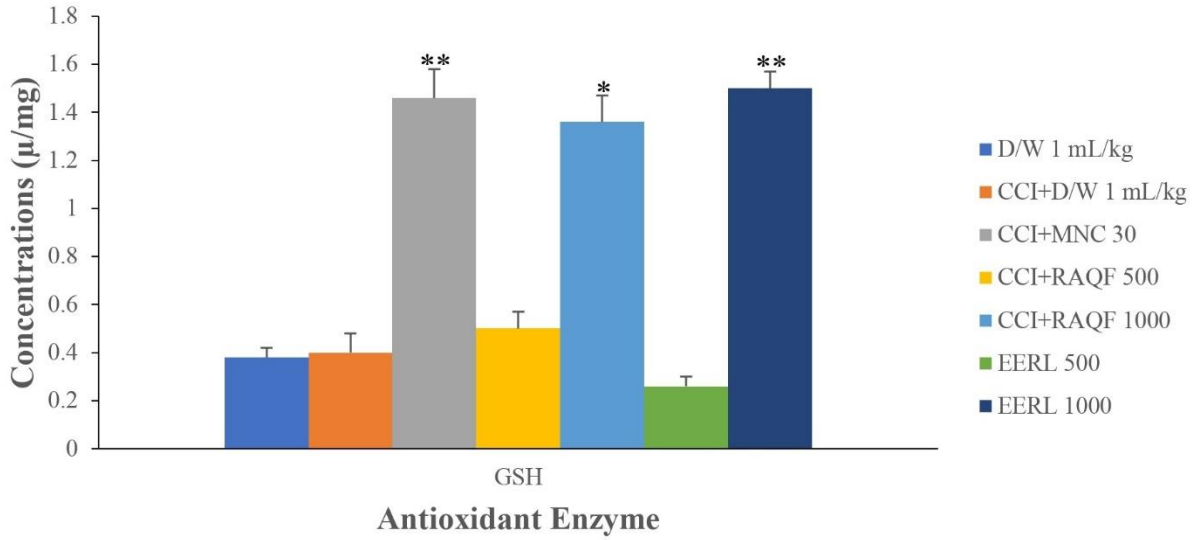

Figure 3: The effect of EERL and its RAQF on reduced glutathione (GSH) following CCI-induced in rats. Values are presented as Mean± S.E.M. Data were analysed using one-way analysis of variance followed by Bonferroni's post hoc test, * = p <0.01 compared to CCI+D/W, ** = p <0.001 compared to D/W, n = 6, D/W = distilled water, EERL = ethanol leaf extract of *Rothmannia longiflora*, RAQFRL = residual aqueous fraction of *Rothmannia longiflora* leaf extract, CCI = chronic constriction injury, S.E.M = standard errors of mean, GSH = reduced glutathione.

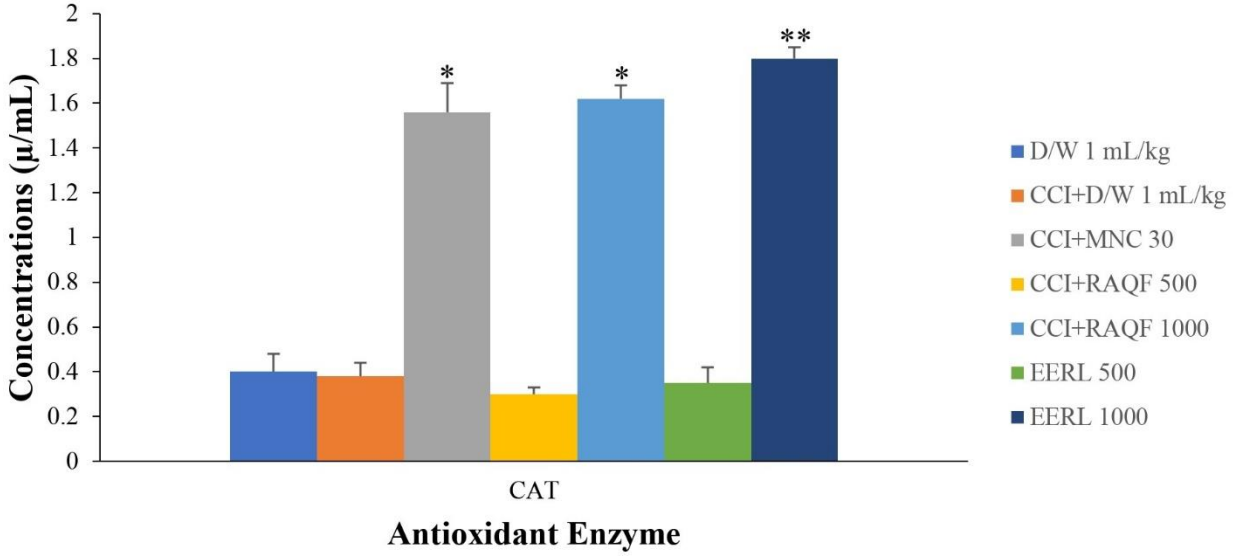

Figure 4: The effect of EERL and its RAQF aqueous fraction on catalase following chronic constriction injury-induced neuropathic pain in rats. values are presented as Mean± S.E.M, Data were analysed using one-way analysis of variance followed by Bonferroni's post hoc test, * = p <0.01 compared to CCI+D/W, ** = p <0.001 compared to D/W. n = 6, D/W = Distilled water, EERL = ethanol leaf extract of *Rothmannia longiflora*, RAQFRL = residual aqueous fraction of *Rothmannia longiflora* leaf extract, CCI = chronic constriction injury, S.E.M = standard errors of mean, CAT = catalase.

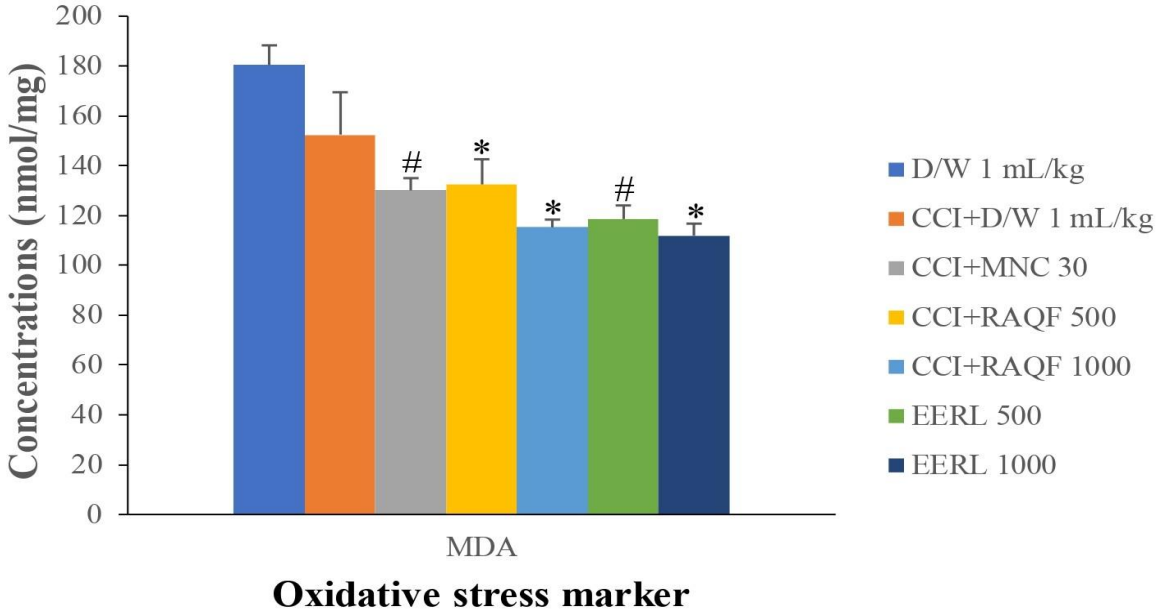

Figure 5: The EERL and its RAQFRL on malondialdehyde (MDA) following CCI-induced neuropathic pain in rats. Values are presented as Mean± S.E.M, * = *p* <0.05, # = p<0.01, ** = *p* <0.001 compared to CCI+D/W. Data were analysed using one-way analysis of variance followed by Bonferroni's post hoc test, n = 6, D/W = distilled water, EERL = ethanol leaf extract of *Rothmannia longiflora*, RAQFRL = residual aqueous fraction of *Rothmannia longiflora* leaf extract, CCI = chronic constriction injury, S.E.M. = standard errors of mean, MDA = malondialdehyde acid.

Experimentation: **DM** and **EGE**. Data collection: **DM**. Data Analysis: JOA. Manuscript writing: **DM**. Proofreading: **MT** and **EB**. Supervision: Nuhu Mohammed Danjuma, **MGM** and **BAC**. Graphics editing: **MT**.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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