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INVIVO AND EXVIVO ANTIOXIDANT ACTIVITIES OF *PERSEA AMERICANA* EXTRACTS ON ALBINO WISTAR RATS

[1]Iloh E. S., [2]C. S. Nworu, [3*]M. A. Omoirri, [4]N. U. Madubogwu, [3]B. C. Ugwu

[1]Department of Pharmacology and Toxicology, Chukwuemaka Odumegu University Egbariam Anambra State, Nigeria.
[2]Department of Pharmacology and Toxicology University of Nigeria Nssuka Enugu State, Nigeria.
[3]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka Anambra State, Nigeria.
[4]Department of Pharmacology and Toxicology, Chukwuemaka Odumegu University Egbariam Anambra State Nigeria.

***Corresponding Author: M. A. Omoirri**

Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka Anambra State, Nigeria.

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ABSTRAC

For many years now, plants and plant products have been used in traditional medicine for treatment of infectious diseases. This study investigated the anti-oxidant activity of *Persia americana* extract under invivo and ex vivo conditions. The antioxidant activity of the leaf extract/fractions of *P. americana* was carried out in vivo using the 1, 1-diphenyl-2-picryl-hydraxyl (DPPH) assay. A total of sixty five (65) healthy adult Albino rats of either sexes, weighing between 200 - 250 g were procured and housed in the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra state, Nigeria. They were maintained under standard environmental conditions with free access to standard diet and water *ad libitum.* They were then grouped into 13 groups of five rats per group. For the aqueous extract unit, group 1 received 10ml/kg of distilled water, while group 2 received 50mg/kg of diclofenac. Groups 3, 4 and 5 received 50mg/kg, 150mg/kg and 400mg/kg body weights of crude extract respectively. Fractions extract unit received 10ml/kg of distilled water (Group 1, control) and 50mg/kg of diclofenac (Group 2). Groups 3 and 4 were then given 150mg/kg of ethyl acetate fraction and 400mg/kg of ethyl acetate fraction respectively, while groups 5, 6, 7 and 8 got 150mg/kg, 400mg/kg of n-hexane fraction and 150mg/kg of butanol fractions respectively. Phytochemical evaluation of the plant confirmed the presence of alkaloids, flavonoids, saponins, tannins, steroids, terpenoids, glycosides, carbohydrates, proteins, and coumarins. In the antioxidant assay, the crude extract and fractions of the *P. americana* leaves were administered to adult albino rats at variable doses. Various degrees of antioxidant activities at the concentration range of 31.25 -500 µg/mL were observed, with inhibition ranging from 4.2 – 41.8%. The crude extract showed best antioxidant activity compared with the fractions with IC_{50} = 587 µg/mL. Thus, the results of this study investigated the antioxidant properties of *P. americana* leaves extract, providing scientific information that validates the ethnobotanical use of *P. americana* leaves in disease management. Findings of this study also reveal that potentially *P. americana* could be a source of pharmacologically active compounds of pharmaceutical importance.

KEYWORDS: Persia Americana, Antioxidant, Phytochemicals, fractions.

INTRODUCTION

The use of medicinal plants remains significant as therapeutic remedies and still plays an important role in primary health care in developing countries such as Nigeria.[1,2] For many years, people depended exclusively on leaves, flowers and barks of plants for medicine. Although synthetic drugs have also come into use, in many instances, they are replicas or modifications of chemicals identified in plants.[3]

Plants, which constitute a major component of foodstuffs in humans, have formed the basis of various traditional medicine systems and folk medicines that have been practiced for thousands of years during the course of human history.[4,5] Until now, plants/herbs are still highly esteemed all over the world as a rich source of therapeutic agents for the treatment and prevention of diseases and ailments; at present, more than 35,000 plant species are used for medicinal purposes around the world.[6] In conventional Western medicine, 50–60% of pharmaceutical commodities contains natural products or are synthesized from them; 10–25% of all prescription drugs contain one or more ingredients derived from plants.[7]

Plants are presented as a promising source for the search of new substances, because they have a higher molecular diversity when compared to products synthetic chemically.[8, 9] There is 1–10% out of 250.000–500.000 plant species have been fully studied for their potential medicinal value.[10]

There is growing interest in correlating phytochemical constituents of plants with their pharmacological activity.[11] Flavonoids have been reported to possess antibacterial activity, in which it has the ability to form complex with extracellular soluble proteins and bacterial cell walls.[12] In the same manner, purified alkaloids, as well as their synthetic derivatives, are used for their various biological effects such as analgesic, antispasmodic and bactericidal as remedies for diseases.[13]

Investigations into the chemical and biological activities of plants during the past two centuries have yielded compounds for the development of modern synthetic organic chemistry and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents.[14, 15]

Many medicinal plant products are used in Africa for the management of pain and inflammation and their efficacy and potency are traditionally acclaimed.[16] Phytochemicals with various bioactivities, including antioxidant, anti-inflammatory and anticancer properties are extensively extracted from many plant species.[17]

Avocado (*Persea americana*), although a native of Central America (Mexico), is found in most tropical and subtropical countries of the world[18], including Nigeria. Avocado is now cultivated commercially as a fruit crop in many countries of the world. In many parts of Africa, the fruits of avocado are much sought after by human beings and some other animals as a valuable foodstuff. Besides the fixed oil, the fruit pulp contains carbohydrates and more protein than any other fruit, while its contents of vitamins A and B are high.

Apart from its use as food, the avocado is traditionally utilized for various medicinal purposes including as hypotensive, hypoglycemic and anti-viral, and is applied for the treatment of ulcers and cardiovascular diseases.[19] The avocados are also known to possess analgesic and anti-inflammatory properties.[20] Also, avocado pulp is also used in various dermatological formulations namely, emulsions for the treatment of dry skin, protective agents against ultraviolet radiation, and anti-aging agents. Given the variety of uses that are assigned to ethnobotanical species *Persea americana* several studies have been conducted in order to unveil their biological activity.
Persea americana has been reported to contain many bioactive chemical compounds, including: polyphenolics, tannins, coumarins, flavonoids, triterpenoids, phytosterols (especially, β-sitosterol), biotin, α-tocopherol, carotene, ascorbic acid, scopoletin, quercetin, oils, organic acids and inorganic substances such as calcium, magnesium, zinc, phosphorus, and so forth.[19] It is used in traditional medicine practice for treatment of injuries, wounds and other diseases that may have significant inflammatory aetiology with claim of success. However, there is paucity of scientific data to support or refute this practice. This is the major motivation for the study. Generally, herbal preparations are used in the treatment of several disease conditions particularly in resource-poor countries. The reasons for the increased popularity of these herbal medicines may include their relative cheapness compared to orthodox medicines, availability (since they are almost always derived from available plants in the locality), and time-trusted efficacy. Besides, orthodox medicines have been known to present adverse effects which affect compliance to therapy and total quality of life of the patient. It is therefore important to continue to explore plants and plant products for their potential medicinal uses.

To further determine the medicinal properties of *P. americana*, present study investigated the antioxidant activities of ethanol leaf extracts of *P. americana*.

AIM OF STUDY

This study evaluates the antioxidant properties of ethanol extract of the leaves of *Persia americana*. Specifically, study;

i. Examined the fractional constituents of *P. americana* leaves.
ii. Determined the antioxidant properties of ethanol leaf extract of *P. americana.*

MATERIALS AND METHODS

Test Animals

Healthy adult Albino Wistar rats of either sex weighing between 200 - 250 g were used for the experiments. The animals were obtained from the animal house of Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra state, Nigeria. They were maintained under standard environmental conditions with free access to standard diet and water *ad libitum*. All animal experiments were conducted in compliance with NIH Guide for Care and Use of Laboratory Animals (Pub. No. 85-23 Revised 1985).

Study Design

A total of 65 rats were used. They were grouped into 13 groups of five rats per group as follows;

Aqueous Extract

Group 1 received 10ml/kg of distilled water
Group 2 received 50mg/kg of diclofenac
Group 3 received 50mg/kg of crude extract
Group 4 received 150mg/kg of crude extract
Group 5 received 400mg/kg of crude extract

Fractions extract
Group 1 received 10ml/kg of distilled water
Group 2 received 50mg/kg of diclofenac
Group 3 received 150mg/kg of ethyl acetate fraction
Group 4 received 400mg/kg of ethyl acetate fraction
Group 5 received 150mg/kg of n-hexane fraction
Group 6 received 400mg/kg of n-hexane fraction
Group 7 received 150mg/kg of butanol fraction
Group 8 received 150mg/kg of butanol fraction

One (1) hour post-treatment, the animals received sub-plantar injection of 0.1 ml of fresh egg albumin in the right hind paw. The paw size was measured before (basal) and at 1, 2, 3, 4 and 5 hour(s) after egg albumin injection.

Sample Collection, Identification and Preparation
Fresh leaves of Avocado (*Persea americana*) were collected from Awka in the month of October, 2018. The identity of the plant was confirmed and authenticated by a plant expert at the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University, Awka, Anambra state, Nigeria and a voucher specimen deposited (N.a.u.h.n-183a). The leaves were air dried, and the dried leaves and seeds were then pulverized to fine powder.

Extraction of *Persea americana*
i. Crude Extraction
Extraction of the plant leaves was carried out as described by Onyegbule *et al.* (2014). The pulverized leaves were cold-macerated in ethanol 48 h and filtered. The filtrates were concentrated in a rotary evaporator at 40°C.

ii. Fractionation
Fractionation of the crude ethanolic leaf extracts of the plant was carried out as described by Onyegbule *et al.* (2014). The extracts were fractionated using the liquid-liquid fractionation method with ethyl acetate, *n*-hexane, and *n*-butanol. The filtrates obtained were concentrated in a rotary evaporator at 40°C.

Determination of Percentage Yield
The percentage yield for the leaf and seed extract and fractions was calculated and recorded.

$$\text{Percentage (\%) yield} = \frac{\text{Weight (g) of concentrated leaf or stem extract}}{\text{Weight (g) of powder leaves or stem}} \times \frac{100}{1}$$

Qualitative Phytochemical Analysis
Screening for presence of secondary metabolites (flavonoids, alkaloids, reducing sugars, tannins and saponins, etc. in *P. americana* leaves was performed following standard phytochemical tests. The test was carried out according to the procedures outlined by Trease and Evans (2002).[21] Ten percent (10%) preparation of the extract in distilled water was considered as the test samples. Distilled water was used as a negative control throughout the phytochemical tests.

Determination of Oxidative Stress Activity
Thiobarbituric Acid (TBARS) Assay
TBARS assay is a well-recognized, established method for quantifying these lipid peroxides. Lipid peroxidation generates peroxide intermediates which upon cleavage releases malonyldialdehyde, a product which reacts with thiobarbituric acid (Gwarzo *et al.,* 2014). The activity of TBARS was determined by the methods of Feldman (2004)

Assay of Superoxide Dismutase (SOD) Activity
The activity of superoxide dismutase (SOD) was determined using serum from the experimental rats. The activity of superoxide dismutase was assessed using the NWLSS, superoxide activity assay which will provide a simple rate method for the determination. The method was based on monitoring the auto-oxidation rate of haematoxylin as described by Martin *et al,* (1987). Sample SOD activity was determined by measuring the ratio of auto-oxidation rate in the presence and absence of the sample and expressed as traditional ‘McCord-Fridovich.

Assay of Catalase (CAT) Activity
The catalase activity of the hemolysate was determined by adopting the method of Brannan *et al.* The assay is based on the disappearance of H_20_2 in the presence of the enzyme source at 26 degree celciou In brief; the hemolysate was prepared from lysed RBC suspension, further diluted by phosphate buffer [pH 7.0]. Here the reaction mixture containing 0.05 M phosphate buffer [pH 7.0] 1.2 mM H_20_2 and 0.2 ml of diluted hemolysate was allowed to stand for 25 minutes. At the end of which reaction was stopped by the addition of 2.5 ml of peroxidase reagent containing peroxidase and the chromogen system. Peroxidase reduced the H_20_2 to give a red coloured compound and absorbance measured at 505 nm. With each assay a suitable blank which contained no H20 and a control which contained 1 ml sodium azide, a catalase inhibitor, were included.

In Vitro Antioxidant Assays
(1, 1-diphenyl-2-picryl-hydraxyl (DPPH) Free Radical Assay)
The antioxidant activity of the leaf extract/fractions of *P. americana* was carried out using the DPPH assay as described by Shen *et al.,* (2010).[22] The percentage inhibition of the samples was determined at concentrations of 31.25 - 500 µg/mL. An equal volume of DPPH solution was added to the samples dissolved in methanol. Ascorbic acid was used as the positive control and solution of DPPH in methanol (0.1 mM) was used as blank. The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was then measured at 517 nm using a UV-VIS

spectrophotometer. The DPPH free radical scavenging effect of the samples was calculated using the following formula;

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Abs of blank } (A_0) - \text{Absorbance of sample } (A_1)}{\text{Abs of blank } (A_0)} \times \frac{100}{1}$$

The inhibitory concentration (IC50) of the plant extracts/fractions needed to inhibit 50% of the DPPH radicals obtained from the standard curve was compared to that of standard (ascorbic acid).

Statistical Analysis

Results were expressed as mean ± SEM. Student's *T-test* was used to analyse the significance of the results. The difference between mean was considered significant at $p<0.05$.

RESULTS

The crude ethanol extract of *P. americana* derived from the cold maceration of the powdered dried plant material was fractionated into ethyl acetate, *n*-hexane, and butanol fractions. The yield from the extraction and fractionation of is *P. americana* shown in Table 1.

Table 1: Percentage Yield of Aqueous Extract and fractions of *P. americana* Leave.

Extract/Fraction	% Yield
Ethanol crude	45%, 219g
Ethyl acetate fraction	10%, 29g
N-hexane fraction	11% 31g
Butanol fraction	13.5%, 35g

Table 2: Results of Phytochemical Analysis

Qualitative phytochemical analysis of *P. americana* leaves was conducted using standard methods. Results are presented in Table 2.

It can be observed from the table, that alkaloids, flavonoids, saponins, tannins, steroids, terpenoids, glycosides, carbohydrates, proteins, and coumarins were detected in the plant leaves; while carotenoids and anthraquinones were absent.

Components	Aqueous extract	n-hexane fraction	Ethyl acetate fr.	Butanol fraction	Water fraction
Saponins	+ + +	+ + +	+++	++	++
Tannins	+++	++	+++	++	+
Carbohydrates	++	+	++	+	+
Reducing Sugars	+	+	+	+	+
Flavonoids	+++	+++	+++	++	+
Alkaloids	++	++	+	++	-
Glycosides	++	++	++	+	+
Steroids	+	+	+	+	+
Fats and oils	++	+	+	+	-
Proteins	++	+	-	-	+
Coumarins	++	-	++	+	-
Terpenoids	+	+	+	+	+

(-) => Not Present, (+) => Present in small concentration, (++) => Present in moderately high concentration, (+++) => Present in high concentration.

The antioxidant activities of the ethanol and crude (aqueous) extracts, and ethyl acetate, *n*-hexane, and butanol fractions of *P. americana* leaves were determined using the DPPH assay method.

From the result, the crude extract and fractions of the plant leaves displayed various degrees of antioxidant activities at the concentration range of 31.25 -500 µg/mL with inhibition ranging from 4.2 – 41.8%. The positive control (ascorbic acid) displayed higher antioxidant activity at same concentrations with inhibition ranging from 50.6 – 65.8% (Table 3).

The IC_{50} of the plant extract and fractions were also calculated and the result is presented in Figure 6. The crude extract (IC_{50} = 587 µg/mL) showed best antioxidant activity compared with the fractions (IC_{50} = ≥1006.3 µg/mL).

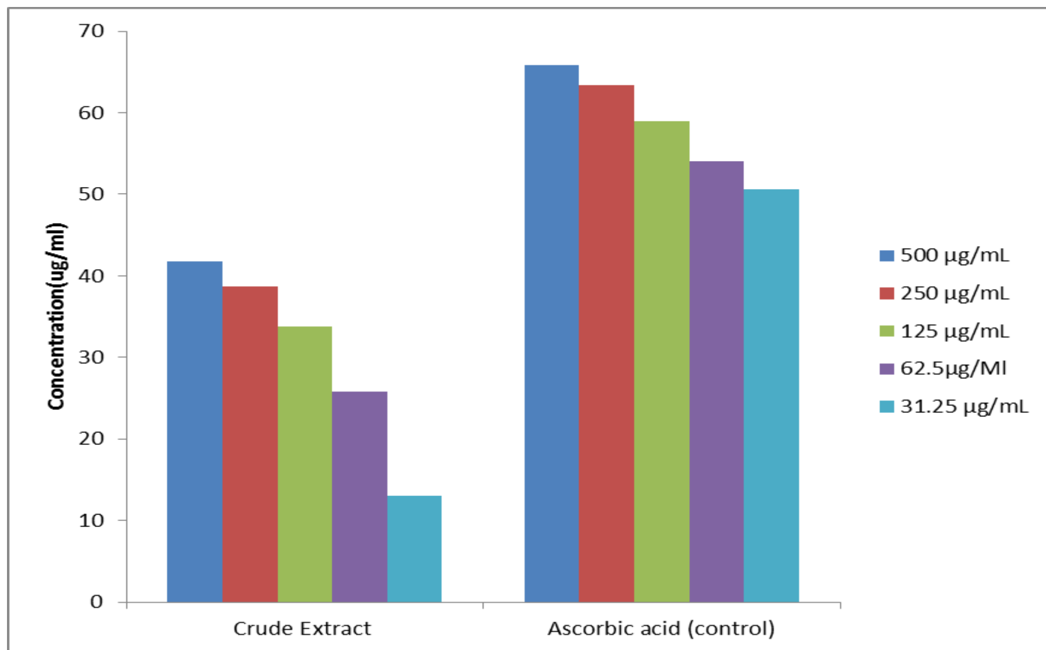

Figure I: DPPH Antioxidant Activities of Crude Extract of *P. americana* Leaves showing IC_{50} (µg/mL).

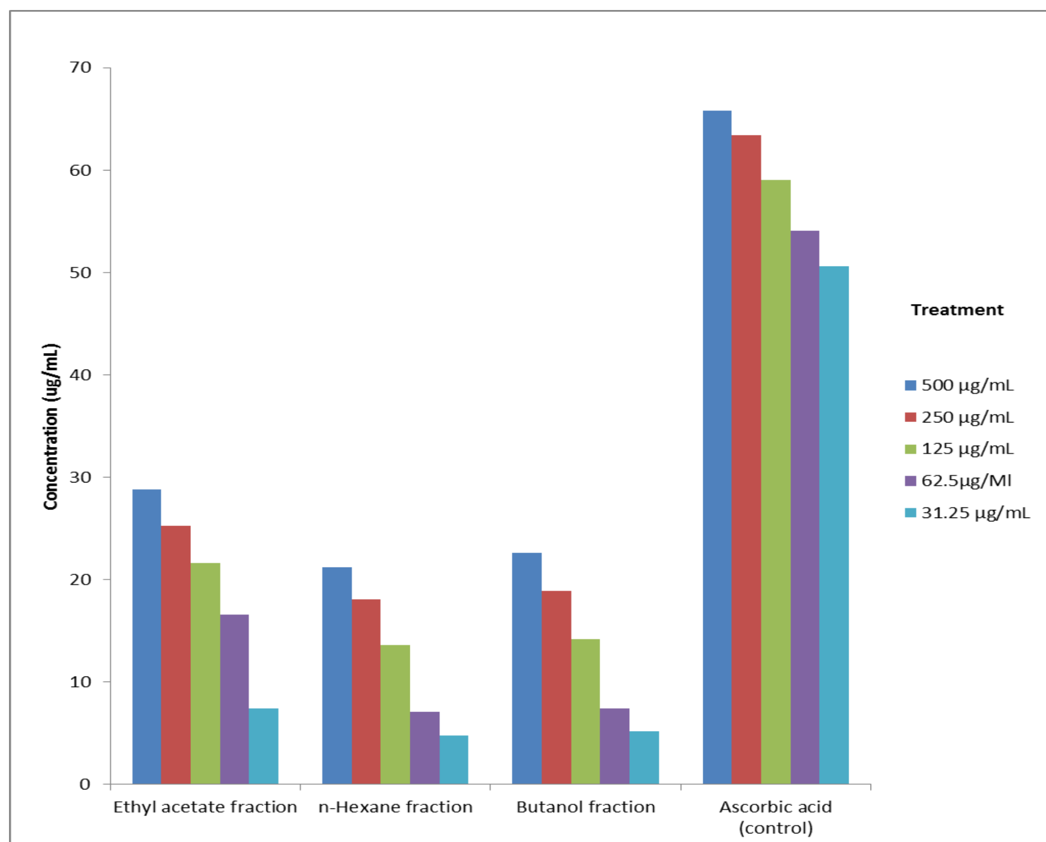

Figure 2: DPPH Antioxidant Activities of Fractions of *P. americana* Leaves showing IC_{50} (µg/mL).

Antioxidant Marker Activities

Figure 3 and 4 represent the effect of administration of the mean invivo Antioxidant activities of crude extract and fraction of *P. Americana* on oxidative stress (MDA, SOD and CAT) parameters. After treatment with crude extract and fraction of *P. Americana* there was significant ($p < 0.05$) increase in SOD, CAT while MDA significantly ($p < 0.05$) reduce in the treated groups when compared to control. In both crude and fractions increase in SOD and CAT was found in the highest doses while reduction in MDA was also seen in the highest dose. Therefore it can be said that the results are dose dependent.

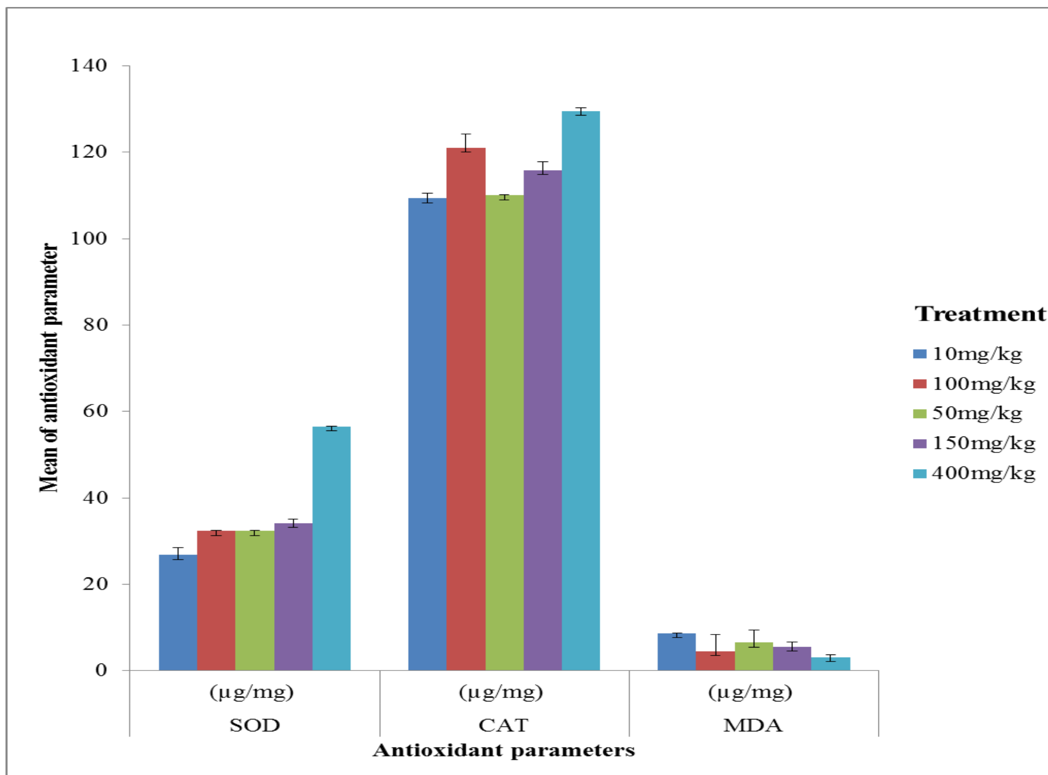

Figure 3: Invivo Antioxidant activities of crude extract of *P. Americana*.

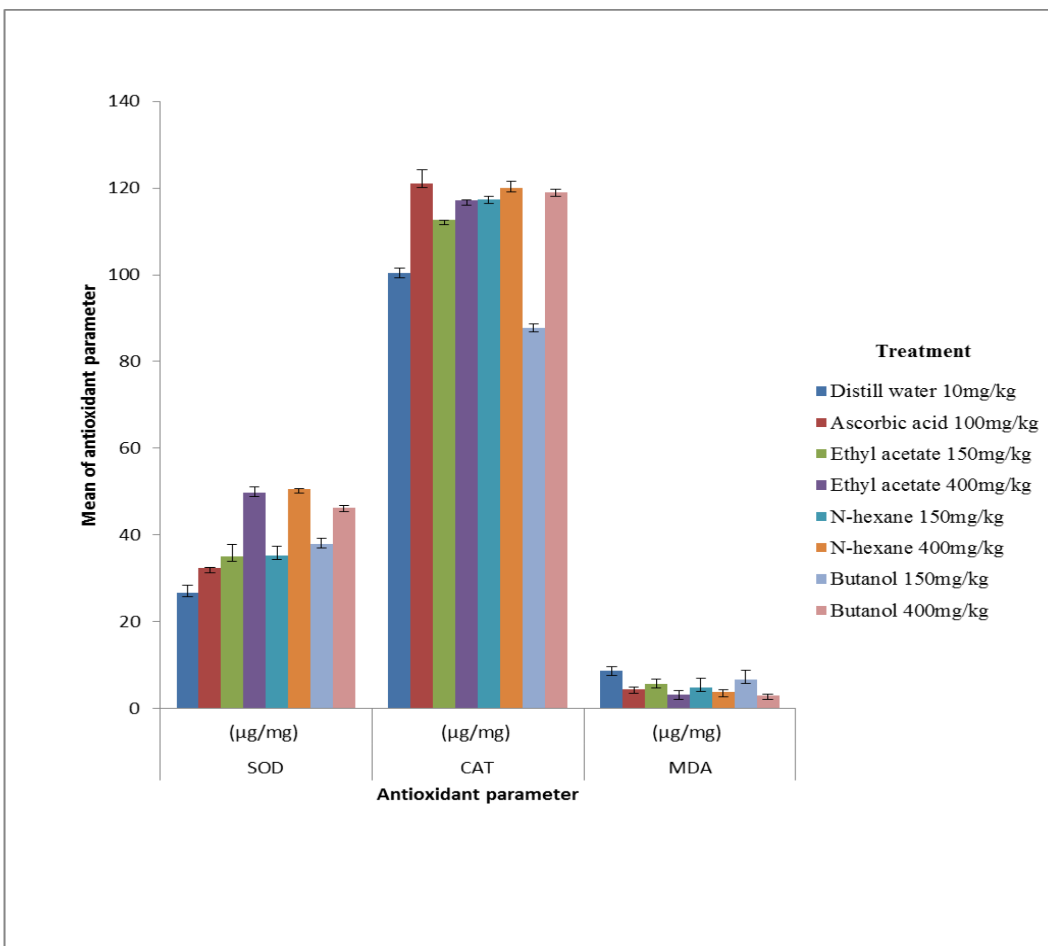

Figure 4: Invivo Antioxidant activities of fraction extract of *P. Americana*.

DISCUSSION

Often time, *P. americana* is reportedly enjoyed as fruit with highly medicinal properties. It is widely accepted in traditional medicines of many African countries and other parts of the world for the management of several disease conditions such as amenorrhoea, anaemia, insomnia, hyperlipidaemia, hypertension, diabetes mellitus, diarrhoea, dysentery, gastritis, peptic ulcers, bronchitis, cough, hepatitis, etc.[23]

In view of this, current study attempted to scientifically confirm and/or validate the entho-medicinal application of *P. Americana*. The study was examined various extracts of the plant for its antioxidant pharmacological properties.

Available reports posit that *Persea americana* contains many bioactive chemical compounds, including: polyphenolics, tannins, coumarins, flavonoids, triterpenoids, phytosterols (especially, β-sitosterol), biotin, α-tocopherol, carotene, ascorbic acid, scopoletin, quercetin, oils, organic acids and inorganic substances such as calcium, magnesium, zinc, phosphorus, etc.[16] This was confirmed by the results of current study, confirming the presence of some of such components as alkaloids, flavonoids, saponins, tannins, steroids, terpenoids, glycosides, carbohydrates, proteins, and coumarins (Table 1).

Theoretically, Antioxidant compounds are known to play a key role in detaining inflammation with the mechanism of capturing free radicals and detaining cyclooxygenase enzyme to hold up the occurrence of prostaglandin. This will impact in the inhibition of inflammatory mediators.[24] From this study, the crude extract and fractions of the *P. americana* leaves displayed various degrees of antioxidant activities at the concentration range of 31.25 -500 µg/mL with inhibition ranging from 4.2 – 41.8% (Table 3). The crude extract showed best antioxidant activity compared with the fractions with an IC_{50} = 587 µg/mL (Figure 3 and 4).

Antioxidants act by scavenging and interacting with free radicals, neutralising them as such to prevent them from causing cellular damage to biological systems.[14] The body makes some of the antioxidants it uses to neutralize free radicals. These antioxidants are called endogenous antioxidants. However, the body also relies on external (exogenous) sources, primarily diets to obtain the rest of the antioxidants it needs.[16] These exogenous antioxidants are commonly called dietary antioxidants. Fruits, vegetables and grains are rich sources of dietary antioxidants.[25] From this study, the in vivo antioxidant assay showed that *P. americana* extract increased the activity of serum superoxide dismutase (SOD) and catalase, and decreased the serum level of TBARS.

Catalase is a ubiquitous enzyme that catalyses the decomposition of hydrogen peroxide, a reactive oxygen species, which is a toxic product of both normal aerobic metabolism and pathogenic ROS production.[5] The SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen, thereby reducing the likelihood of superoxide anion reacting with nitric oxide to form reactive peroxynitrite.[13] The increased serum activities of catalase and SOD as observed in this study suggest that the extract has an in vivo antioxidant activity and is capable of ameliorating the effect of ROS in biologic system. Also, ROS react with all biological substance; however, the most susceptible ones are polyunsaturated fatty acids. Reactions with these cell membrane constituents lead to lipid peroxidation (LPO). Increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptor. Thiobarbituric acid reactive substance (TBARS) levels were measured as a marker of LPO and malondialdehyde (MDA) production. Malondialdehyde is an endogenous genotoxic product of enzymatic and ROS-induced LPO whose adducts are known to exist in DNA isolated from healthy human being.[18]

In this study, the level of TBARS in the extract treated groups decreased in a dose dependent manner when compared to control. This decrease in the TBARS levels may indicate increase in the activities of glutathione peroxidase and hence inactivation of LPO reactions.[11] Some of the phytochemical constituents of the extract may be responsible for the antioxidant activity as demonstrated in our study. Flavonoids or bioflavonoids and saponins are a ubiquitous group of polyphenolic substances which are present in most plants, concentrated in seeds, fruit skin or peel, bark, and flowers. Numerous studies have shown that flavonoids, saponins and tannis possess potent antioxidant activities capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals attributed the pharmacological activities (anti-inflammatory, antiviral, antibacterial, antiulcer, antiosteoporotic, antiallergic, and anti-hepatotoxic actions) of flavonoids to their potent antioxidant activity.[13]

CONCLUSION

This study observed the leaf extract and fractions of *Persia americana* for antioxidant activity in the blood. Plant leaves were first obtained, pulverized, and subjected to qualitative screening of phytochemical constituents. Next, crude extract of the plant leaves was fractionated using ethyl acetate, n-hexane and butanol. The crude extract and fractions of the plant leaves were administered to albino rats at variable concentration. This then necessitated the evaluation of *P. americana* for antioxidant potency using standard methods. Results of the qualitative phytochemical evaluation of the plant confirmed the presence of phytoconstituents such as alkaloids, flavonoids, saponins, tannins, steroids, terpenoids, glycosides, carbohydrates, proteins, and coumarins (Table I). The crude extract and fractions of *P. americana* leaves displayed various degrees of antioxidant properties, confirming and validating their

supposed ethnobotanical use in disease management; plus their potency as a source of pharmacologically active compounds of pharmaceutical importance.

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