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Article

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Abstract: Purpose: Oxidative stress is responsible for over seventy five diseases and other harmful cytological challenges including cancer, type 2 diabetes mellitus, lipid peroxidation, distortion of cell membrane integrity, shortening of telomerase among others. Antioxidant potency of any plant is a very positive remedy to the debilitating effects of prooxidants and oxidants. The study was aimed at investigating the antioxidant potency of methanol extract of *Pentaclethra macrophylla* stem bark- *in vitro* and *in vivo*. **Methods:** Different phytochemicals were determined using standard methods. Nitric oxide, 1, 1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity, total antioxidant capacity and ferric reducing/anti-radical power were used to investigate the *in vitro* antioxidant activity. Catalase assay, superoxide dismutase, malondialdehyde and glutathione peroxidase were used to determine the *in vivo* antioxidant efficacy using six groups of five rats. **Results:** The result showed various quantities of phytochemicals, including antioxidant phytochemicals-flavonoids, tannins and total phenolics. The *in vitro* assay showed that 250 µg/ml of extract had the highest percentage inhibition of DPPH radical (93.93%). The 125 µg/ml had the highest scavenging activity of nitric oxide radical (49.47%) while 250µg/ml showed the least with 41.73%. Other methods showed various degrees of activity. The *in vivo* antioxidant showed that the principal mechanism of antioxidant activity is by modulating the iron-dependent catalase activity. **Conclusion:** *Pentaclethra macrophylla* has both *in vitro* and *in vivo* antioxidant activities and therefore is useful in ameliorating the debilitating effects of oxidants.

Keywords: *Pentaclethera macrophylla* stem bark; methanol extract; bergenin; acute toxicity; antioxidant; DPPH

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1. Introduction

Oxygen plays a significant role in living things in a sequence of oxidation-reduction and enzymatic processes. It may also transmit electrons from one atom to another and is a vital component of mammalian metabolism and aerobic life as it is the system's primary electron acceptor in the electron transport chain that results in the production of ATP [1]. However, when the electron flow decouples (transfer of unpaired single electrons), resulting in the production of free radicals, issues may occur. Free radicals are highly unstable atoms, molecules, or ions with unpaired electrons that are eager to interact chemically with other molecules. Three elements—oxygen, nitrogen, and sulfur—are their sources. Reactive oxygen species (ROS), which include superoxide ($O^{2-\cdot}$), hydroxyl ($OH\cdot$), peroxyl (ROO), alkoxyl (RO), and nitric oxide ($NO\cdot$), are examples of oxygen-centered free radicals. The molecules in neighboring cells are swiftly attacked by the highly reactive free radicals.

Living creatures are able to maintain a balance between oxidative stress and antioxidant defense because of their systems of antioxidant defense. The maintenance of cell homeostasis depends on the production of these ROS [2,3]. Living creatures produce ROS as part of their normal cellular metabolism, which can be hazardous to some macromolecules such as lipids, carbohydrates, nucleic acids, and proteins. Living organisms constantly contain ROS, which are created as waste products of metabolism, regular respiration, the autoxidation of xenobiotics, or as a result of stress caused by a range of illnesses [4]. An imbalance between ROS and antioxidant defenses leads to oxidative stress. This oxidative stress disrupts a number of cellular processes and causes a variety of pathological conditions where the body's antioxidative defenses are overwhelmed by ROS, resulting in tissue damage, accelerated cell death, and oxidative modification of biological macromolecules as the basis of many diseases [5].

Antioxidants are essential for reducing oxidative processes and the negative effects of ROS in both food systems and the human body [6]. Antioxidants aid in decreasing amino acids, protein oxidation, and the interaction of carbonyls produced from lipids with proteins that alters protein function [7]. Free radical scavengers include phenolic compounds, ligands, flavonoids, and phenolic acids. Generally, antioxidants carry out these functions primarily through two mechanisms: electron transfer and the inactivation of free radicals [8]. Those oxidants known as natural antioxidants can be found in foods such as fruits, vegetables, and meats. Phenolic acids, Vitamin C (ascorbic acid), Vitamin E (tocopherols), Vitamin A (carotenoids), various polyphenols, including flavonoids and Anthocyanins (a type of flavonoid), Lycopene (a type of carotenoid), and Coenzyme Q10, also known as Ubiquitin, which is a type of protein, are the most prevalent natural antioxidants found in everyday foods [9]. All sections of plants contain natural antioxidants. These anti-oxidant substances operate as reductants, scavenge free radicals, and prevent Fe^{+3}-induced oxidation [10].

The African oil bean tree, *Pentaclethra macrophylla* Benth (Leguminosae, subfamily Mimosoidae), is a large leguminous, nodule-forming multipurpose tree species occurring naturally in the humid lowlands and some parts of the sub-humid zones of West and Central Africa [11]. They have a self-supporting growth form. They have compound broad leaves. Individuals can grow to 3.5 m. The edible part of the plant is the seed which can be consumed as roasted snack, or as fermented condiment in soups and porridges [11,12]. It contains appreciable amounts of thermo-stable amylases and lipases [13]. The seeds also have high phytase activity to take care of the high phytate content that decreases divalent minerals' absorption [14].

A number of pharmacological activities of *Pentaclethra macrophylla* have been reported in literature. These pharmacological activities include: antioxidant activity, anti-diabetic activity, anticancer activity, anti-hyperuricemia activity, anticonvulsant activity and cholesterol-lowering effect [15–18]. *In vitro* antioxidant and radical scavenging activities of the methanol extracts of the stem and seed of *Pentaclethra macrophylla* were evaluated and confirmed by a few studies [15,16]. Methods employed included ABTS (2,2′-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activities as well as ferric-reducing antioxidant power, FRAP assays among others. Also, the stabilization of lipid oxidation by the extract was analyzed using fish oil emulsion model. It was discovered that the sample reduced oxidation of polyunsaturated fatty acid in fish oil. Furthermore, the plant showed a decrease in serum total

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bilirubin and hepatic malondialdehyde levels in treated groups' level while glutathione-S-transferase activity was significantly increased in the liver. The hypoglycemic effects of *Pentaclethra macrophylla* leaf (50% and 100%) extracts on the blood glucose level of alloxan-induced diabetic albino rats was reported by [17]. It showed significant hypoglycemic effect. The effect of aqueous leaf extracts of *Pentaclethra macrophylla* on 2-aminoanthracene (2-AA) and 4-nitroquinoline-N-oxide (4-NQO)-induced mutation and oxidative damage was evaluated [19]. The leaf extracts caused concentration-dependent inhibition of the mutagenicity of 2-AA (an indirect mutagen) and 4-NQO (a direct mutagen) toward *Salmonella typhimurium* TA 98 and TA 100. Cytotoxic activity of *Pentaclethra macrophylla* stem extracts on colorectal cancer cells (HCT-116, HT-29) and its effect on the expression of early apoptotic genes, c-fos and c-jun have also been investigated [18]. The extract significantly up-regulated the expression of c-fos and c-jun leading to a cytotoxic effect on colorectal cancer cells.

Hyperuricemia can lead to several diseases [20]. The anti-hyperuricemia activity of *Pentaclethra macrophylla* fruit extract in xanthine oxidase and monosodium urate (MSU)-treated RAW264.7 macrophages has been reported [16]. Butanol extract also inhibited xanthine oxidase activity in monosodium urate (MSU)-treated RAW264.7 macrophages [16]. Jeremiah *et al.* [21] evaluated the anticonvulsant potential of *Pentaclethra macrophylla* seed aqueous fraction in mice subjected to pentylene tetrazole (PTZ)-, strychnine- and maximal electroshock (MES)-induced seizures. There was 33.33% defense against transience in PTZ- and strychnine-induced convulsion with significant reduction in the time of recovery from MES-induced seizure in animals' pre-administered *Pentaclethra macrophylla* seed. According to the findings, specific phytochemicals in *Pentaclethra macrophylla* seed extract possess anticonvulsant activity and the inhibitory neurotransmission was due to mild affinity for PTX binding site of the GABA receptor. Cholesterol-lowering activity of *Pentaclethra macrophylla* was evaluated by Huang et al., [22]. They administered 2% of ethanolic and water extract of *Pentaclethra macrophylla* seed, leaves and dry pulp. Experimental diets containing the seed extract of *Pentaclethra macrophylla* reduced the plasma total cholesterol while lupeol acetate and β-amyrin acetate (triterpenoids isolated from the seed extract) decrease plasma total cholesterol by 15%–20% in hamsters. It was concluded that the ethanol extract of the seed decreased plasma total cholesterol which could be associated with active triterpenoids in the extract. This study was aimed at investigating the antioxidant potency of methanol extract of *Pentaclethra macrophylla* stem bark- *in vitro* and *in vivo* with the view to ascertaining its contribution in mitigating the deleterious effects of oxidants especially in biological systems. This is most important because Nigerian people liberally consume the water infusion of the stem bark extract for many biological activities. That is why we tried to isolate the ingredient (Bergenin) and study the stem bark extract to know what antioxidant effect it has.

2. Materials and Methods

2.1. Quantitative Phytochemical Determinations

2.1.1. Determination of Total Phenolic Compounds:

The method described by Harborne. [23] was used to estimate the amount of total phenolics present in the extract. A quantity of 0.2 g of the extract sample was weighed and dissolved in 45% ethanol. 100 microlitre this solution was transferred to triplicate test tubes, then 0.5 ml of Folin-Ciocalteu reagent and 1.5 ml 2 % of Na_2CO_3 solution was added and finally the blend was incubated in the dark at room temperature for 15 mins after which the absorbance was taken at 765 nm. The total phenolic composition was calculated using a standard calibration curve obtained from various diluted concentrations of gallic acid.

2.1.2. Determination of Concentration of Alkaloids

The method described by Harborne, [23] was used to determine the amount of alkaloids present in the extract. A quantity 0.2 g of the sample was weighed into a container and 2 ml of 20 % H_2SO_4 and 2 ml absolute ethanol were added and mixed with 1ml of 60 % H_2SO_4. Five mins later 1 ml of 0.5

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% formaldehyde in 60 % H_2SO_4 was added and the solution was allowed to stand for 3 h. The absorbance of the sample was taken at 565 nm.

2.1.3. Determination of Total Tannins

The method described by Harborne, [23] was used to determine the amount of total tannins present in the extract. A quantity 0.2 g of the sample was weighed into a sample container. Aqueous ethanol was used to macerate the sample. 0.5 ml of the supernatant was transferred to triplicate tubes. 0.5 ml Folin-Ciocalteau's reagent was added. Five minutes later, 0.5 ml of 2 % Na_2CO_3 was added followed by the addition of 4 ml distilled water. The solution was incubated for 30 minutes, centrifuged for 10 minutes at 3000 rpm and the supernatant was read at 725 nm.

2.1.4. Determination of Concentration of Flavonoids

Flavonoids content of *Pentaclethra macrophylla* was determined by the aluminum chloride colorimetric assay. A 0.2 g of sample was weighed and macerated with 6 ml ethyl acetate. The supernatant was shaken with 6 ml aqueous ammonia for five minutes. The supernatant was discarded and the infra-natant was read at 490 nm. The flavonoids contents were determined using standard curve generated from quercetin.

2.1.5. Determination of Concentrations of Steroids

The Steroids contents of the sample was determined. A quantity 0.2 g of the sample was weighed and macerated with 5 ml ethanol. The supernatant (0.8 ml) was mixed with 0.8 ml of chromogen and 0.3 ml concentrated H_2SO_4 and allowed to stand for 30 mins. The absorbance was taken at 550 nm.

2.2. Acute Toxicity Studies (LD_{50}) (Lorke's Method)

The acute toxicity test of the extract was estimated in mice using the method of Lorke, [24]. Briefly, the tests involved two phases. The first phase was determination of the toxic range. The mice were placed in three groups (n = 3) and respectively were given 10, 100 and 1000 mg/kg; p.o. solubilized in a solution of 3 %, v/v Tween 80. The treated mice were observed for 24 h for number of deaths. The death pattern in the first phase determined the doses used for the second phase. Since there was no deaths recorded in the first phase, a fresh batch of three mice received respectively 1600, 2900, and 5000 mg/kg of the extract orally. The treated animals were observed for lethality or signs of acute intoxication for 24 h.

2.3. Identification of Bergenin

2.3.1. Bergenin Isolation by Preparative HPLC

The isolation of bergenin from plant extract was performed by a DIONEX UltiMate 3000 UHPLC+ focused (Thermo Scientific), containing pump, diode array detector, and automated fraction collector. For this analysis a VP 250/10 NUCLEODUR C18 Gravity, 5 µm (Macherey-Nagel) column was used. Briefly, the sample of extract (25 mg) was dissolved in methanol and injected into the system followed by the elution with a gradient from 5% to 50% solvent B over 30 min (solvent A: H_2O (0.05% formic acid), solvent B: ACN (0.05% formic acid)) at a flow rate 10 mL/min. Pure fractions were checked by LC-MS, combined and lyophilized. The obtained information was compared with the available data [45,46].

2.3.2. NMR Measurement

The extracted bergenin was characterized by ^{1}H NMR, ^{13}C NMR and ^{13}C DEPT-135 spectra recorded on Bruker Avance III 600 MHz spectrometer with the use of methanol-d_4 as a solvent. The chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane, with *J* values in Hertz. The splitting patterns in ^{1}H NMR spectra are reported as follows: s = singlet; d = doublet; t = triplet; dd = doublet of doublet; bs = broad singlet; m = multiplet.

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2.3.3. Analytical LC-MS Analysis

For analytical LC-MS, the extracted bergenin was dissolved in methanol. The measurements were conducted with a DIONEX UltiMate 3000 UHPLC+ focused (Thermo Scientific), containing RS Pump, RS Autosampler, Diode Array Detector, Column Compartment (heated to 40 °C) and ISQ EC Mass Spectrometer. Hypersil GOLD C18 Column 100 x 2.1mm 1.9UM column (Thermo Scientific) was used for analysis. The gradient was 5-100 % solvent B over 5 min (solvent A: H_2O containing 0.05 % formic acid, solvent B: ACN containing 0.05 % formic acid) with a flow rate of 0.6 mL/min.

2.3.4. Infrared Spectroscopy:

A few crystals of the off-white powder (biomarker) was placed on the top plate and fastened to a PerkinElmer Attenuated Total Reflectance (ATR) Fourier Transform (FT) IR spectrometer equipped with diamond crystal surface. The IR spectrum was obtained over the wavenumber range of 4000 to 650 cm-1.

2.4. In Vitro Antioxidant Assay

2.4.1. DPPH (1, 1-diphenyl-2-picrylhydrazyl) Radical Scavenging Assay

In the DPPH radical scavenging assay, antioxidants reduce the free radical 1, 1-diphenyl-2-picrylhydrazyl radical, which has an absorption maximum between 515 nm and 520 nm. The free radical scavenging activity (RSA) of extract was tested using a 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) technique. The radical solution was prepared by dissolving 2.4 mg DPPH in 100 ml methanol to make a stock solution. Filtration of DPPH stock solution using methanol yielded a usable mixture with an absorbance of around 0.973 at 517 nm. In a test tube, 0.5 ml DPPH workable solutions were combined with 1000 µL of the extract. Three milliliters of solution containing DPPH in methanol only was used as a control. After that, the tubes were kept in complete darkness for 30 min. The absorbance was therefore determined at 517 nm. The following formula was used to compute the percentage of antioxidants or RSA

$$\% \text{ of antioxidant activity} = \frac{(Ac - As)}{Ac} \; x \; 100 \ldots\ldots\ldots\ldots (1)$$

Where Ac is Control reaction absorbance and As is Testing specimen absorbance.

A curve of % inhibition (antioxidant activity) against sample concentration was plotted and the concentration of the sample required for 50 % inhibition was determined.

2.4.2. Nitric Oxide ($NO\cdot$) Radical Scavenging Activity:

To 50 µl of the experimental sample, 1500 µl of sodium nitropruside in phosphate buffered saline (PBS) was added. After 150 mins of incubation at room temperature exposed to light, 250 µl of the NED (N-(1- Naphthyl) ethylenediamine) solution (0.1% NED in ethanol) were also added. The resulting solution was well mixed and then incubated at room temperature for 30 mins exposed to light. The absorbance of the colored azo compound formed within 30 mins was measured at 546 nm. The Percentage Inhibition of the extract and standard IC_{50}, which is an inhibitory concentration of each extract required to reduce 50% of nitric oxide formation, was determined.

The nitric oxide radicals scavenging activity was calculated using the equation

$$\%\text{Inhibition of Nitric oxide activity} = \frac{(A_O - A_1)}{A_O} \times 100 \ldots\ldots\ldots\ldots (2)$$

Where A_0 is the absorbance value of the blank sample or control reaction and A_1 is the absorbance value of the test sample. Ascorbic acid was used as standard.

2.4.3. Total Antioxidant Capacity (TAC) Assay:

The total antioxidant capacity of the methanol extract was determined by phosphomolybdate method using ascorbic acid as a standard. The stock solution (1mg/ml) of extract was diluted to 6 lower concentrations of 15.63, 31.25, 62.5, 125, 250, and 500 µg/ml respectively. An aliquot of 0.15ml of sample solution was mixed with 1.5 ml of reagent solution (0.6 M sulphuric acid, 28mM sodium

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phosphate and 4mM ammonium molybdate). The sample tubes were capped and incubated in a water bath at 95^{0}C for 90 mins. After the sample has been cooled to room temperature, absorbance of the mixture was measured at 765nm against a blank on a spectrophotometer. A typical blank contained 1ml of the reagent solution along with appropriate volume of the solvent and incubated under similar conditions. The antioxidant capacity of extract was estimated using the formula:

$$\text{TAC (\%)} = \frac{Control_{Abs} - Sample_{Abs}}{Control_{Abs}} \; x\; 100 \quad \ldots\ldots\ldots (3)$$

2.4.4. FRAP (Ferric Reducing/Antioxidant Power) Assay:

The FRAP assay "Ferric reducing/antioxidant power as described by Benzie and Strain [25] was used with minor modification. Briefly, the antioxidative activity of the sample and standard was estimated by using the increase in absorbance caused by the generated ferrous ions. 250 microliter of phosphate buffer (pH 6.6) was mixed with 250 µl of different concentrations of sample (15.63, 31.25, 62.5, 125, 250, and 500 µg/ml). Thereafter, 250 µl of 0.1 % potassium ferricyanide was added and the mixture incubated at 50 ˚C for 20 mins. 250 µl of 10 % TCA (trichloro acetate) was used to stop the conversion. 1 ml of water was added to each tube and 200 µl of 0.1% ferric chloride was added. The reduction kinetics was determined after 10 mins at 700 nm.

2.5. In Vivo Antioxidant Activity:

2.5.1. Experimental Design

The *in-vivo* activity was carried out using rats, grouped from I to VI. Group I represents Normal Control (No induction, no treatment), Group II induction only using 2000 mg/kg body weight paracetamol, Group III induction (2000 mg/kg body weight paracetamol) + standard drug (ascorbic acid) (20 mg/kg body weight), Group IV induction (2000 mg/kg body weight paracetamol) + extract (100 mg/kg body weight), Group V induction (2000 mg/kg body weight paracetamol) + extract (200 mg/kg body weight), Group VI induction (2000 mg/kg body weight paracetamol) + extract (400 mg/kg body weight). The animal experimental protocols were in accordance with the guidelines for conducting animal experiments stipulated by our Institution's Animal Ethics Committee and in compliance with the Federation of European Laboratory Animal Science Association and the European Community Council Directive of November 24, 1986 (86/609/EEC). Briefly, the rats were divided into groups and acclimatized to the laboratory environment a week before the study.

2.5.2. Catalase Assay

Catalase was assayed using the method of Aebi [26]. The ultra-violet absorption of hydrogen peroxide (H_2O_2) can easily be measured at 240 nm. On the decomposition of hydrogen peroxide (H_2O_2) with catalase the absorption decreases with time and from this decrease, catalase activity can be measured. The reagent used is made up of phosphate buffer (pH 7.0), H_2O_2, (0.2 M) Potassium dichromate (5 %) and 5 % glacial acetic acid. The Phosphate buffer (2.5 ml) was pipetted into a test tube. This was followed by addition of 2.0 ml H_2O_2 and 0.5 ml of the blood sample. To 1.0 ml portion of the reaction mixture was added 2.0 ml of dichromate acetic acid reagent. Then the absorbance was read at 240 nm at a minute interval into 4 places. Catalase activity was calculated using the following equation:

$$\text{Catalytic Concentration } \left(\frac{\text{U}}{\text{L}}\right) = \frac{0.23\text{x logAbs1/2}}{0.00693} \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Where Abs= absorbance; 0.23 and 0.00693 = constants

2.5.3. Superoxide dismutase (SOD)

This was determined using the method of Xin *et al* [27]. Superoxide dismutase reduces superoxide to hydrogen peroxide. The theory of this method is based on the competition between superoxide dismutase activity and iodonitrazolium violet in reacting with superoxide which is generated by xanthine oxidase (XOD) reaction. The reagents used are phosphate buffer pH 7.0;

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carbonate buffer pH 10.2 and xanthine oxidase 0.3 U/ml. Superoxide dismutase activity is then calculated.

2.5.4. Malondialdehyde (MDA)

Malondialdehyde reacts with thiobarbituric acid to form a red or pink coloured complex which in acidic solution, absorbs maximally at 532nm. It gives a measure of lipid peroxidation. Lower malondialdehyde level signifies reduction in lipid peroxidation. The reagents required are 1% thiobarbituric acid (TBA), 0.3% sodium hydroxide, 2.5% trichloro acetate and distilled water. MDA level is calculated from the quantity of the TBA:MDA adduct formed.

2.5.5. Glutathione Peroxidase (GPx)

Glutathione peroxidase activity was determined based on the method of Paglia and Valentine [28]. Glutathione peroxidase catalyzes the oxidation of reduced glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized gluthathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured in spectrophotometer and the glutathione peroxidase activity is determined.

3. Results

3.1. Quantitative Evaluation of Phytochemicals

The quantitative evaluation of phytochemical constituents of *Pentaclethra macrophylla* is shown in Table 1. The results obtained from the quantitative analysis of *Pentaclethra macrophylla* showed the presence of phytochemicals in varying concentrations. Total phenolic (4407.5300 mg/100g) represents the highest concentration of phytochemical while the least concentration of phytochemical present in the study plant was recorded for glycosides (2.1067 mg/100g). In a decreasing order of concentration, the quantity of Phytochemicals present is as follows; Total phenolic >Alkaloids > Flavonoids >Terpenoids > Reducing sugar> Tannins> Steroids > Glycosides. The variations in mean concentration of these phytochemicals is statistically significant at p- value < 0.05.

Table 1. Quantitative Phytochemical Composition of Methanol Extract of *Pentaclethra macrophylla*.

Phytochemicals	Mean Quantity ± SD (mg/100 g)
Alkaloids	961.94±3.76
Flavonoids	230.56±0.21
Tannins	16.74±0.81
Total phenolics	4407.53±3.72
Steroids	3.38±0.05
Terpenoids	135.87±17.28
Reducing sugar	99.78±5.23
Glycosides	2.11±0.03

3.2. Acute Toxicity

Table 2 showed the result of the acute toxicity testing. No mice died after ingesting doses up to 3200 mg/kg bw. Table 2 shows the result of the Lorke's model which credited a higher dose of 5,000 mg/kg as safe for mice since no death was recorded after ingestion. The treated animals were observed for lethality or signs of acute intoxication for 24 h. There was still no death up to 5,000 mg/kg dose of crude extract.

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Table 2. Acute Toxicity and Lethality (LD_{50}) Test.

First stage of investigation			
Group	Number of mice	Dose (mg/kg)	Mortality
I	3	10	0/3
II	3	100	0/3
III	3	1000	0/3
Second stage of investigation			
Group	Number of mice	Dose (mg/kg)	Mortality
I	1	1,600	0/3
II	1	2,900	0/3
III	1	5000	0/3

3.3. Identification of Bergenin

The melting point of Bergenin (BGN) was observed to be 237 °C. The UV–visible spectrum of BGN dissolved in methanol showed λmax at 274 nm. The structure of BGN is shown in Figure 1 following its spectroscopic fingerprints (Figures 1–6).

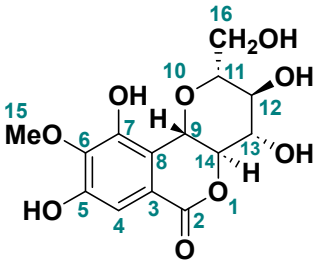

Figure 1. Bergenin structure.

^{1}H NMR (500 MHz, CD_3OD-d_4): δ 7.11 (s, 1H, H-4); 4.98 (d, 1H, H-9); 4.09 (dd, 1H, H-14); 4.04 (dd, 1H, H-16); 3.93 (s, 3H, OMe); 3.83 (t, 1H, H-13); 3.70 (m, 1H, H-11); 3.70 (m, 1H, H-16); 3.45 (t, 1H, H-12). ^{13}C NMR (125 MHz, CD_3OD-d_4): δ 164.4 (C-2); 151.1 (C-5); 148.1 (C-7); 140.9 (C-6); 118.1 (C-3); 115.8 (C-8); 109.6 (C-4); 81.7 (C-11); 80.1 (C-14); 74.2 (C-13); 72.9 (C-9); 70.5 (C-12); 61.3 (C-16); 59.5 (C-15). ^{13}C-DEPT (125 MHz, CD_3OD-d_4): δ 109.6 (CH, C-4); 81.7 (CH, C-11); 80.1 (CH, C-14); 74.2 (CH, C-13); 72.9 (CH, C-9); 70.5 (CH, C-12); 61.3 (CH_2, C-16); 59.5 (CH_3, C-15). MS (ESI) *m*/*z*: 327.0 $[M–H]^-$. HPLC: t_r = 1.55 min.

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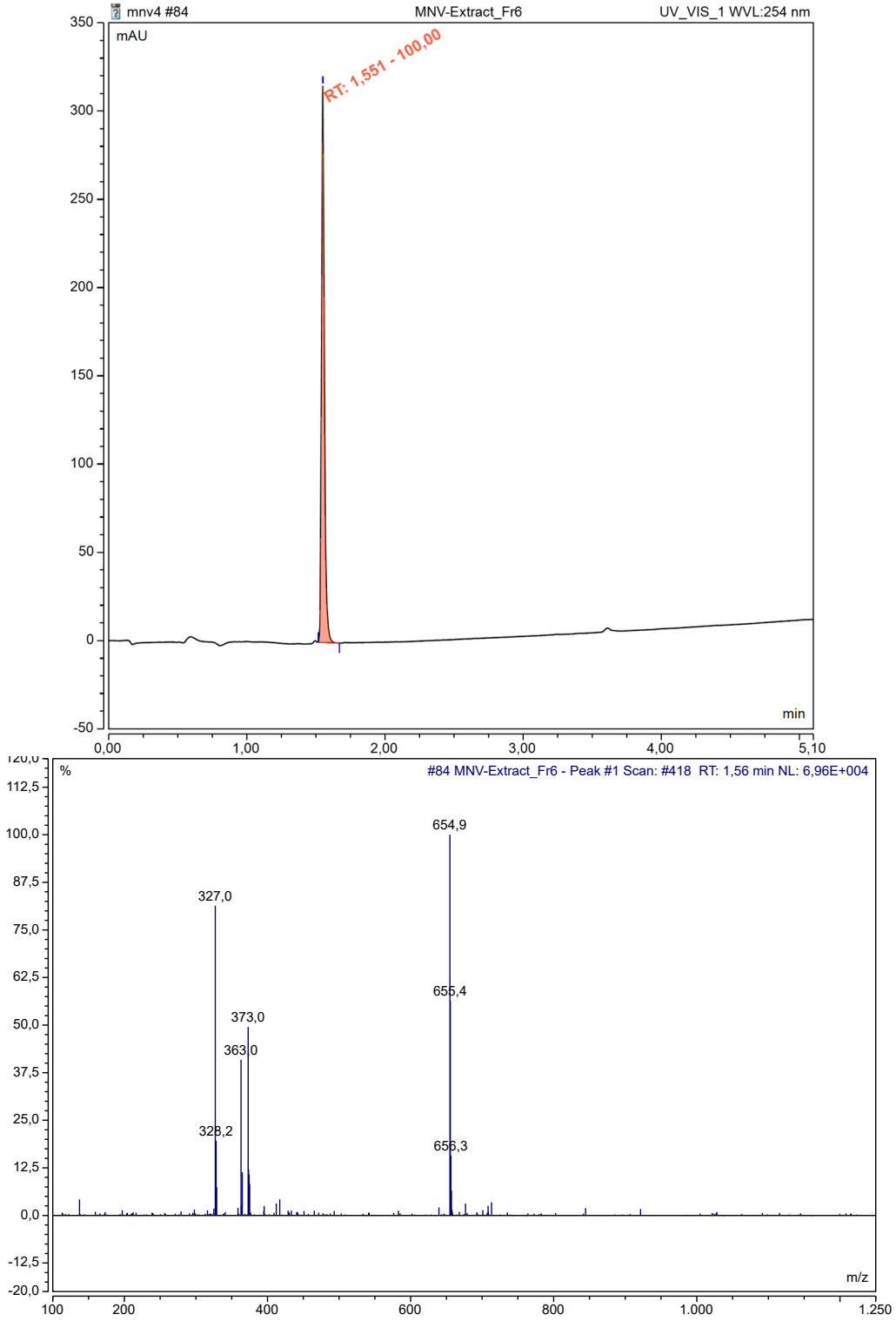

Figure 2. LC-MS spectra of bergenin.

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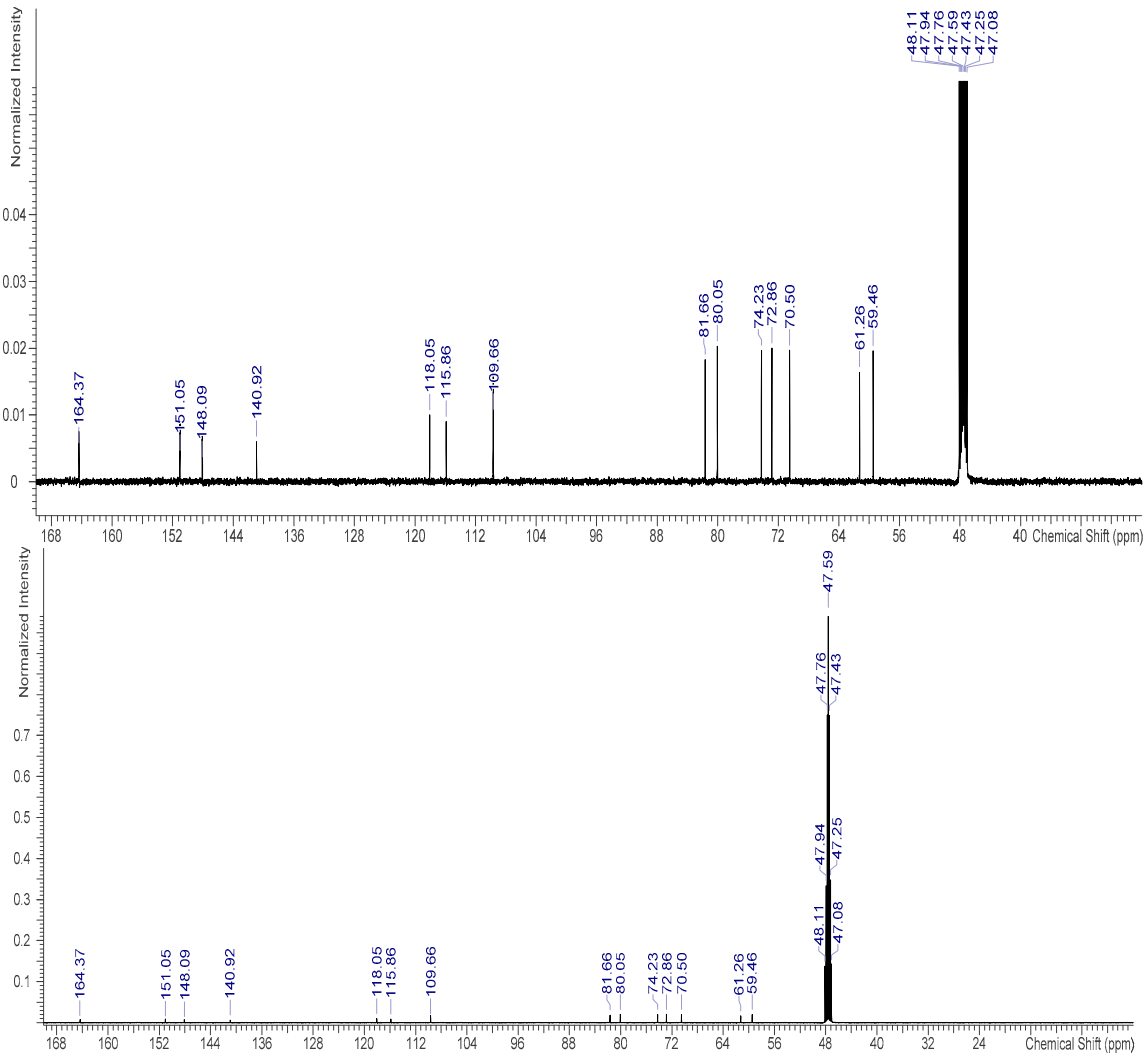

Figure 3. ^{13}C NMR spectrum of bergenin.

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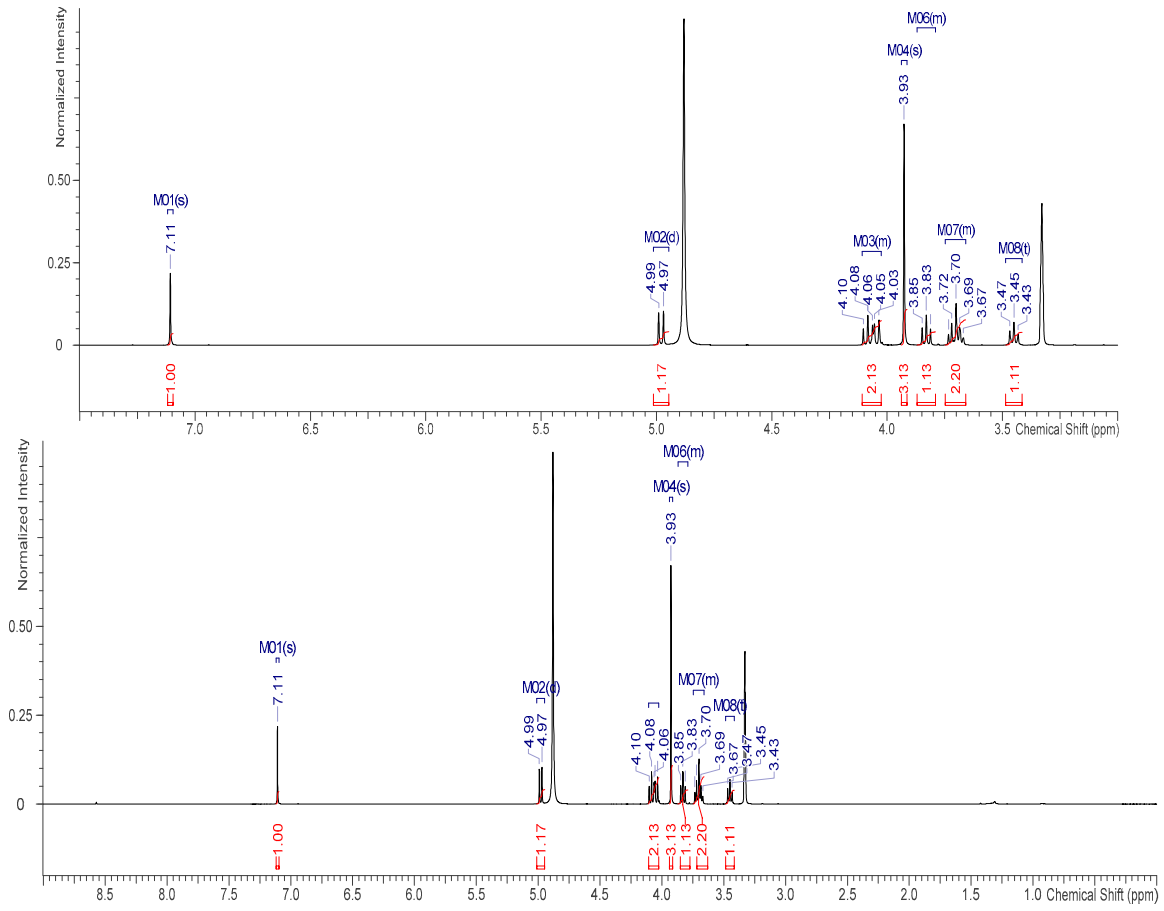

Figure 4. [1]H NMR spectrum of bergenin.

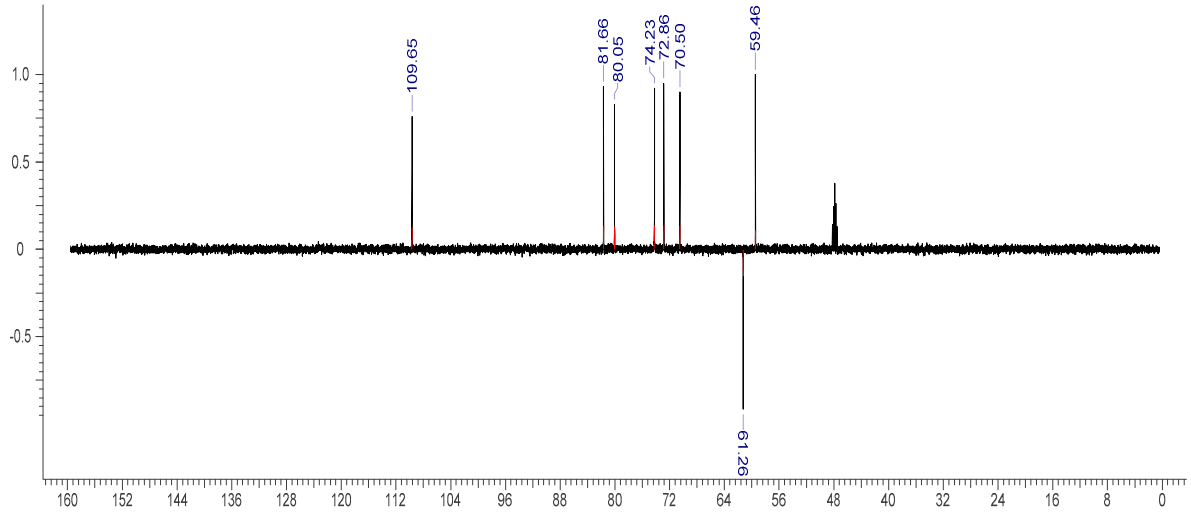

Figure 5. [13]C-DEPT NMR spectrum of bergenin.

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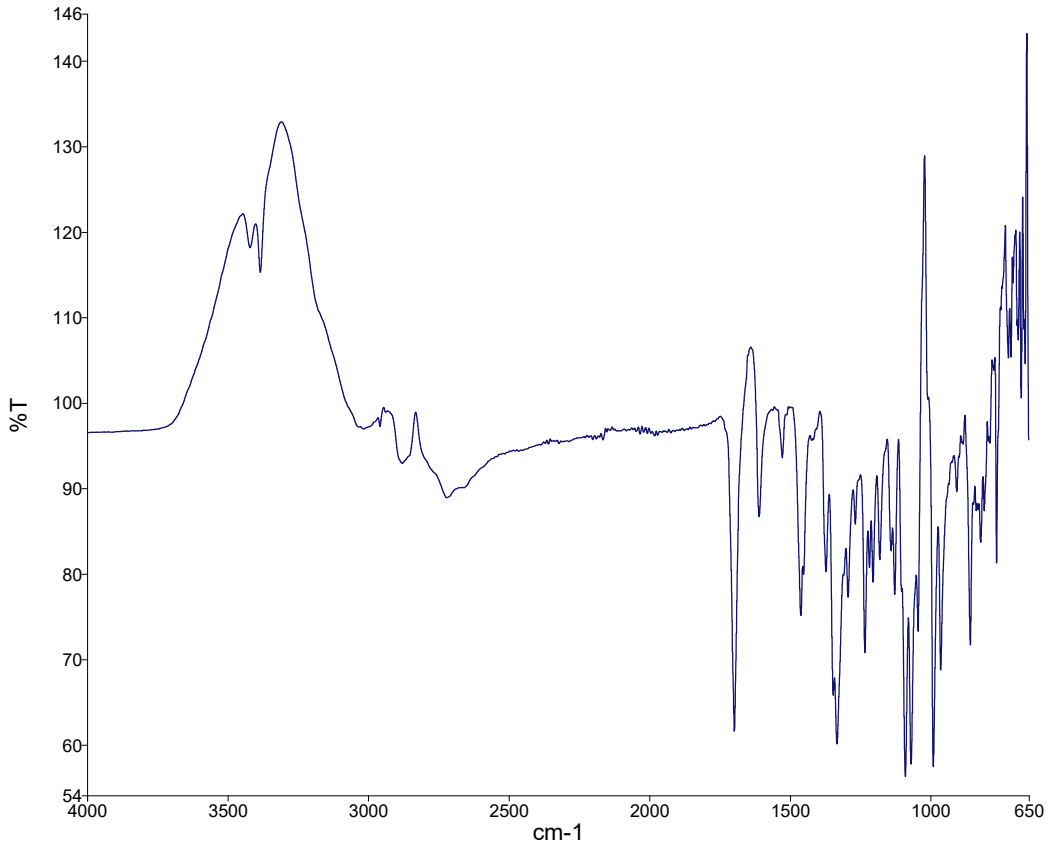

Figure 6. FTIR spectrum of isolated BGN.

3.3.1. FTIR Spectroscopy of Bergenin

The FTIR spectra of isolated BGN (Figure 6) shows characteristic 3387 (O-H); 3244 (C-H stretching, aromatic); 2960, 2894 (C-H stretching, alkyl); 1700 (C=O stretching); 1609, 1526, 1463 (C=C stretching, aromatic); 1373 (CH bend, CH2, CH3); 1333 (C-O-C stretching, ether); 1090, 765 (C6H6OH). The isolated crystalline compound was confirmed as BGN when the melting point, UV and FTIR spectra were compared with the data reported for standard bergenin [29].

3.4. The In-Vivo Antioxidant Effect of Different Concentrations of Pentaclethra macrophylla Extracts, Determined by Using Malondialdehyde (MDA), Glutathione Peroxidase (GPx), Superoxide Dismutase (SOD), and Catalase (CAT), Is Shown in Table 3

The effect of treatment on serum concentration of malondialdehyde revealed an increase in negative control compared to the normal control. However, a significant decrease in levels of MDA ($p<0.05$) following treatment with increasing concentrations of *P. macrophylla* was observed. This increase persisted in the levels of GPx and SOD in negative control groups compared to the normal group but decreased not significantly ($P>0.05$) with the administration of different doses of P. *macrophylla* extract. We observed a decrease in catalase activity in all the groups compared to normal control.

However, treatment with the various doses of extract resulted in lower serum catalase level when compared to the negative control, except for the group treated with 100mg/kg with higher catalase level (1.8 U/mg).

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Table 3. *In vivo* antioxidant activities of *Pentaclethramacrophylla* extracts.

Groups	MDA (mg/ml)	GPx U/mg	SOD U/mg	Cat U/mg
One	1.04±0.03	47.67±4.73	11.44±0.03	1.76±0.93
Two	1.16±0.07	59.03±11	11.39±0.04	0.71±0.39
Three	1.2±0.05	48.33±4.44	11.42±0.06	1.61±0.98
Four	1.34±0.00	47.61±0.00	11.37±0.00	1.8±0.00
Five	1.26±0.02	48.09±5.61	11.16±0.45	1.11±0.23
Six	1.25±0.12	47.02±4.28	11.08±0.71	1.42±0.50

Key: Group One: Normal control- no induction no treatment; Group Two Induction (2000mg/kg bw PCM) only; Group Three Induction (2000mg/kg bw PCM) + Std (Ascorbic acid) 20 mg/kg bw; Group Four Induction (2000mg/kg bw PCM) + Treatment 100 mg/kg bw; Group Five Induction (2000mg/kg bw PCM) + Treatment 200 mg/kg bw; Group Six Induction (2000mg/kg bw PCM) + Treatment 400 mg/kg bw.

In Vitro Antioxidant

The antioxidative potential of the extract as shown by scavenging of DPPH radical, revealed a significant decrease ($P< 0.05$) in inhibition activity as the concentration increased (Table 4). Nitric oxide radical scavenging assay, showed increase in scavenging activity of the plant in concentration dependent manner. Percentage total antioxidant capacity (%TAC) was observed as the concentration of the extract increases. FRAP assay showed a non-significant ($p>0.05$) variation in values with respect to varying concentration of the extracts, with an increased ferric reducing/ antiradical power recorded at 125µg/ml and 500µg/ml.

Table 4. *In vitro* antioxidant activities of *Pentaclethramacrophylla* extracts.

Concentration (µg/ml)	ASC Acid	DPPH	% Inhibitory NO	%TAC	% FRAP
15.63	84.54±1.9	93.40±0.62	46.67±7.39	77.17±2.37	125.17±2.84
31.25	85.05±1.14	93.77±0.12	44.80±12.31	50.27±6.35	122.6±8.94
62.5	84.63±1.78	94.10±0.10	48.27±12.66	51.5±3.29	120.2±9.88
125	82.1±0.51	93.90±0.26	49.47±11.03	90.03±6.29	128.2±8.15
250	77.21±5.37	93.93±0.15	41.73±14.61	134.1±9.18	123.2±1.3
500	60.4±8.06	92.17±0.06	44.57±7.56	168.3±27.31	128.4±6.55
Total	78.99±9.63	93.54±0.71	45.92±9.81	95.23±45.55	124.63±6.65
F(p value)	16.15(0.000)	18.034(0.000)	0.186(0.962)	43.265(0.000)	0.643(0.672)

4.0. Discussion

Oxidative stress is responsible for over seventy five diseases and other harmful cytological challenges including cancer, type 2 diabetes mellitus, lipid peroxidation, distorting of cell membrane integrity, shortening of telomerase among others. Antioxidant potency of any plant is a very positive remedy to the debilitating effects of prooxidants and oxidants. The study was aimed at investigating the antioxidant potency of methanol extract of *Pentaclethra macrophylla* stem bark- *in vitro* and *in vivo.*

The *in vivo* and *in vitro* test systems for analyzing the antioxidant activity showed varying results that confers antioxidative potentials to *P. macrophylla* stem bark extract. *P. macrophylla* stem bark contains bergenin. Bergenin is an isocoumarin compound, which was first extracted from plants of the genus Bergenia [32]. Bergenin exerts a strong antioxidant effect [33,34].

The increased levels of MDA and GPx in serum of negative control group compared to the normal control are probably due to oxidative stress occurring after infection. Similar results on higher MDA and Gpx levels in parasitic diseases have been reported [35]. The increase of malondialdehyde (MDA) level in serum induced by infection suggests increased membrane peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals [35].

P. macrophylla extract generally showed an increase in CAT activity. This reaction has been reported to be responsible for the curative effect of the extract. Elsewhere, the administration of the methanolic extract to treated rabbits enhanced catalase and peroxidase profiles, dose dependently, by acting as a strong free radical quencher and protecting the tissues [35]. Peroxidase and catalase are essential for the endogenous antioxidative defense system to scavenge reactive oxygen species and maintain the cellular redox balance [36].

The observed decrease in SOD activity following infection and treatment with different doses of plant extract might be due to the oxidation of CAT and GSH-Px enzymes. In agreement with our study, Pawel *et al*. [36] showed a decrease in SOD and CAT activities in infected rats compared to control rats. Farombi *et al*. [35] suggested that superoxide radicals by themselves, or after their transformation to hydrogen peroxide, caused oxidation of CAT and GSH-Px enzymes and thus decreases SOD activity.

Our results showed good and significant antioxidant activity in the DPPH free radical assay, which is consistent with the finding of Oyinloye and Enujiugha [37]. The decrease in absorbance of DPPH caused by antioxidants is due to the reaction between antioxidant and radical, which results in scavenging of the radical by hydrogen donation. The effect of *Pentaclethra macrophylla* on DPPH was thought to be based on the hydrogen donating ability of the extract. As shown in the result, the IC_{50} of *Pentaclethra macrophylla* on DPPH was 683.6938µg/ml while that of the standard was 43.4781µg/ml, which shows that the standard had higher activity than the extract *Pentaclethra macrophylla.*

Sodium nitroprusside is a great source of free radicals. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine is used as a marker for $NO^{\cdot}$ scavenging activity. Upon addition of extract, it was observed a colour change from green to slightly yellow also indicative of antioxidation activity. Decreased absorbance showed an increase in activity as concentration of the plant extract increased giving an IC_{50} of 178.83µg/ml and ascorbic acid 1219.2577µg/ml. This shows there is higher activity than the standard. Using the anti-radical power it shows that *Pentaclethra macrophylla* has more potency than ascorbic acid in $NO^{\cdot}$ scavenging assay. The presence of phytochemicals with pharmacological activities, in large quantities may have been responsible for the reported *in vivo* and *in vitro* antioxidant activity.

4.1. Conclusion

Pentaclethral macrophylla stem bark extract is rich in phytochemicals with pharmacological potentials. The extracts at varying concentrations, showed significant antioxidant properties. The presence of phytochemicals (e.g. Bergenin) is responsible for these pharmacological effects. The antioxidant activity of Bergenin in *P. macrophylla* may help to protect against oxidative stress and reduce the risk of certain diseases, such as heart disease, cancer, ulcer and neurological disorders. This tries to provide proof why the Nigerian people liberally consume the bark extract infusion of this plant. It further reflects a hope for the development of many novel therapeutic agents or templates from this plant, which in future may serve for the production of synthetically improved therapeutic agents.

Author Contributions: Petra Nnamani: Conceptualization, Methodology, Original draft preparation, Edith Diovu: Plant extraction, Peter Ikechukwu: Plant collection, Onwuka Akachukwu: Plant assay, Ibezim Obinna Chibueze: Plant Extraction and methodology, Abonyi Obiora: Animal study and statistical analysis, Birgitta Loretz: Conceptualization and supervision, Mariia Nesterkina: Methodology, Claus-Michael Lehr: Conceptualization, supervision and review.

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Conflicts of Interest : The authors declare no conflict of interest.

Authors' Declaration: The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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