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(Research Article)

Free Radical Scavenging Activity of Methanolic Extract of *Calotropis procera* Roots in Ethanol-Induced Oxidative Stress Male Wistar Rats

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ABSTRACT

The generation of free reactive radicals and the biochemical strategy underlying their mopping are paramount to healthy cellular activities. The scavenging activity of the methanolic extract of Calotropis procera roots on the hydroxyl radicals, nitric oxide and hydrogen peroxide was investigated in ethanol-induced oxidative stressed male Wistar rats and a concentration dependent percentage radical scavenging activity was observed against these free radicals with IC_{50} of 54.9 mg/ml, 54.2 mg/ml and 37.3 mg/ml respectively.

The rats were treated with 250 mg/ml and 500 mg/ml per kg body weight after 5-6 hours of inducing oxidative stress. The blood serum was analysed for the activities of superoxide dismutase (SOD) and catalase (CAT). A positive in vivo modulatory effect on the catalytic turnover rate of SOD and CAT was observed. SOD activity reduced from 0.057±0.003 U/ml in induced oxidative stress rats to 0.023±0.003 U/ml when treated with 250 mg/ml/kg root extract and 0.014±0.003 U/ml when treated with 500mg/ml root extract, a value which was not statistically different ($p<0.05$) from the control. In addition, the CAT activity reduced from 77000±140 µmolmin^{-1} in induced oxidative stress rats to 37000±130 µmolmin^{-1} when treated with 250 mg/ml/kg roots extract, a value which was not statistically different ($p<0.05$) from the control, and there was a further reduction in the CAT activity when treated with 500 mg/ml/kg root extract. Hence, the methanolic extract of the root of Calotropis procera may serve as a good non-enzymatic radical scavenger.

These results have shown a concentration dependent radical scavenging activity of the methanolic extract of the roots of Calotropis procera against reactive oxygen and nitrogen free radicals with positive modulatory action in the biochemical rescue of plasma antioxidant enzymes in scavenging these deleterious free radicals, which are constantly generated in aerobic cellular activities.

Key Words: *Calotropis procera*, Oxidative stress, SOD, CAT, Free radicals

INTRODUCTION

Calotropis species are perennial herbs with a long history of use in traditional medicine especially in the tropical and subtropical regions. A wide range of chemical compounds including cardiac glycosides, flavonoids, phenolic compounds and terpenoids have been isolated from this plant[1,2,3,4]. Of the large number of plant species reported on the Ethnobotanical interest, the two species of *Calotropis* R. Br viz *Calotropis procera* and *Calotropis gigantean* hold a pride of place largely because of their geographical distribution and economic values[5,6,7]. *Calotropis procera* is well known for its medicinal uses in traditional system of medicine for the treatment of variety of disease conditions including leprosy, ulcers, tumors, piles, cancer, inflammation, constipation, parasitic/microbial infections, pyretic, analgesic[8,9,10]. These plants have been found to show *in vitro* antioxidant activity[11], antimyocardial properties[12,13], anti-inflammatory and analgesic[14,15] and also elicited immunological response[16]. For its popularity, it is called *"ewe bomu-bomu"* in the Western part of Nigeria. Traditionally, it is popularly found around houses where it is believed to keep away all forms of snakes.

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism[17] and they are continually formed as a result of oxidative stress imposed on metabolizing cells/tissues[18,19]. The most common reactive oxygen species (ROS) include superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\cdot}$) and hydroxyl radicals ($OH^{\cdot}$) while the reactive nitrogen species (RNS) include nitric oxide radicals ($NO^{\cdot}$) and peroxynitrite anion ($ONO_2^{\cdot}$). Free radical species have been implicated in several diseases[20], which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infections and

acquired immunodeficiency syndrome (AIDS). Ineffective antioxidant activities in the body have probably been traced to the one of the factors responsible for autoimmune reactivity, which could lead to terminal abortion[21]. In preventing these diseases, antioxidant therapy has gained an immense importance in ethnomedicine through phytopreventive measures[22].

The use of *Calotropis procera* plant products as anti-infertility and for abortifacient in Nigeria has brought a new dimension to the nature of this plant[23]. Novel natural antioxidants from some medicinal plants have been extensively studied in the past few years for their free radical scavenging properties[24] using aqueous and mixtures of organic solvents but little or no information has really been published recently on the free scavenging activity of pure methanolic extract of the roots of *Calotropis procera* alongside its *in vivo* effect in induced oxidative stress male Wistar rats. It was therefore, the interest of this work to look at the effect of methanolic extract of the roots of *Calotropis procera* against hydroxyl radicals, nitric oxide and hydrogen peroxide and its role on the activity of plasma antioxidant enzymes in alcohol-induced oxidative stress male Wistar rats.

MATERIALS AND METHODS

Plant Roots

Calotropis procera roots were purchased from Iyana-Iba market in Ojo Local Government of Lagos State, Nigeria. The roots were identified and authenticated at the herbarium room of the Botany department, Faculty of Science, University of Lagos, Akoka Lagos State, Nigeria. The plant roots were peeled and washed thoroughly with distilled water and oven-dried at 50 ^{o}C for four days.

Laboratory Animals

Twelve male Wistar rats, weighing 100-150 g, were obtained from the Animal Centre Laboratory of the College of Medicine, Idi-Araba, University of Lagos, Akoka Lagos State, Nigeria. The animals were kept in metal cage with ample space to avoid restrained oxidative stress. They were acclimatized for two weeks and fed with standard rat feeds. Water was given *ad libitum.* Every other procedures guiding the laboratory animal cares according to the Animal Laboratory Manuals from the Animal House, College of Medicine of the University of Lagos, Idi Araba Lagos State, Nigeria were carefully followed. The rats were divided into four groups of three each represented as *control, placebo, 250 mg/ml/kg* and *500 mg/ml/kg* body weight treatments (A, B, C and D respectively)

Methanolic Extraction of the Plant Roots

After four days of over-dried, 200g of chopped dried root were soaked for 48 hours in 800 mL of methanol (99.8 % Assay, BDH). The extract was oven-dried at 45 ^{o}C for 48 hours.

Experimental Design

Group A (control), was given 1.0 mL of distilled water orally for 6 hours at every 2 hours intervals. Group B, C and D were given 1.0 mL each of 50 % absolute ethanol (BDH) three times in 6 hours to induce oxidative stress. All rats were allowed to rest for 5 hours after the last dosage. Then, group B (placebo) was given 1.0 mL of distilled water three times in 24 hours while groups C and D were treated with 250 mg/ml/kg and 500mg/ml/kg body weight three times in 24 hours. Each rat was sacrificed by mild anaesthesia using acetone and the blood was quickly collected through jugular puncture into the heparinized bottles. The blood was centrifuged at 3000 rpm for 5 minutes and the serum was refrigerated at 4^{o}C until it was used.

Hydroxyl Radical Scavenging Activity Assay

The scavenging activity for hydroxyl radicals was measured through Fenton Reaction[24]. The reaction mixture contained 60 µL of 1.0 mM $FeCl_3$, 90 µL of 0.1 mM of 1,10-phenanthroline, 2.4 mL of 0.2 M phosphate buffer (pH 7.8) and 150 µL of 0.17 M H_2O_2. Then, 1.5 mL of the root extract was quickly added to the mixture and the reaction was incubated at room temperature for 5 minutes. The absorbance of the reaction was measured at 560 nm. The hydroxyl radicals scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = [{}^{Ao- A1}/_{Ao}] \times 100$$

Where A_o was the absorbance of the blank sample and A_1 was the absorbance of test sample.

Nitric Oxide Scavenging Activity Assay

Nitric oxide radical scavenging activity was determined according to the method described by Garrat[25]. Briefly, 2.0 mL of 10 mM sodium nitroprusside in 0.5 mL of 0.05 M PBS (pH 7.4) was mixed with 0.5 mL of the extract and the mixture was incubated at 25 ^{o}C for 150 minutes. After incubation, 0.5 mL of the mixture was added to 1.0 mL sulfanilic acid solution (33 %v/v in 20 %v/v glacial acetic acid) and incubated at room temperature for 5 minutes. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1 %w/v) was introduced and incubated at room temperature for 30 minutes. The absorbance was read at 540 nm. The nitric oxide radicals scavenging activity was calculated according to the following equation:

$$\%\text{inhibition} = [{}^{Ao- A1}/_{Ao}] \times 100$$

Where A_o was the absorbance of the control and A_1 was the absorbance of the test sample.

Hydrogen Peroxide Scavenging Activity Assay

The H_2O_2 scavenging activity of the extract was estimated by replacement titration[26]. Briefly, 1.0 mL aliquot of freshly prepared 0.1 mM H_2O_2 and 1.0 mL of the plant root extract were mixed, followed by 2 drops of 3 % ammonium molybdate, 10 mL of 2 M H_2SO_4 and 7.0 mL of 1.8 M KI. The mixed solution was titrated with 5.09 mM $Na_2S_2O_3$ until yellow colour disappeared. Percentage scavenging activity of hydrogen peroxide was calculated as:

$$\%\text{inhibition} = [{}^{Ao- A1}/_{Ao}] \times 100$$

Where A_o was the absorbance of the control and A_1 was the absorbance of the test sample.

Superoxide Dismutase Assay

The activity of the superoxide dismutase (SOD) assay was done using a method described by Buyukokuroglu[27]. The assay contained 20 µL of serum sample, which was added to 3.0 mL of 50 mM Na_2CO_3 buffer, pH 10.2 (2.33 g Na_2CO_3, 2.35 g $NaHCO_3$, 37.22 g EDTA in 1 L distilled water) with 30 µL of epinephrine. The reaction mixture was shaken and the absorbance was read at 480 nm at 3rd and 5th minutes of the reaction against a reaction blank. The amount

of oxygen generated within the three minutes of reaction was estimated by the formula stated below:

$$[{}^{\Delta A480nm}/_{\varepsilon} \times {}^{V}/_{v}]$$

Where "ε" represents the molar extinction co-efficient (4020 $M^{-1}cm^{-1}$), "V" represents the total volume of the reacting sample, "v" represents the volume of the sample and "ΔA" represents changed in the optical density of the reaction mixture. 1.0 unit (U) of SOD activity was defined as the amount of oxygen generated per minute of the reaction sample and was expressed as U/ml.

Catalase Assay

The activity of catalase was determined by the method described by Zámocký[28]. Briefly, 200 µL of the methanolic extract of *Calotropis procera* root was added to 1.8 mL of 30 mM H_2O_2 (1.84 mL of 50% H_2O_2 per Litre of 50 mM of phosphate buffer, pH 7.0; 6.81 g KH_2PO_4, 8.90 g $Na_2HPO_4.2H_2O$ in 1 L of distilled water). The absorbance of the reaction mixture was read at 280 nm against reagent blank at 30 seconds and 60 seconds respectively. Catalase activity was estimated using the formula stated below:

$$[{}^{\Delta A280nm}/_{t} \times {}^{V}/_{\varepsilon v}]$$

Where "V" represents the total volume of the reacting sample, "v" represents the volume of the sample, "t" represents the time, "${}^{\Delta A280nm}/_{t}$" is expressed as enzyme activity and "ε" represents the molar extinction co-efficient (40 $M^{-1}cm^{-1}$). 1.0 unit (U) of CAT activity was defined as the amount of enzyme required to transform 1.0 µmol of substrate to product(s) in 60 seconds at 25 ^{o}C under optimum condition of measurement. CAT activity was measured in µmol/min. All chemical reagents used in this work were of Analar grade.

Statistical Analysis

One-way analysis of variance (ANOVA) for multiple comparisons of parameters was carried out using Tukey's Multiple Comparison Test (TMCT) with GraphPad Prism Version 5. The results were expressed as mean±SEM. The IC_{50} of the extract against the hydroxyl radicals, nitric oxide and hydrogen peroxide was graphically determined using nonlinear regression analysis. The mean difference was considered significant at $p<0.05$.

RESULTS AND DISCUSSION

There are several reports on the use of the extracts and latex of *Calotropis procera* as antioxidant, antibacterial, antifungal, antidiabetes and antiinflammatory[19,29,30,31]. This has degenerated into the cytotoxic, hepatoprotective, nephroprotective and cardioprotective studies of this plant extracts[32,33,34].

The percentage radical scavenging effect of the methanolic extract of the roots of *Calotropis procera* against hydroxyl radicals, nitric oxide radicals and hydrogen peroxide and its effect on the activity of SOD and CAT were studied. The radical scavenging activity of the extract is shown in **Figure 1**.

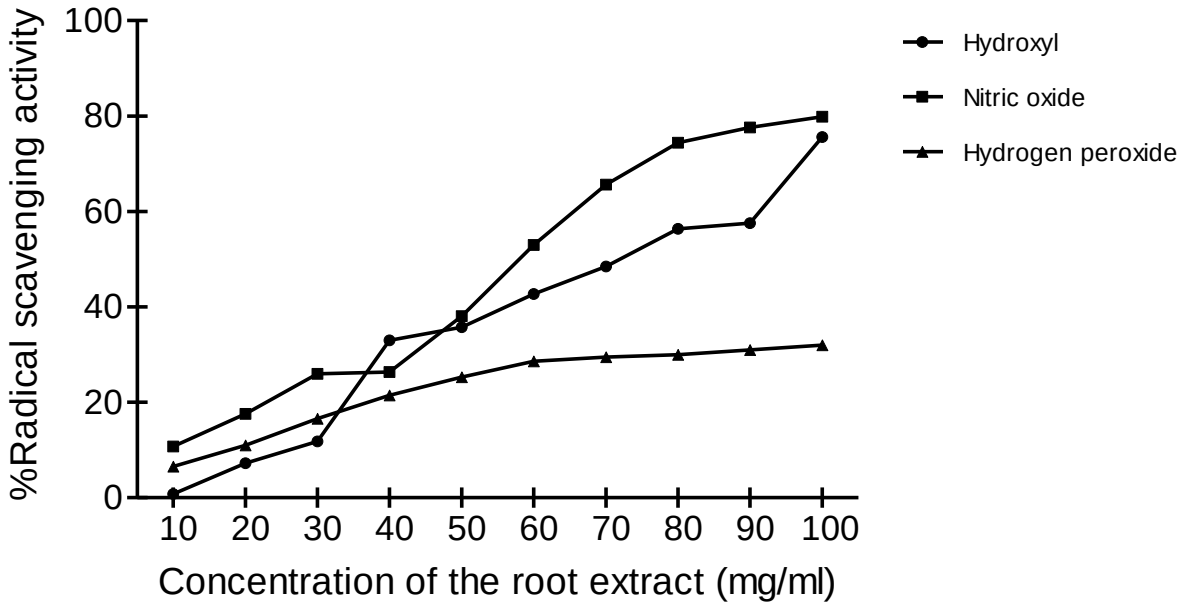

Figure 1: Concentration dependent radical scavenging activity of methanolic extract of the root of *Calotropis procera*

The maxima percentage scavenging activities against nitric oxide = 79.86, hydroxyl = 75.57 and hydrogen peroxide = 32.0 corresponding to 100 mg/ml concentration of the extract. The highest scavenging activity was observed against nitric oxide.

The root extract showed a dose dependence effect on each of the radical tested. The highest percentage scavenging activity of 100 mg/ml root extract against hydroxyl, nitric oxide and hydrogen peroxide were 75.57 %, 79.86 % and 32.0 % respectively. The highest peak was observed in activity against nitric oxide. The average percentage scavenging activity is presented in **Figure 2.** Highest average scavenging activity of 46.93±8.36 was observed against nitric oxide. This value was not statistically different from hydroxyl radicals (36.91±7.67) but statistically higher than the hydrogen peroxide (23.19±2.85). The inhibitory concentration of this extract resulting in 50 % scavenging activity (IC_{50}) is shown in **Figure 3.** The IC_{50} observed for hydroxyl, nitric oxide and peroxide were 54.9 mg/ml, 54.2 mg/ml and 37.3 mg/ml respectively. Hydrogen peroxide has the lowest IC_{50}.

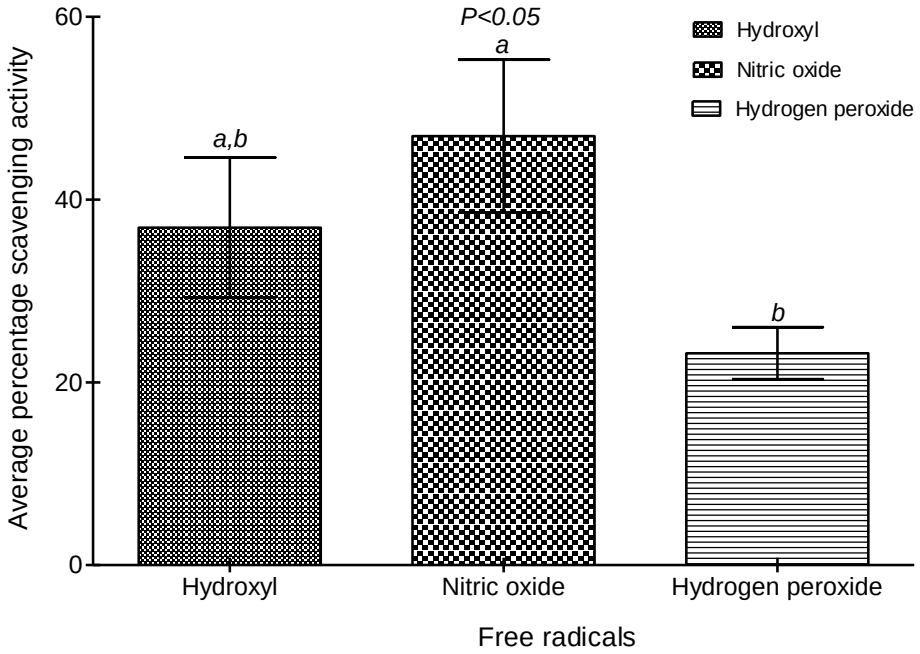

Figure 2: Average percentage scavenging activity of the methanolic root extract of *Calotropis procera*

The values were presented as mean±SEM representing the average percentage scavenging activity of the root extract against the free radicals. Bars with the same alphabets were not statistically different, otherwise they were statistically difference ($^{a,b}p<0.05$).

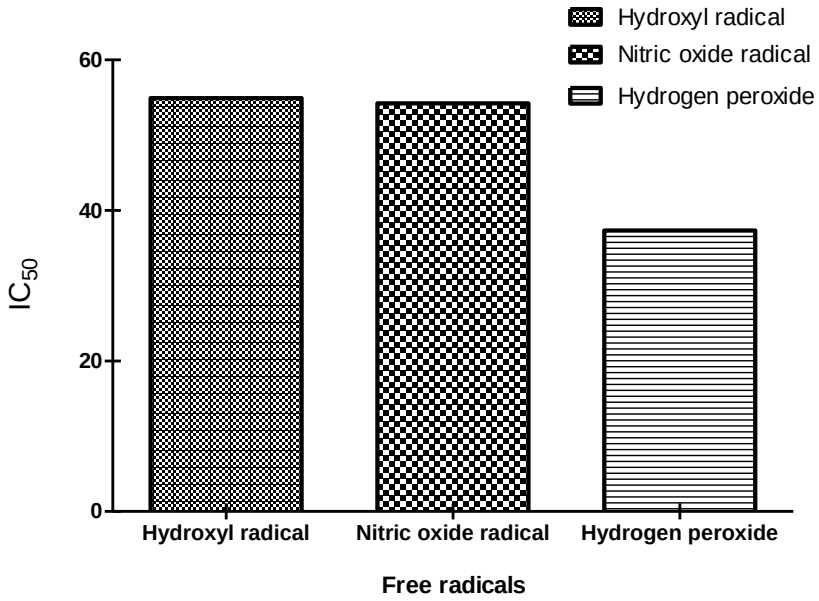

Figure 3: Inhibition concentration of the methanolic extract of *Calotropis procera* root resulting in 50% radical scavenging activity

The values were presented as mean±SEM representing the concentration of the methanolic root extract of Calotropis procera causing 50 % scavenging radical activity. Hydrogen peroxide has the lowest IC_{50} hence the most sensitive to the root extract.

The activities of SOD and CAT are shown in **Tables 4 and 5** respectively. The activities of these enzymes in the placebo were generally reduced to competitive values with control group by both 250 mg/ml/kg and 500 mg/ml/kg body weight treatments. The SOD activity reduced from 0.057±0.003 U/ml in the placebo to 0.023±0.003 U/ml and 0.014±0.003 U/ml when treated with 250 mg/ml/kg and 500 mg/ml/kg root extracts respectively. The control group has SOD activity of 0.013±0.002 U/ml. There was a significant difference *(p<0.05)* when comparing the SOD activity in both the placebo and either of the treated groups of rats. There was no difference between the activity of SOD in the control and either of the treated group. There was no significant difference *(p>0.05)* when the activity of SOD in the treated groups were compared. Likewise, the activity of CAT reduced from 77000±140 µmol/min in the placebo to 37000±130 µmol/min and 24000±770 µmol/min when treated with 250 mg/ml/kg and 500 mg/ml/kg root extracts respectively. The activity of CAT in the control group was 37000±440 µmol/min. There was a significant difference *(p<0.05)* when CAT activity in the placebo and either of the treated groups were compared. There was no significant

difference in the CAT activity between the control and 250 mg/ml/kg treated rats but 500mg/ml/kg treated rats had a lower CAT activity comparing to the control. This invariably showed that higher concentration of the extract elicited more impact than lower concentration, nevertheless the threshold concentration will be of great important to this effect.

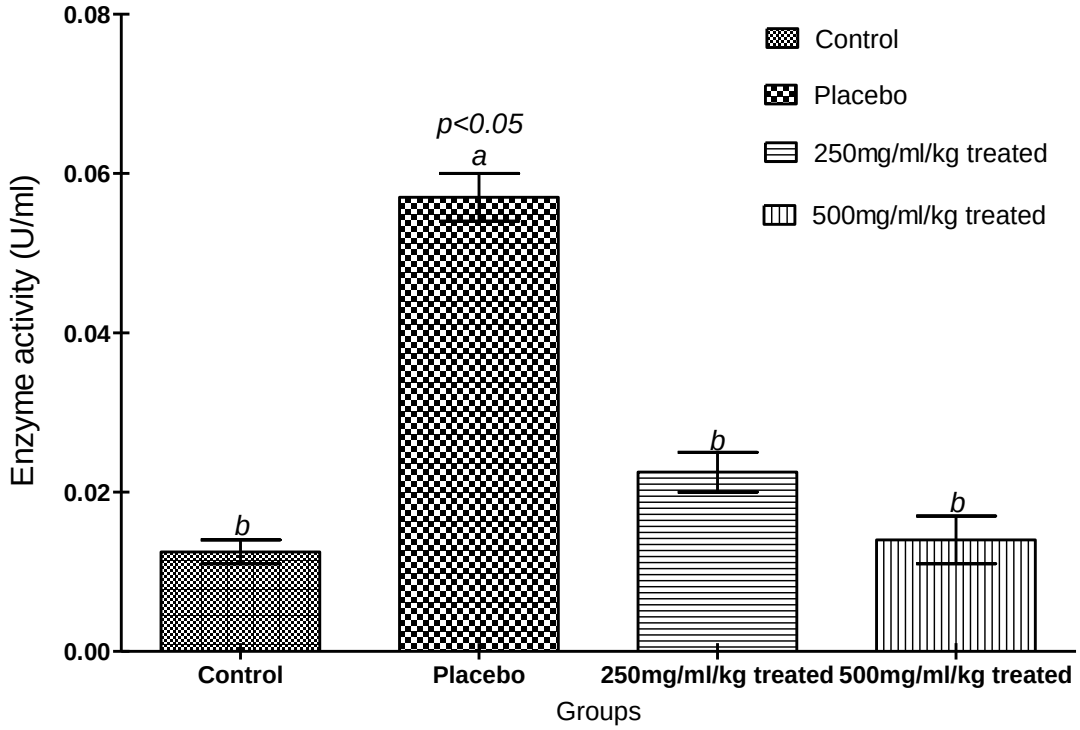

Figure 4: Superoxide dismutase (SOD) activity in the ethanolic-induced oxidative stress male Wistar rats when treated with methanolic extract of the roots of *Calotropis procera*

The values were presented as mean±SEM (n=3) representing the amount of oxygen produced/ml of reaction sample. Bars of the same alphabet were not statistically different from each other, otherwise they were statistically different ($^{a,b}p<0.05$).

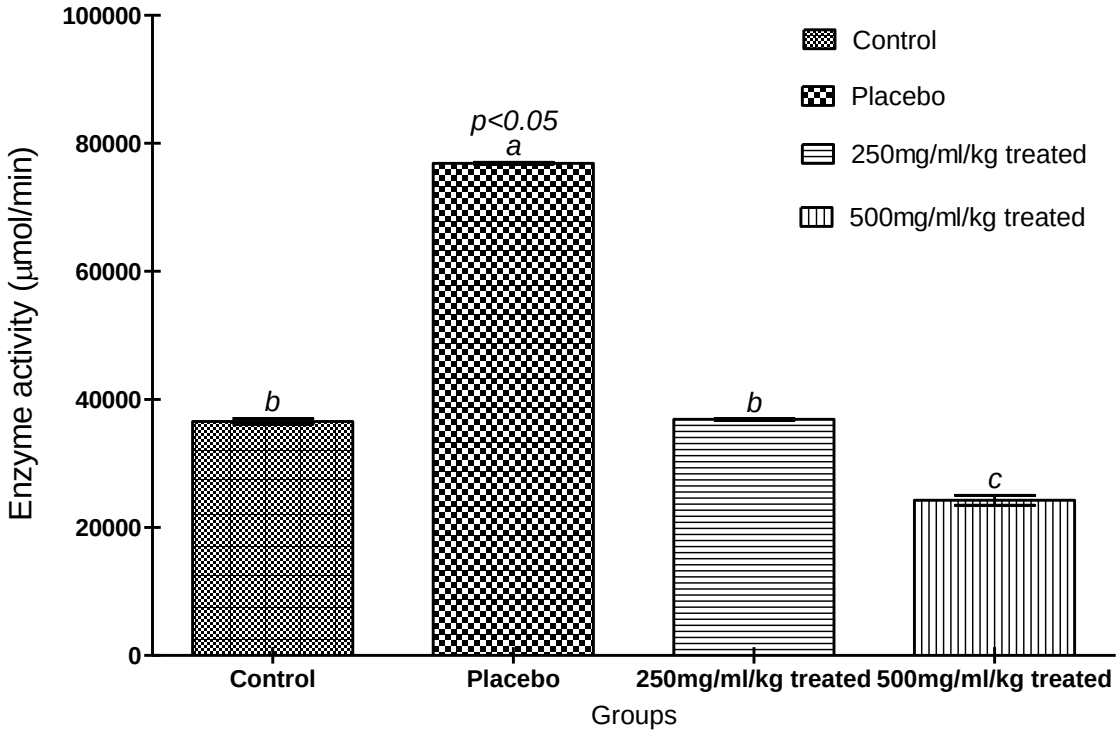

Figure 5: Catalase (CAT) activity in the ethanolic-induced oxidative stress male Wistar rats when treated with methanolic extract of the roots of *Calotropis procera*

The values were presented as mean±SEM (n=3) representing the activity of the catalase. Bars of the same alphabet were not statistically different from each other, otherwise they were statistically different ($^{a,b}p<0.05$).

Free oxygen and nitrogen radicals are usually and increasingly generated from induced/oxidatively stressed cells during cellular respiration and failure to designing mechanisms that will neutralize this upset has led to the formation of several diseases like arthritis, necrosis, atrophy, fatigues, cancer, dystrophy and many more and these are manifested in ageing, skin wrinkling/folding, tissue degeneration, cardiac failure and hepatic malfunctions. Many reports of natural antioxidants of plant origin have been published and their importance in health, food and preventive medicine has been well documented[33]. The antioxidant scavenging activity of the methanolic extract of the roots of *Calotropis procera* was investigated in oxidatively induced male Wistar rats. A significant impact of the methanolic extract of *Calotropis procera* in curbing reactive oxygen and nitrogen radicals, and positively modulated the catalytic efficiency of superoxide dismutase and catalase, was observed. Generally, there was a direct relationship between the concentration of the plant extract and the percentage scavenging activity on each of the tested radicals. The IC_{50} result has shown that the methanolic extract of the roots of *Calotropis procera* was much more active in scavenging hydrogen peroxide compared to nitric oxide and hydroxyl radicals, probably because of the instability of hydrogen peroxide. The potential antioxidant properties of this plant might probably due to the active hydrogen donor ability of hydroxylated substitution of calotropogenin, calotropin and calotoxin. Similarly, high molecular weight isolated compounds and the proximity of many aromatic rings of coroglaucegenin, glucofrugoside, calotroposide A and B could be more important for the free radical scavenging activity of this plant extract.

Presumably, the extract may act by reducing the amount of the radicals available for SOD and CAT to mop thereby lessen the burden on these antioxidant enzymes or probably constitute a sort of organic inhibitor to the activity of these enzymes. However, reduction in the activities of these enzymes in the treated rats as compared to the placebo has revealed that the methanolic root extract of *Calotropis procera* did not hinder the activities of these enzymes but rather complemented the scavenging activity of the enzymes by acting as non-enzymatic radical scavenging effector. The results for the positive modulation of this extract on the activities of SOD and CAT probably suggest the reason why *Calotropis procera* may be a good source of natural antioxidant. Flavonoids, phenols and terpenoids are but few phytoconstituents that may contribute to its antioxidant property[34,35,36,37].

CONCLUSION

The antioxidant activity of the methanolic extract of the roots of *Calotropis procera* through the scavenging of free radicals presented as hydroxyl, nitric oxide and hydrogen peroxide has been demonstrated. The extract showed a more significant effect on the hydrogen peroxide radicals. Furthermore, the extract has also shown to play a positive *in vivo* modulatory role to the activities of SOD and CAT. Although, the plant contained some inimical constituents like alkaloids, cardiac and cardenolide glycosides[38] nevertheless a systematic screening of these plants with special reference to its active phytoconstituents may be of greater benefit to explore the nutraceutical advantages of this plant.

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