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Antioxidant Enhancing Effect of *Azadirachta indica* A. Juss. Leaf Fractionated Extracts on *Naja nigricollis* Reinhardt Venom in Albino Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author IS designed and managed the analyses of the study and performed the statistical analysis. Authors RAU, SWH and UZF wrote the protocol and managed the literature searches. Author FB wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The lethality of snake venom is mainly attributed to its phospholipase A2 component that hydrolyzes cellular phospholipids, leading to the release of arachidonic acid that generates potentially toxic reactive oxygen species (ROS). Imbalance between excessive generation and poor removal of ROS causes lipid peroxidation leading to cellular damage. Hence, this research was aimed at evaluating the antioxidant-enhancing effect of *Azadirachta indica* leaf fractionated extracts on *Naja nigricollis* venom in albino rats. *A. indica* leaf was collected, authenticated and extracted using 95% methanol followed by fractionation using hexane and ethyl acetate. Ferric

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reducing antioxidant power assay was used for the *in vitro* test, while, *in vivo* experiments were conducted using Albino rats. The *in vitro* antioxidant effect of the hexane and ethyl acetate fractions presented ferric reducing power of 68.80 ± 1.40% and 71.54 ± 2.12% respectively. This are closely related to those of ascorbic acid (78.50 ± 2.80%) and α-tocopherol (75.00 ± 1.85%). The results of the *in vivo* tests indicated that a single injection (0.195 mg/kg b. wt.) of *N. nigricollis* venom caused significant ($P<0.05$) elevation of hepatic and renal ROS levels (7 and 8 folds respectively) with a concomitant increase in lipid peroxidation (LPO) compared to the control group. The ROS levels were decreased significantly leading to the decrease in the level of LPO in the envenomed rats treated with the hexane and ethyl acetate fractions compared to the venom control. The treatments significantly ($P<0.05$) increased the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in both the hepatic and renal homogenates compared to the venom control. The degree of protection against LPO by reducing the levels of ROS as well as increasing the activities of the antioxidant enzymes has significantly ($P<0.05$) increased when combine treatment of standard antivenin and any of the hexane or ethyl acetate fractions was considered compared to when each of them was used alone. Based on these findings, it has been established that, the tested extracts have antioxidant as well as antioxidant-enhancing effects against the oxidative toxicity of *N. nigricollis* venom.

Keywords: Azadirachta indica; Naja nigricollis; antioxidant; antivenom; enhancing effect.

1. INTRODUCTION

Snakebite envenomation is mediated by subcutaneous or intramuscular injection of venom into the human victims resulting in complicated pharmacological effects that depend on the synergistic action between venom proteins with enzymatic and non-enzymatic activities [1]. The pathophysiology of snake envenomation includes both local and systemic effects [2].

The lethality of snake venom is mainly attributed to its highly active enzymatic component, phospholipase A2 (PLA2) [3] that hydrolyzes cellular phospholipids, resulting in the release of arachidonic acid [4,5]. Oxidative metabolism of arachidonic acid generates potentially toxic Reactive Oxygen Species (ROS) including superoxide and hydroxyl free radicals [6]. An imbalance between the excessive generation and poor removal of ROS causes lipid peroxidation leading to cellular damage [7]. PLA2 from snake venom has been implicated in multiple pathologies including hepatotoxicity and nephrotoxicity [8,9]. Snakebite envenomation is also accompanied by signs of inflammation and local tissue damage [10]. Neutrophils and macrophages are induced to produce superoxide radical anion (O_2^-) which reacts with cellular lipids leading to the formation of lipid peroxides necrosis [11].

Plants secondary metabolites such as vitamins (A, C and E), flavonoids, terpenoids, tannins, other polyphenols and some minerals, like Selenium have the capability of neutralizing free radicals; hence they are valuable natural antioxidants that scavenge and remove oxygen free radicals, stabilize cell membranes [12], act as immune-modulators [13] and neutralize snake venom toxicities [14,15]. These classes of compounds are known to be powerful antioxidants both in hydrophilic and lipophilic environments. They can prevent, stop or reduce oxidative damage as a result of PLA2 activity by selectively binding to the active sites or modify conserved residues that are critical for the catalysis of the PLA2 [3,16]. Vitamin E (α-tocopherol, an antioxidant molecule) decreases both enzymatic and inflammatory activities of an isolated PLA2. It also has the ability to bind to the hydrophobic pocket of PLA2, inhibiting free access of substrate to the catalytic site [17].

After snakebite envenomation, inorganic cations in snake venom, such as, iron and zinc can generate highly reactive $OH^{\bullet}$ radicals by Fenton reactions and superoxide ($O_2^{\bullet -}$) by Haber-Weiss reaction [18]. Chelating agents, which stabilize pro-oxidative transition metal ions by complexing them [10], are regarded as secondary antioxidants. Lopes et al. [19] reported antioxidant properties of tannic acid to result from forming stable complexes with Fe (II). Polyphenols (tannins) remove Fe (III) from other iron/ligand complexes [20].

Azadirachta indica A. Juss. (Neem tree) belongs to the Meliaceae (mahogany) family. It is known as '*Dogon yaro or Darbejiya'* in Hausa language. The tree can grow up to 30 m tall with spreading

branches covering some 10 m across [21]. The tree has long been recognized for its unique properties in improving human health [22]. It is grown in most tropical and sub-tropical areas of the world for shade, reforestation and for the production of raw material for natural insecticides and medicines [23]. Different parts of the Neem tree such as leaf, bark, root, seed, and flower show role in disease management through modulation of various biological activities [24].

A research by Sithisarn et al. [25] evaluated the antioxidant activity of different extracts obtained from various parts of the neem tree. The results suggest that extracts from leaf, flower and stem-bark hold high antioxidant activity. In another study, ethanol extracts of flower and seed oil were also found to have free radical-scavenging activity [24].

In a comparative study, it was noticed that the neem stem-bark possessed complex phenolic contents than the leaf with higher antioxidant activity [26]. Flavonoids in the root methanol extract were estimated and their free radical-scavenging properties have also been evaluated [27]. Furthermore, in another study between the methanol and chloroform extracts of the neem leaf, it has been observed that relatively methanol extract possesses significantly more antioxidant properties [28]. Therefore, this research was aimed at evaluating the antioxidant adjuvant effect of *Azadirachta indica* leaf fractionated extracts against *Naja nigricollis* venom in albino rats.

2. MATERIALS AND METHODS

2.1 Study Area

This research work was conducted within Aliero town, Nigeria. It was performed in Biochemistry Research Laboratory, Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria.

2.2 Experimental Animals

Adult Wistar albino rats of both sexes aged 3 – 4 months and weighing between 150 – 200 g were used for the experiments. They were purchased from National Veterinary Research Institute, Vom, Nigeria and kept under standard laboratory conditions (22–24^{o}C; 12:12 h dark/light cycle). The animals were allowed free access to both food (commercial rodents pellets) and water *ad libitum* [29], they were allowed to acclimatize for 2 weeks. Weight of each rat was taken before the commencement of the experiment. All animal experiments were conducted in accordance with the guidelines for the use and care of experimental animals [30].

2.3 Standard Snake Venom Antiserum (Antivenin)

The lyophilized polyvalent snake venom antiserum (Batch No.: 01AS83659, Manufacture Date: March, 2018, Expiry Date: February, 2021) was used as a standard to compare with the efficacy of the plant extracts. It was produced by a standard pharmaceutical company (VINS Bioproducts Limited, Andhra Pradesh, India).

2.4 *Naja nigricollis* Reinhardt

The snake species (*Naja nigricollis* Reinhardt) used was captured and housed in a wooden cage with the help of a snake-charmer. After collection, it was duly identified and authenticated by a Zoologist at the Department of Animal and Environmental Biology, Kebbi State University of Science and Technology, Aliero, Nigeria. Its venom was milked and used for the experiments.

2.5 Milking of Venom

The venom was collected between 5.00 pm to 6.00 pm, in a low light condition at an ambient temperature according to the method of Goswami et al. [1] with modification by using a short-acting general anesthesia; halothane (Piramal Healthcare Limited, U.K.). The glands below the eyes of the snake were compressed to release the stored venom into a cleaned and sterilized container.

2.6 Preparation of Venom

After milking, the venom was lyophilized using a freeze-dryer (Millrock Technology, USA) and kept inside a refrigerator (HR135A, Haier-Thermocool, Lagos, Nigeria) in a light-resistant and air-tight container. Before use, the lyophilized venom was reconstituted in 0.9% saline (regarded as the venom) and kept at 4°C. The venom concentration was expressed in terms of dry weight (mg/ml) [31].

2.7 Dosing of the Venom

The dose of the venom used (0.190 mg/kg b. wt.) for the *in vivo* experiments was based on the

LD_{50} of the venom (0.380 mg/kg b. wt.) as reported by Sani et al. [32].

2.8 Collection and Authentication of the Plant Material

Azadirachta indica leaf was collected within Aliero town, Kebbi State, Nigeria. It was authenticated at the herbarium of the Department of Plant Science and Biotechnology, Kebbi State University of Science and Technology, Aliero, Nigeria and voucher specimen; VN:083 was deposited there.

2.9 Preparation of Crude *A. indica* Leaf Methanol Extract

The extract was prepared according to the method of Dupont et al. [33]. The collected leaf was cleaned with water and air-dried under shade, pulverized using pestle and mortar. One kilogram (1 kg) of the powdered leaf was measured and soaked in 2.5 L of 95% methanol. The mixture was then kept at room temperature for 24 h and filtered twice; initially with a muslin cloth and later with a Whatman filter paper No.1. The filtrate was evaporated to dryness at 45^{o}C using rotary evaporator. The residue was further fractionated.

2.10 Solvent-Fractionation of Crude *A. indica* Leaf Methanol Extract

The crude methanol extract of the *A. indica* leaf was fractionated by liquid-liquid extraction using n-hexane and ethyl acetate in increasing order of polarity. Two hundred grams (200 g) of the dried methanol extract were reconstituted in 400 ml of distilled water in a 1 liter separating funnel. This was then partitioned sequentially with equal volume of n-hexane and ethyl acetate to yield the n-hexane and ethyl acetate fractions. The fractions were concentrated to dryness and the residues were kept in a refrigerator in an air-tight container for further use. Before use, each fraction was reconstituted in 1% aqueous solution of Tween-80 (polysorbate) and was expressed in terms of dry weight (mg/ml).

2.11 *In Vitro* Antioxidant Activity (Ferric Reducing Antioxidant Power Assay)

The ferric reducing antioxidant power **(**FRAP**)** of the hexane and ethyl acetate extracts was determined using potassium ferrocyanide-ferric chloride method [34]. Two milliliters (2 ml) of extract (10 mg/ml) were added to 2.5 ml of potassium ferrocyanide, then, incubated at 50^{o}C for 20 minutes. Trichloroacetic acid (2.5 ml) was added to the mixture which was then centrifuged at 650x g for 10 minutes. To 2.5 ml of the supernatant, 2.5 ml of distilled water and 0.5 ml of ferric chloride were added. The absorbance was then read at 700 nm spectrophotometrically. The same procedure was followed for the FRAP assay of ascorbic acid (vitamin C) and α-tocopherol (vitamin E). Aqueous solution (1%) of tween-80 was used as control. Higher absorbance indicated greater reducing capacity which was calculated as follows.

$$\%\ \text{Reducing Power} = \frac{\text{Absorbance of Test} - \text{Absorbance of Control}}{\text{Absorbance of Test}} \times 100$$

2.12 *In Vivo* Antioxidant Activity Screening

Fifty five (55) rats were randomly distributed into eleven (11) groups of five (5) rats each as follows:

Group 1: Received (orally) 1% aqueous solution of tween-80 and served as normal control.

Group 2: Injected intraperitoneally (*i.p*) with 0.190 mg/kg b. wt. of the snake venom and served as venom control.

Group 3 and 4: Injected (*i.p*.) with 0.190 mg/kg b. wt. of the snake venom, then after 30 min they were administered (orally) with the n-hexane and ethyl acetate extracts at the dose of 100 mg/kg b. wt. respectively.

Group 5: Injected (*i.p*.) with 0.190 mg/kg b. wt. of the snake venom, then after 30 min, they were administered orally with Ascorbic acid (Vitamin C) at the dose of 15 mg/kg b. wt.

Group 6: Injected (*i.p*.) with 0.190 mg/kg b. wt. of the snake venom, then after 30 min, they were injected (i.v.) with α-tocopherol acetate (Vitamin E) at the dose of 10 mg/kg b. wt.

Group 7: Injected (*i.p*.) with 0.190 mg/kg b. wt. of the snake venom, then after 30 min, they were administered (*i.v*.) with standard conventional serum-based antivenin, at the dose of 1 ml/0.6 mg venom.

Group 8 and 9: Injected (*i.p*.) with 0.190 mg/kg b. wt. of the snake venom, then after 30 min, they were administered (*i.v*.) with the conventional serum-based antivenin (1 ml/0.6 mg venom) and

orally with the n-hexane and ethyl acetate extracts at the dose of 100 mg/kg b. wt. respectively.

Group 10: Injected (*i.p.*) with 0.190 mg/kg b. wt. of the snake venom, then after 30 min, they were administered (*i.v.*) with the conventional serum-based antivenin (1 ml/0.6 mg venom) and orally with Ascorbic acid (Vitamin C) at the dose of 15 mg/kg b. wt.

Group 11: Injected (*i.p.*) with 0.190 mg/kg b. wt. of the snake venom, then after 30 min, they were administered (*i.v.*) with the conventional serum-based antivenin (1 ml/0.6 mg venom) and also intravenously with α-tocopherol acetate (Vitamin E) at the dose of 10 mg/kg b. wt.

Six (6) hours later, the animals were sacrificed by cervical decapitation after anaesthetizing with chloroform. Liver and kidneys were collected, weighed and tissue homogenates were prepared by weighing 1 g of tissue, minced with fine sterile laboratory sand and homogenized with 10 ml of 10 mM ice cold phosphate buffer (pH 7.4). The resultant mixtures were centrifuged at 8000 rpm for 10 minutes at 4^{o}C.

2.12.1 Biochemical analyses

After homogenates were centrifuged as mentioned above, the supernatants (10%) were used for the various biochemical analyses.

2.12.1.1 Determination of Lipid Peroxidation (MDA)

Malondialdehyde (MDA) as an index of lipid peroxidation was determined using the method of Buege and Aust [35]. One milliliter (1.0 ml) of the supernatant was added to 2 ml of (1:1:1) TCA-TBA-HCl reagent (Thiobarbituric acid 0.7%, 0.24 N HCl and 15% TCA) then boiled at 100^{o}C for 15 min, and allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 min. The supernatant was removed and the absorbance was read at 532 nm against a blank. MDA was calculated using the molar extinction coefficient for MDA-TBA complex of 1.56 x 10^5 $M^{-1}cm^{-1}$.

$$\mathrm{MDA} = \frac{\Delta \mathrm{A} \times \mathrm{V_T}}{\sum \times \mathrm{V_S}}$$

ΔA= Change in Absorbance, V_T = Total volume, Vs = Sample volume, ∑ = Molar extinction

2.12.1.2 Quantification of Reactive Oxygen Species (ROS)

The method of Vrablic et al. [36] was used. A 0.2 ml of nitro blue tetrazolium (NBT) (1 mg/ml) was added to the hepatic or renal homogenate, followed by additional incubation for 1 h at 37^{o}C. The solution was then treated with 0.1 ml KOH (2 M). The absorbance was measured at 570 nm and expressed as mmol NBT reduced/g tissue.

2.12.1.3 Analysis of Superoxide Dismutase (SOD) Activity

Superoxide Dismutase activity was determined using the method of Sun and Zigma [37]. The reaction mixture (3 ml) contained 2.95 ml of 0.05M sodium carbonate buffer (pH 10.2), 0.02 ml of the liver or kidney homogenate and 0.03 ml of epinephrine in 0.005 N HCl was used to initiate the reaction. The reference cuvette contained 2.95 ml of buffer, 0.03 ml of substrate (epinephrine) and 0.02 ml of distilled water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 min. ∑ = 4020 $m^{-1}cm^{-1}$.

$$\mathrm{SOD} = \Delta A/\mathrm{min} \times V_T/\sum \times Vs$$

ΔA/min = change in Absorbance per minute, V_T = Total volume, Vs = Sample volume, ∑ = Molar extinction

2.12.1.4 Analysis of Glutathione Peroxidase (GPx) Activity

Principle: GPx assay measures GPx activity indirectly by coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) produced upon reduction of hydroperoxide by GPx is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the absorbance is directly proportional to the GPx activity in the sample [38].

A reagent kit was used. A 120 µl of assay buffer (containing; 50mM Tris-HCl, pH 7.6 and 5 mM EDTA) was added to the background of non-enzymatic wells and 50 µl of co-substrate mixture to three wells. A 100 µl of assay buffer was added to positive control wells (bovine erythrocyte GPx), 50 µl of co-substrate mixture and 20 µl of diluted GPx (control) to three wells. A 100 µl of assay buffer was added to sample

wells, 50 µl of co-substrate mixture and 20 µl of sample to three wells. The samples were diluted with sample buffer. The mixture was mixed by shaking the plate and the reaction was initiated by adding 20 µl of Cumene hydroperoxide to all the wells. The absorbance was read once every minute at 340 nm using a plate reader to obtain at least 5 time point. The following formula for the calculation of GPx activity was used. The reaction rate at 340 nm was determined using the NADPH extinction coefficient of a 0.00373 μM^{-1} cm^{-1}. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP per minute.

$$\text{GPx Activity} = \frac{\frac{\Delta A340}{\text{min}}.}{0.00373\ \mu\text{M}^{-1}} \times \frac{0.19\ \text{ml}}{0.02\ \text{ml}} \times$$

$$\text{Sample dilution} = \text{nmol/min/ml}$$

$$\text{Where; } \Delta A_{340}/\text{min} = \frac{\text{A34 0(Time 2)} - \text{A34 0(Time 1)}}{\text{Time 2 (min)} - \text{Time 1 (min)}}$$

ΔA_{340}/min. = Change in absorbance at 340 nm per minute

2.12.1.5 Analysis of Catalase (CAT) Activity

The procedure is based on decomposition of H_2O_2 to water and oxygen in the presence of CAT [39]. An aliquot (50 µl) of clear supernatant from the tissue homogenate was mixed with 1.95 ml of 50 mM potassium phosphate buffer (pH 7.0) and 1.0 ml of 20 mM H_2O_2. The change in the absorbance at 240 nm was recorded immediately and after every 30 s for 3 min. CAT activity was determined using the rate of decomposition of H_2O_2, which is proportional to the reduction of the absorbance at 240 nm. One unit of CAT activity was defined as the amount of CAT decomposing 1.0 µM H_2O_2 per min and was calculated using the molar extinction coefficient of H_2O_2 (43.6 $M^{-1}cm^{-1}$ at 240 nm).

$$H_2O_2 = \Delta A/\text{min} \times V_T/\sum \times Vs$$

Where; ΔA/min = change in Absorbance per minute, V_T = Total volume, Vs = Sample volume, ∑ = Molar extinction

2.13 Data Analysis

The data generated from the study are presented as mean ± SEM and subjected to one way analysis of variance (ANOVA) and statistical difference between the means were separated using New Duncan's Multiple Range Test at $P<0.05$ with the aid of a statistical package (IBM SPSS Statistics 20)

3. RESULTS

3.1 *In Vitro* Antioxidant Activity

Table 1 presents *in vitro* antioxidant activities of the hexane and ethyl acetate fractions as well as those of the Ascorbic acid and α-tocopherol. Using the ferric reducing antioxidant power (FRAP) assay, Ascorbic acid demonstrated the highest percentage reducing power (78.50 ± 2.80%), followed by α-tocopherol (75.00 ± 1.85%). Interestingly, the hexane and ethyl acetate fractions presented percentages of the reducing power close to those of the standard antioxidants. Hexane fraction had 68.80 ± 1.40%, while ethyl acetate fraction had 71.54 ± 2.12%.

3.2 *In Vivo* Antioxidant Activity

The results of the *in vivo* screening clearly indicated that a single injection of *Naja nigricollis* venom at a dose of 0.195 mg/kg b. wt. caused significant ($P<0.05$) elevation in hepatic and renal ROS levels (7 and 8 folds respectively) with a concomitant increase in lipid peroxidation (LPO) in the hepatic and renal homogenates compared to the control group (Table 2). In addition, the results of the venom control showed a significant decrease in SOD, GPx and CAT activities in both the hepatic and renal homogenates when compared to the normal control group. The levels of the ROS were decreased significantly with concomitant decrease in the LPO in the envenomed rats administered with hexane and ethyl acetate fractions as well as ascorbic acid and α-tocopherol compared to the venom control group. The treatments significantly ($P<0.05$) increased the activities of the SOD, GPx and CAT in both the hepatic and renal homogenates compared to the venom control group (Table 2). The degree of protection against LPO by reducing the levels of ROS as well as increasing the activities of the antioxidant enzymes has significantly increased when combine treatment of standard antivenin and any of the hexane or ethyl acetate fractions or with ascorbic acid or α-tocopherol was conducted compared to when each of them (standard antivenin, hexane fraction, ethyl acetate fraction, ascorbic acid or α-tocopherol) was used alone (Table 2).

Table 1. Ferric reducing antioxidant properties of hexane and ethyl acetate fractions of *A. indica* leaf

Test Material	Reducing Power (%)
Hexane Fraction	68.80 ± 1.40
Ethyl acetate Fraction	71.54 ± 2.12
Ascorbic Acid (Vitamin C)	78.50 ± 2.80
α-Tocopherol (Vitamin E)	75.00 ± 1.85

Values are presented as mean ± SEM of triplicates

4. DISCUSSION

Ferric reducing antioxidants power (FRAP) assay measures the reducing ability of antioxidants against oxidative effect of reactive oxygen species (ROS) [34]. Therefore, the significant percentage reducing power of the hexane and ethyl acetate fractions of *Azadirachta indica* leaf indicated their antioxidant power. Electron donating antioxidants can be described as reducing agents, and inactivation of oxidants by reductants can be described as antioxidant activity [40]. Total antioxidant power may be referred analogously to total reducing power [41].

Among the numerous naturally occurring antioxidants; ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoid and phenolic compounds are among the most effective [42]. They are known to inhibit species by propagating a reaction cycle and to chelate heavy metal ions [43]. Studies on medicinal plants and vegetables strongly support the idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems [44,45].

The results of the *in vivo* study of this research demonstrated that envenomation by *Naja nigricollis* venom caused a significant increase in free radicals and other reactive oxygen species levels with a concomitant increase in lipid peroxidation (LPO) in the hepatic and renal homogenates of the venom control rats compared to the normal control group. The lethal effects of snake venom were largely attributed to its active ingredient of phospholipase A_2 (PLA2) [46-48]. Phospholipid hydrolysis by PLA2 enzyme releases arachidonic acid whose metabolism results in the formation of potentially toxic ROS and lipid peroxides [3,7,49].

The significant reduction in the levels of ROS with concomitant reduction of LPO levels in the hepatic and renal homogenates of the hexane and the ethyl acetate fractions treatment groups might be attributed to the high level of tannins in these plant fractions [50]. Tannins are water soluble phenolic substances with the ability to form complexes with proteins. Among its various biological activities are enzyme inhibition, inhibition of lipid peroxidation, scavenger of free radicals and anti-tumor action [51,52].

Antioxidant enzymes are capable of stabilizing or deactivating free radicals before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. In addition, they may also interrupt the oxidizing chain reaction to minimize the damage caused by free radicals. The repair enzymes that can inactivate some antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) and other metalloenzymes [53].

SOD is the antioxidant enzyme that catalyzes the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. Hydrogen peroxide (H_2O_2) can be chemically altered by CAT or GPx reactions [54]. CAT catalyzes the conversion of H_2O_2 to water and molecular oxygen [55]. GPx catalyzes the reduction of hydrogen peroxides (H_2O_2) using reduced glutathione (GSH), thereby protecting mammalian cells against oxidative damage. In fact, glutathione metabolism is one of the most essential antioxidative defense mechanisms [56].

The result of the *in vivo* study showed that, *N. nigricollis* venom significantly reduced the activities of SOD, GPx and CAT in the hepatic and renal homogenates of the venom control group compared to the normal control rats (Table 2). Reduction in the activities of the antioxidant enzymes leads to the accumulation of oxidants which results to oxidative stress. Hence, the result indicated the oxidative effect of *N. nigricollis* venom in rats. The decrease in antioxidant enzymes activities is directly proportional to the increase in reactive oxygen species (ROS) and malondialdehyde (MDA) [57]. These findings are supported by previous reports on snake venom induced lipid peroxidation and tissue injury in different organs [58]. Al-Asmari et al. [59] reported a significant increase in lipid peroxidation in the kidney and brain within 1 h of *Echis pyramidum* venom (EPV) injection, whereas the significant changes in the liver, lung and heart were observed after 3 h. Hence, this

Table 2. *In-vivo* antioxidant activities of hexane and ethyl acetate fractions of *A. indica* leaf in rats

Treatment	Biochemical Analyses									
	MDA (μmol/g tissue)		ROS (mmol NBT/g tissue)		SOD (U/g tissue)		GPx (U/g tissue)		CAT (U/g tissue)	
	Hepatic	Renal	Hepatic	Renal	Hepatic	Renal	Hepatic	Renal	Hepatic	Renal
Normal control	1.04 ± 0.81^{a}	1.22 ± 0.43^{a}	0.39 ± 0.03^{a}	0.26 ± 0.09^{a}	39.89 ± 3.92^{b}	37.88 ± 1.04^{b}	162.49 ± 12.01^{c}	139.95 ± 11.01^{c}	22.04 ± 3.13^{b}	14.90 ± 1.02^{ab}
Venom control	5.29 ± 0.78^{c}	3.80 ± 0.14^{c}	2.85 ± 0.49^{c}	1.97 ± 0.03^{c}	11.76 ± 2.06^{a}	6.28 ± 0.18^{a}	17.29 ± 8.03^{a}	23.16 ± 5.02^{a}	7.58 ± 0.65^{a}	5.04 ± 0.12^{a}
Venom + Hexane fraction	2.42 ± 0.90^{b}	1.79 ± 0.67^{b}	1.72 ± 0.21^{b}	1.11 ± 0.48^{bc}	47.97 ± 1.22^{c}	38.52 ± 0.92^{b}	80.63 ± 11.94^{b}	114.10 ± 10.04^{bc}	25.10 ± 1.12^{b}	23.46 ± 2.04^{b}
Venom + Ethyl acetate fraction	2.10 ± 0.62^{b}	1.19 ± 0.39^{a}	1.01 ± 0.05^{b}	0.63 ± 0.02^{a}	64.07 ± 6.05^{d}	47.52 ± 1.31^{c}	111.21 ± 21.06^{bc}	105.65 ± 4.10^{b}	28.83 ± 2.16^{b}	24.68 ± 1.01^{b}
Venom + Ascorbic acid	1.36 ± 0.38^{a}	1.40 ± 0.58^{ab}	1.54 ± 0.11^{b}	0.70 ± 0.03^{b}	55.13 ± 2.30^{c}	37.89 ± 0.37^{b}	167.69 ± 4.82^{cd}	88.63 ± 9.36^{b}	30.05 ± 4.06^{b}	26.13 ± 0.08^{b}
Venom + α-Tocopherol	2.71 ± 1.39^{b}	1.85 ± 0.72^{b}	1.09 ± 0.33^{b}	0.93 ± 0.11^{b}	44.58 ± 2.60^{bc}	38.77 ± 4.10^{b}	120.72 ± 3.07^{bc}	116.55 ± 10.62^{bc}	21.91 ± 3.14^{b}	15.10 ± 1.02^{ab}
Venom + Antivenin	1.02 ± 0.39^{a}	1.43 ± 0.27^{ab}	0.72 ± 0.00^{ab}	0.43 ± 0.11^{a}	39.90 ± 4.77^{b}	37.61 ± 1.94^{b}	183.94 ± 22.04^{d}	113.24 ± 16.86^{bc}	11.48 ± 1.04^{a}	25.40 ± 2.01^{b}
Venom + Antivenin + Hexane fraction	1.37 ± 0.92^{a}	1.40 ± 0.29^{ab}	0.41 ± 0.07^{a}	0.72 ± 0.02^{b}	31.37 ± 0.92^{b}	47.69 ± 3.01^{c}	125.65 ± 6.94^{bc}	90.21 ± 5.01^{b}	43.37 ± 8.05^{c}	30.41 ± 6.13^{bc}
Venom + Antivenin + Ethyl acetate fraction	0.96 ± 0.21^{a}	1.22 ± 0.43^{a}	0.69 ± 0.04^{ab}	0.39 ± 0.06^{a}	65.35 ± 2.94^{d}	45.30 ± 1.96^{bc}	177.69 ± 8.22^{cd}	117.57 ± 13.11^{bc}	54.18 ± 10.04^{c}	34.42 ± 5.01^{c}
Venom + Antivenin + Ascorbic acid	1.58 ± 0.55^{ab}	1.05 ± 0.73^{a}	0.49 ± 0.10^{a}	0.35 ± 0.06^{a}	64.05 ± 3.77^{d}	62.00 ± 0.00^{d}	193.04 ± 12.82^{d}	143.62 ± 13.50^{c}	47.60 ± 3.21^{c}	68.42 ± 2.11^{d}
Venom + Antivenin + α-Tocopherol	0.99 ± 0.21^{a}	1.45 ± 0.44^{ab}	0.55 ± 0.08^{a}	0.23 ± 0.02^{a}	44.55 ± 4.02^{bc}	39.30 ± 2.04^{b}	159.48 ± 6.91^{c}	148.04 ± 8.33^{c}	35.18 ± 2.04^{bc}	23.84 ± 2.99^{b}

Results are presented as Mean ± SEM (n = 5), values carrying different superscript(s) (a, b, c, d, ab, bc or cd) from the normal control for each parameter (across a column) are significantly (P<0.05) different using ANOVA and Duncan multiple range test

research proved that, *N. nigricollis* venom increases the oxidative stress level due to the presence of the active enzymatic component, phospholipase A_2 (PLA2) that hydrolyses cellular phospholipids, resulting in the release of arachidonic acid. Therefore, oxidative metabolism of the arachidonic acid generated potentially toxic ROS leading to weakening of the activities of these natural antioxidant defense enzymes [60]. Hence the reason for the reduction in the activities of SOD, GPx and CAT in group administered with venom only.

The significant increase in the activities of these natural antioxidant enzymes in the envenomed rats when hexane and ethyl acetate fractions of the *A. indica* leaf were administered, suggests their possible free radical scavenging and antioxidant activity which may be as a result of the presence of phenolic compounds in the plant fractions [50].

5. CONCLUSION

This research has validated the antioxidant properties of *Azadirachta indica* leaf against *Naja nigricollis* venom induced oxidative effect. Additionally, the antioxidant enhancing effect of the plant extracts tested has been identified. The plant extracts have effectively enhanced the neutralization of the effects of the snake venom generated reactive oxygen species in the presence of serum-based antivenin, which is another advantage. It may be opined that the extracts having shown serum antivenin potentiating antioxidant action as seen in this study might be considered for further studies. It is now obvious that, combination of serum-based antivenin and herbal remedies may provide a suitable alternative for the treatment of snakebite envenomation in the near future. Hence, these findings would be of importance in the area of drug development with a view to maximizing the effectiveness of snakebite therapeutic options.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the Nigerian Tertiary Education Trust Fund (TETFund) through Institution-Based Research Grant intervention.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All animal experiments were conducted in accordance with the guidelines for the use and care of experimental animals, National Veterinary Research Institute, Vom, Nigeria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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