Antioxidant and Hepatoprotective Properties from the Extract and Fractions of *Annona senegalensis* Pers (Annonaceae) Stem Bark Grown in Nigeria

ABSTRACT

Aim: This study was carried out to assess the antioxidant and hepatoprotective properties of the extract and fractions of *Annona senegalensis* stem bark through *in vitro* and *in vivo* experimental models.

Study design: The study followed a completely randomized design (CRD) of groups of treatments and control samples for all the tests.

Place and Duration of Study: Department of Pharmacognosy and Environmental Medicines, University of Nigeria, Nsukka, between January and September 2016.

Methodology: Phytochemical constituents and *in vitro* antioxidant activities using different models (reducing power, DPPH free radical scavenging, ABTS radical scavenging, Hydroxyl radical scavenging, Hydrogen peroxide scavenging, β-carotene bleaching, FRAP scavenging and superoxide radical scavenging assays) were carried out. *In vivo* antioxidant activity was determined from the assays of lipid peroxidation, superoxide dismutase and total protein while hepatoprotective activity was evaluated against CCl_4 induced liver damage and elevated serum marker enzymes.

Results: The results showed that the extract and fractions of stem bark of *A. senegalensis* had appreciable amounts of total flavonoids (845.67±93.62 mg/g) and total phenols (866.67±8.41), and exhibited good antioxidant activities at higher concentrations. Doses of the extract and fractions administered at 400 mg/kg protected the CCl_4–induced lipid peroxidation and significantly (P = .05) reduced the elevated serum marker enzymes - aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphate (ALP), and bilirubin level on a dose and solvent dependent fashion. At 200 and 400 mg/kg extract, the serum AST was reduced (by 40.34% and 45.66% respectively) as much as the MeOH fraction (43.88%) and control (43.44%), whereas EtOAc fractions gave significantly the best reduction (52.49%). The ethyl acetate fraction gave the best activity among all the fractions. **Conclusion:** The results showed that the stem bark is a potential source of natural antioxidants and hepatoprotective agents, and justifies its use in traditional herbal practice.

Keywords: antioxidant models, CCl_4 induced liver damage, serum marker enzymes

1. INTRODUCTION

Free radicals and highly reactive oxygen species (ROS) are formed by normal body physiological activities, but they build up and give rise to oxidative stress when not efficiently eliminated by the endogenous systems [1, 2]. They are cytotoxic, cause abnormal enzyme activation and tissue damage. Oxygen derived free radicals such as hydrogen peroxide, superoxide anions and hydroxyl radicals give rise to oxidative stress which, in turn, could initiate serious health issues such as cardiovascular disease, cancer, Alzheimer's disease,

Parkinson's disease, liver disease, and numerous disorders such as ulcerative colitis, neural disorders and ageing [3, 4]. The liver is the largest organ and arguably the most important organ in human body because of its pivotal role in various metabolic activities. It secretes bile, produces blood-clothing factors and is involved in detoxification of the body system. However, liver diseases (such as hepatitis, jaundice and cirrhosis) have contributed to major causes of high rate of morbidity and mortality in recent days, largely owing to the intake of hepatotoxic drugs. Several other exogenous factors of free radicals such as air pollution, smoking, pesticide poisoning and so on, contribute to liver damage [5].

Plants contain chemical substances in them that make it possible for them to carry out the responsibilities of maintaining good health and curing diseases in humans. These substances are known as the phytochemicals or phytoconstituents [6, 7]. Among the phytochemicals utilized for their antioxidant properties are the polyphenols and flavonoids. From the days of earlyman, the use of plants as medicine has been recorded [8]. Majority of indigenous people, especially in developing countries, use plant based medicines, which make up the traditional system of medicine for healthcare delivery [9]. Early documentations of cultures of the ancient Chinese, Indians and Africans provide reliable evidences of man's dependence on plants for the treatment and management of wide array of ailments [10]. Furthermore, a glance into many archeological documentaries shows that even pre-historic men made use of plant based drugs in management and treatment of illnesses, and today the World Health Organization (WHO) estimated that about 80% of the world's population relies on traditional herbal remedy, in one form or the other, for the management of several health issues [11].

Many Nigerian ethno botanic traditions propose a rich repository of medicinal plants used by the population for treatment of oxidative stress-related diseases, including liver diseases. However, there were not enough scientific investigations to validate these claims. One of such plants from Nigerian flora is *A. senegalensis* Pers. It is a multipurpose medicinal plant which several parts are used in ethnomedicine by various peoples of tropical Africa for the management and treatment of diseases and symptoms such as malaria, cancer, dysentery, filariasis, convulsions, diarrhea, dysentery, impotency, inflammations, pains [12, 13], snake bites and sexually transmitted diseases [14]. It is also reported that decoction of the plant is used in folkloric medicine to treat kwashiorkor; marasmus, eyelid swelling and body ache [15]. In Northern Nigeria, the stem bark is used to treat hepatitis, gastroenteritis, guinea worms, toothache, pneumonia and respiratory infections [16]. Apart from the medicinal values, the leaves of *A. senegalensis* are sometimes eaten as vegetables [17] and stem bark produces an effective insecticide. The following phytoconstituents have also been reported on the plant: tannins, glycosides, flavonoids, resins, alkaloids (for example, (-) roemerine, an aporphine) saponins and carbohydrates. Sterols, fatty acids, sesquiterpenoids and monoterpenoids have been isolated from the leaves and fruits, while amino acids and ent-kaurenoids have been isolated from the stem bark and root back respectively [14].

2. MATERIAL AND METHODS

2.1 Plant collection

Stem bark of *Annona senegalensis* was collected from Nsukka, Nigeria (6º51'24"N 7º23'45"E), after proper identification and authentication by Mr. Alfred Ozioko, a Plant Taxonomist at the International Centre for Ethno medicine and Drug Development (InterCEDD) Nsukka, Enugu State. A voucher sample (INTERCEDD 0314) was prepared and deposited in the herbarium of the same institution.

2.2 Experimental animals

White albino Wistar rats weighing 86 - 100 g and mice were purchased from the Laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were caged and fed with commercial animal feed (Guinea Feed®) and clean tap water. They were maintained in accordance with the Guide for the Care and Use of Laboratory Animals

(DHHS, NIH Publication No. 85-23, 1985). They were allowed 2 weeks to acclimatize before the start of the experiments.

2.3 Extraction and fractionation

The plant material was air-dried under shade (at 35 – 40 ºC) and thereafter pulverized. A measured quantity (2 kg) was cold-macerated with 1:1 dichloromethane/methanol (Sigma-Aldrich analytical grade) for 48 hours. The liquid extract was concentrated using rotary evaporator (Buchi Rotavapor® R-215) under a reduced pressure to obtain the extract. A 400 g of the crude extract was fixed on Silica gel (Kieselgel 60 $PF_{254+366}$) and subjected to column chromatography using n-hexane, ethyl acetate and methanol (Sigma-Aldrich analytical grade) as eluents to obtain n-hexane, EtOAc and MeOH fractions respectively. The different fractions were obtained and air dried at room temperature.

2.4 Phytochemical screening

The preliminary phytochemical test was carried out following standard methods as described by Trease and Evans [18].

2.4.1 Determination of total phenolic and flavonoid contents

Total phenolic compound was determined by the Folin-Ciocalteu method described by Charoensin [3]. A 100 µL of each of the dissolved crude extract and fractions were put in chamber containing 2.8 mL of deionized water and 2 mL of 50% Folin-Ciocalteau's phenol reagent. The mixtures allowed standing at room temperature for 30 min after which the absorbance was read at 765 nm. The total phenolic content was expressed as milligram gallic acid equivalent per gram extract/fraction (mg GAE/g extract/fraction).

Total flavonoid content was determined using the aluminum chloride colorimetric method according to Chang et al. [19]. Briefly, 100 µL of each extract were mixed with 1.5 mL of 95% ethanol, 100 µL of 10 % AlCl3, and 100 µL of 1 M potassium acetate and 2.8 mL of deionized water. The absorbance of the reaction mixture was measured at 415 nm. The total flavonoid content was expressed as milligram rutin equivalent per gram extract/fraction (mg RU/g extract/fraction).

2.5 Acute Toxicity Study

The Lorke [20] procedure of LD_{50} determination was used. The experimental mice were divided into three group (n=3) and were orally administered 10, 100 and 1000, and later, 1000, 1600, 2900 and 5000 mg/kg of the extract of *A. senegalensis* stem bark. The animals were constantly observed for mortality over a period of 24 hours.

2.6 *In Vitro* Antioxidant Analysis

2.6.1 Test for reducing power

The crude extract was subjected to reducing power assay following the method of Alam *et al.* [21]. A 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1% w/v) were added to 1.0 mL of sample dissolved in distilled water. The resulting mixture was incubated at 50 ºC for 20 min, followed by the addition of 2.5 mL of Trichloro acetic acid (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700 nm against blank sample.

2.6.2 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

The DPPH scavenging activity was carried out following Mensor et al. [22]. Varying concentrations (800 - 25µg/ml) of the extract/fraction each was mixed (2:1 v/v) with 0.5 mM DPPH (in methanol) in a cuvette. After an incubation period of 30 minutes, the absorbance was read at 517 nm using a UV-Vis spectrophotometer, and the percentage antioxidant activity was calculated. Ascorbic acid served as a positive control.

2.6.3 Ferric reducing antioxidant power (FRAP) potential assay

The total antioxidant potential of the samples was measured by ferric reducing ability of plasma (FRAP) assay as described by Benzie and Strain [23]. Different concentrations (100-1000 µmol/L) of $FeSO_4.7H_2O$ were used to prepare the standard curve, and the antioxidant capacities of the samples were determined according the reaction signal given by Fe^{2+} solution of known concentration at absorbance of 593 nm. Ascorbic acid was measured

within 1h after preparation. All tests and readings were carried out in triplicates. Calculations were made by a calibration curve:

FRAP value (µM) = Changes in absorbance from 0-4 min x FRAP value of standard (1000 µM)
Changes in absorbance of standard from 0-4 min

2.6.4 Hydrogen peroxide scavenging assay

The method of Bokhari et al. [24] was followed to investigate hydrogen peroxide scavenging capacity of samples. Hydrogen peroxide (2 mM) solution was prepared in phosphate buffer (50mM, pH 7.4). Samples (100µg) were pipette into flasks and their volume made up to 400 µL with 50 mM phosphate buffer (pH 7.4). H_2O_2 solution (600 µL) was added and absorbance at 230 nm was taken 10 min after vortexing the flasks. Percent scavenging activity was determined by following formula;

$$H_2O_2\% \text{ scavenging activity} = \left(1 - \frac{\text{absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Ascorbic acid served as standard.

2.6.5 Hydroxyl radical scavenging assay

The antioxidant activity was evaluated by method reported by Halliwell and Gutteridge [25]. The reaction mixture comprised of 2-deoxyribose (2.8 mM, 500 µL) in 50 mM of phosphate buffer, 100 µL of 0.2 M hydrogen peroxide solution, 200 µL of 0.1M ferric chloride, 0.1M EDTA and 100µl of test sample. The reaction was initiated by the addition of 100 µl of ascorbic acid (0.3M). The mixture was incubated at 37 ^{o}C for 60 min. Trichloroacetic acid (TCA) (2.8% w/v, 1 mL) and 1 mL of thiobarbituric acid (TBA) solution in 50 mM of sodium hydroxide (1% w/v) were added. This reaction mixture was heated for 15 min in boiling water bath and then allowed to cool. Absorbance was recorded at 532 nm.

$$\text{Hydroxyl scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

2.6.6 ABTS radical cation scavenging activity

Re *et al.* [26] methodology with slight modification was followed for ABTS (2,2-azino-bis, 3-ethylbenzthiazoline-6-sulfonic acid) radical cation scavenging activity. ABTS (7 mM) solution was reacted with 2.45 mM potassium persulfate and kept overnight in dark for generation of dark colored ABTS radicals. For the assay, the solution was diluted with 50% ethanol for an initial absorbance of 0.7 at 745 nm. Activity was determined by adding 100 µl sample of different dilution with l ml of ABTS solution in glass cuvette. Decrease in absorbance was measured after one min and 6 min of mixing. The difference was calculated and compared with control. Percent inhibition was calculated by formula:

$$\text{\% ABTS scavenging effect} = \left(\frac{\text{control absorbance} - \text{sample absorbance}}{\text{Control absorbance}}\right) \times 100$$

2.6.7 β-Carotene bleaching assay

Elzaawely *et al.* [27] modified method was used for β-carotene bleaching assay. B-Carotene (2 mg) was dissolved in 10 ml of chloroform and blended with 20 mg of linoleic acid and 200 mg of Tween 20 followed by removal of chloroform under nitrogen with subsequent addition of 50 ml of distilled water and vigorous shacking to prepare β-carotene linoleic acid emulsion. An aliquot of each sample (50 µl) was mixed with 1 mL of the emulsion, vortexed and absorbance was determined at 470 nm immediately against the blank solution. Capped tube was then kept in a water bath at 45 ^{o}C for 2 h and the difference between the initial readings was calculated by measuring the reading after 2 h. β-Carotene bleaching inhibition was estimated by the following equation:

$$\text{\% bleaching inhibition} = \frac{(A_{ot} - A_{120t})}{A_{ot} - A_{120t}} \times 100$$

2.6.8 Superoxide anion radical scavenging assay

Riboflavin light NBT system assay was followed for superoxide radical scavenging activity as described by Nishikimi [28]. The reaction mixture containing 0.5 ml of phosphate buffer (50

mM, pH 7.6), 0.3 ml riboflavin (50 mM), 0.25 ml phenazine methosulfate (PMS) (20 mM), and 0.1 mL NBT (0.5 mM), prior to the addition of 1 mL sample in methanol. Florescent lamp was used for starting the reaction. Absorbance was recorded at 560 nm after incubation for 20 min under light. The percent inhibition of superoxide anion generation was calculated using the following formula:

% scavenging activity = (1 – Absorbance of sample/Absorbance of control) x 100

2.7*In Vivo* Anti-Oxidant Analysis

2.7.1 Total proteins determination (direct biuret method)

The method used for the determination of total protein was that described by Tietze [29]. Three test – tubes were labeled blank (BL), standard (ST) and sample (SA) respectively before the set of the experiment. To the test-tube labeled SA, 0.02ml of serum was added. To the test –tube labeled ST, 0.02 mL of standard (CAL) was added and 0.02 mL was added to the test tube labeled BL. Then, 1 mL of the biuret reagent was added to all the three test tubes. After the addition, it was mixed well and incubated at 30 minutes at room temperature (20-25^{o}C). The absorbance of the sample and standard was measured against the reagent blank at 546 nm.

2.7.2 Lipid peroxidation assay

Lipid peroxidation in the serum from animals of Day 28 was estimated colorimetrically as thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust [30]. A principal component of TBARS is malondialdehyde (MDA), a product of lipid peroxidation. In brief, 0.1 mL of tissue homogenate (Tris-HCL buffer, pH 7.5) was treated with 2 mL (1:1:1 ratio) of TBA-TCA-HCI reagent (thiobarbituric acid 0.37 %, 0.25 N HCL and 15% TCA). The mixture was placed in a water bath for 15 min; it was then allowed to cool. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was expressed as nmol/ mL.

2.7.3 Assay of Superoxide Dismutase

Superoxide dismutase in the serum from animals on day 28 was estimated using the procedure of McLord and Fridovich [31]. A given volume (0.1 mL) of the serum was pipette into cuvette containing 1M KOH buffer of pH 7.8 at 25^{o}C. Reaction was started by the addition of 1.0 mL of freshly prepared 30 % (v/v) superoxide Dismutase (SOD). The rate of decomposition of SOD was measured spectrophotometrically from changes in absorbance at 550 nm. The enzyme activity was expressed as units/ mL protein.

2.8 Hepatoprotective Assay

The rats were divided into eight groups (n=5). Group 1 served as the hepatotoxic group. Group 2 received the reference drug, Silymarin (25 mL/kg body weight), and groups 3, 4 and 5 received the methanol-methylene chloride (1:1) extract (100, 200 and 400 mg/kg body weight respectively). Groups 6, 7 and 8 received 400 mg/kg of the n-hexane, ethyl acetate and methanol fractions respectively. These were given to the animals once daily for 3 days. On day 3, one hour after administration of the appropriate drug for each group, carbon tetrachloride was given to the different groups, and 48 hours after carbon tetrachloride administration, blood was collected from the retro-orbital plexus to be used for the assessment of biochemical parameters.

2.8.1 Liver function tests

Blood was collected from all the groups via the retro-orbital plexus at room temperature and the serum was separated by centrifuging at 2500 rpm for 10 minutes. The serum was used for estimation of the biochemical parameters to determine the functional state of the liver. Serum aminotransferase activities including AST, ALT, alkaline phosphatase, total bilirubins were assayed using the 'RANDOX' commercial Enzyme kit according to the methods of Reitman and Frankel [32], and Schmidt and Schmidt [33].

2.9 Statistical analysis

All data were expressed as Mean ± S.E.M. or % mean. Data were analyzed using one way analysis of variance (ANOVA) at 5% level of significance on the SPSS version 20 software. Bar chart was processed on Microsoft Excel 2007 spread sheet.

3. RESULTS AND DISCUSSION

Oxidative stress as a result of the accumulation of free radicals and reactive oxygen species in the body remains the cause of premature ageing and so many disease conditions facing man today. The use of modern drugs on daily basis has exacerbated the condition due to hepatotoxic properties of most drugs. In this study, we investigated the potential of the stem bark of *A. senegalensis* in the management of oxidative stress and liver disorders. Table 1 shows the respective percentage yields of 2 kg starting plant material into crude extract and fractions. It clearly that among the solvents used for fractionation methanol had the highest yield while n-hexane was the lowest.

Table 1: Percentage yield of the extract and fractions from 2 kg powdered plant material

Plant materials	Weight (g)	Yield (%)
Crude Extract	400	20
n-hexane fraction	20.1	5.01
EtOAc fraction	120.66	30.17
MeOH fraction	180.20	45.05

3.1 Acute Toxicity Test (LD_{50})

The acute toxicity test recorded no mortality even at 5000 mg/kg. This shows that the *A. senegalensis* extract was safe at high dose.

3.2 Phytochemical Analysis

A number of phytoconstituents are attributed to antioxidant activities include the polyphenols, flavonoids and triterpenoids [21]. The results of the qualitative phytochemical screening of the extracts and fractions are presented in Table 2 below. Quantitative analysis of the total flavonoids and total phenolic compounds was also carried out and presented in Table 4. The ethyl acetate fractions had more total flavonoids and phenolics than the rest of the fractions. This is in agreement with the reports of previous authors [14, 19].

Table 2: Qualitative phytochemical screening of the stem bark of *A. senegalensis*

Phytochemical	Crude extract	n-hexane fraction	EtOAc fraction	MeOH fraction
Carbohydrate	++	-	+	+
Alkaloids	++	-	-	++
Tannins	++	-	-	++
Flavonoids	++	+	++	+
Terpenoids	++	+	+	+
Proteins	+	+	-	-
Reducing sugars	+	-	+	+
Glycosides	+	+	+	+
Saponins	+	-	-	+

Resins	+	+	+	+
Steroids	+	-	+	-

- absent; + present; ++ highly present (scored based on colour intensity)

Table 3: Total flavonoids and total phenolic contents

Extract/Fraction	Total flavonoid content of *A. senegalensis* (mg rutin equivalent/g extract or fraction)	Total phenolic content of *A. senegalensis* (mg Gallic acid equivalent/g extract or fraction)
Crude Extract	845.67±93.62	866.67±8.41
n-hexane fraction	78.45 ± 6.54	88.64 ± 8.33
EtOAc Fraction	587.33±50.83	582.00±1.73
MeOH fraction	113.67±9.28	115.33±3.84

Values expressed in mean ± SEM; n = 3

3.3 Antioxidant Assays

The antioxidant reducing power of the crude extract was found to be higher than that of the control (ascorbic acid) at the various concentrations tested (Figure 1). The increase in the absorbance values signifies increased antioxidant reducing power, which often correlates with change in sample concentration [21]. Similar results are reported by Tchimene *et al.* [4] who screened the antioxidant reducing power of the crude extract of *Combretum obanense.*

3.3.1 *In Vitro* Antioxidant Assay

Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of substances [21]. The most commonly used is the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. The DPPH test is a method that estimates the free radical scavenging activity of antioxidants through de-colourisation of the DPPH solution. The concentration and efficacy of the antioxidant is determined from the degree of colour change. A drastic decrease in the absorbance of the reaction mixture therefore signifies high free radical scavenging activity of the test sample [22]. In this study, we also analysed the antioxidant activity of the samples via several other models such as FRAP - which was based on the samples' ability to reduce Fe^{3+}/Fe^{2+} [23], hydrogen peroxide scavenging assay, hydroxyl radical scavenging assay, ABTS cation radical scavenging assay, β-carotene bleaching assay and superoxide anion radical scavenging assay.

In the present study, the results showed that in all the *in vitro* antioxidant models tested, the crude extract and the fractions from n-hexane, ethyl acetate and MeOH exhibited appreciable levels of antioxidant activities when compared to ascorbic acid. However, the ethyl acetate fractions exhibited the greatest activity than others and ascorbic acid in most cases, judging from their lower IC_{50} values (Table 4). Ethyl acetate fraction exhibited greater activity than other extract/fractions with IC_{50} value of 121.67µg/ml. The crude extract from FRAP assay indicated more inhibition than other fractions. Hydrogen radical scavenging capacity of ethyl acetate fraction with IC_{50} value of 75.67 ± 6.33 µg/mL indicates higher activity than others when compared with the standard with IC_{50} of 62.00 ± 1.73 µg/mL. Ethyl acetate indicated higher activity than others in beta-carotene bleaching activity, hydrogen peroxide, superoxide radical and ABTS scavenging activities.

Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidants present, it can be reported that extract/fractions of *A. senegalens is* may act as free radical scavenger, capable of transforming reactive free radical species into stable non radical products [23]. Hydroxyl radical scavenging capacity of an extract or compound is directly proportional to its antioxidant activity which is depicted by the low intensity of red colour. The sample of *A. senegalensis* when added to the reaction mixture actively scavenged the hydroxyl radicals and prevented the degradation of 2-deoxyribose [25]. The extract and fractions of *A. senegalensis* possessed strong ABTS scavenging activity as noticed by their ability to generate a blue/green ABTS + chromophore from the reaction of ABTS and potassium persulfate [26]. Because β-carotene is extremely sensitive to free radical medicated oxidation of linoleic acid, it is commonly used to analyse antioxidant potential of test samples [27]. Our results show that the tested samples inhibited β-carotene oxidation which could be attributed to free hydroxyl groups found in them. Superoxide and hydroxyl radicals are important mediators of oxidative stress that play vital role in some clinical disorders. Any compound, natural or synthetic with antioxidant activities might contribute towards the total/partial alleviation of such damage. Therefore, removing superoxide and hydroxyl radical could contribute to defense of a living body against disease [31].

Table 4: IC_{50} Values of different antioxidant Assays of *A. senegalensis* (IC_{50}, µg/ml)

Test Method Activity	Crude Extract	n-hexane fraction	EtOAc fraction	MeOH fraction	Standard (Ascorbic acid)
DPPH Scavenging activity	752.67±15.60^{a}	355.67±4.49^{b}	121.67±2.40^{d}	293.67±16.91^{c}	69.67±1.86^{e}
FRAP activity	63.00±12.22^{b}	281.67±15.34^{a}	81.00±1.00^{b}	275.67±6.33^{a}	71.66±0.33^{b}
Hydrogen peroxide scavenging activity	390.33±0.88^{a}	437.00±29.05^{a}	256.33±0.88^{b}	404.00±37.70^{a}	107.00±2.52^{c}
Hydroxyl radical scavenging activity	418.00±5.29^{a}	371.67±36.25^{a}	75.67±6.33^{b}	370.67±7.80^{a}	62.00±1.73^{b}
Superoxide radical scavenging activity	333.67±2.40^{a}	140.67±7.97^{c}	79.33±1.45^{d}	235.33±0.88^{b}	68.00±0.58^{d}
B-Carotene Bleaching activity	351.67±4.33^{a}	358.33±7.22^{a}	145.67±5.55^{c}	254.33±4.98^{b}	57.00±1.15^{d}
ABTS Scavenging activity	372.00±3.46^{a}	391.00±14.57^{a}	126.67±1.20^{c}	217.00±4.04^{b}	78.33±1.76^{d}

Values with different letters as superscript across a row are significantly different at $p < 0.05$
Values expressed as mean ± standard error; n = 5.

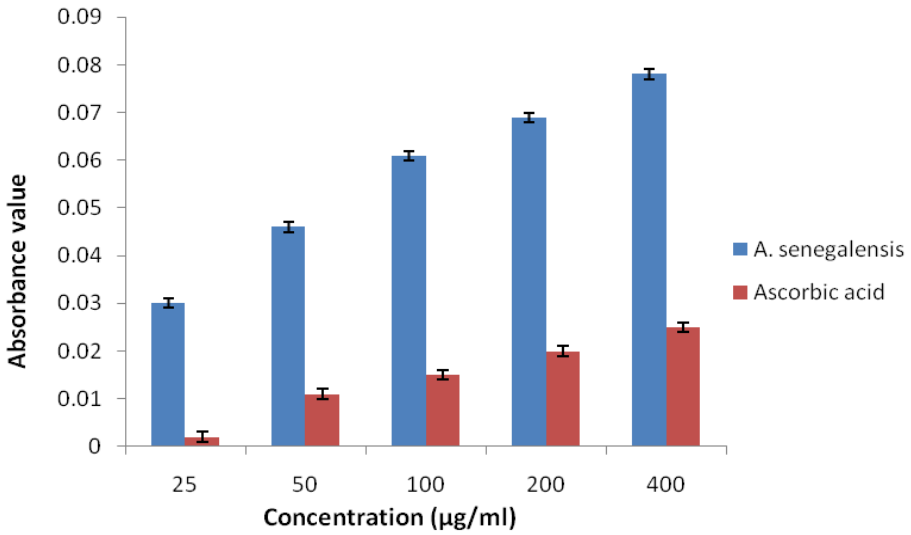

Figure 1: Reducing power activity of the extract compared with ascorbic acid

3.3.2 *In Vivo* Antioxidant Study

The antioxidant activity *in vivo* was dose dependent. It was observed that the crude extract at 100 mg/kg showed no significant activity on the parameters tested when compared to the control. However, higher doses of 200 mg/kg and 400 mg/kg and all the fractions significantly reduced the superoxide dismutase and lipid peroxide, and increased the total protein of the tested animals. Overall, the ethylacetate fraction exhibited the best antioxidant activity among all the samples (Table 5). Our findings agree with previous results by Balne et al. [34] and Sabbani *et al.* [35] who reported significant reduction in superoxide dismutase and lipid peroxidation in the blood serum of rats treated with extracts and fractions of *Marsilea minuta* and *Gardenia gummifera* respectively.

Table 5: Result of *in vivo* antioxidant study

Ext./fraction	Dose (Mg/kg)	SOD (Mg/dl)	L.H_2O_2 (mmol/l)	Vit. C (Mg/dl)	T. Protein (Mg/dl)
Ext.	100	38.00±4.56^{a}	0.32±0.04^{a}	14.40±2.25^{b}	7.66±0.54^{b}
"	200	30.40±3.66^{b}	0.26±0.02^{ab}	18.60±1.57^{a}	8.42±0.43^{a}
"	400	23.00±2.00^{c}	0.20±0.012^{b}	20.00±2.24^{a}	10.46±0.53^{a}
FRACTIONS					
MeOH	400	24.80±2.71^{c}	0.18±0.02^{c}	18.40±2.01^{a}	9.60±0.58^{a}
EtOAc	400	19.80±0.80^{c}	0.17±0.014^{c}	20.40±1.12^{a}	10.84±0.56^{a}
n-Hex	400	34.20±3.57^{ab}	0.27±0.03^{ab}	17.60±1.57^{a}	8.54±0.38^{a}
Crude Ext.	400	23.00±2.00^{c}	0.20±0.02^{b}	20.00±2.24^{a}	10.46±0.53^{a}
3% Tween 80	5ml/kg	40.00±2.30^{a}	0.37±0.02^{a}	10.40±0.51^{c}	6.38±1.12^{b}

Values with different letters as superscripts along a column are significantly different at $P = .05$

Values expresses as mean ± S.E. (standard error) n = 5; SOD = superoxide dismutase, L.H_2O_2 – lipid peroxide, T. protein = total protein.

3.4 Biochemical Tests (Liver Function Tests)

Table 6 below shows the liver function tests of *A. senegalensis.* It shows that higher blood serum enzyme values were recorded for the animals treated with CCl_4 only, which signify CCl_4 intoxication and liver damage in normal rats. A reduction in the blood serum enzyme by the plant drug was also dose and solvent dependent. The 100 mg/kg extract and n-hexane fraction had the least reductions (by 27.34% and 29.95% respectively) in the serum AST. At 200 and 400 mg/kg extract, the serum AST was reduced (by 40.34% and 45.66% respectively) as much as the MeOH fraction (43.88%) and control (43.44%), whereas EtOAc fractions gave significantly the best reduction (52.49%). Similarly, the MeOH and EtOAc fractions compared favorably with the control based on the values of the serum ALP and ALT, and total biluribin levels. Patrick-Iwuanyanwu et al. (2010) also reported hepatoprotective property of ethyl acetate fraction of *Tapinanthus bagwensis* to be higher than methanol and n-hexane fractions. This could be attributed to their different polarity and degrees of extractability [37].

The hepatoprotective study was undertaken to demonstrate the protective ability of the methanol-methylene chloride (1:1) extract of *A. senegalensis* on liver damage induced by Carbon tetrachloride (CCl_4) and the toxic effects of the similar doses in rats. Carbon tetrachloride has been reported to be metabolized to CO_2 in the liver homogenates. It produces free radicals, which affect the cellular permeability of hepatocytes leading to elevated levels of serum biochemical parameters such as alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) [38]. The levels of serum AST, ALT and ALP were taken as indices for oxidativxe stress induced by CCl_4. The serum activities in concentrations of these enzymes decreased significantly in the animals treated with the samples when compared to the untreated group, and this suggests that the extract and fractions of *A. senegalensis* have hepatoprotective activities. This is in agreement with the commonly accepted view that serum levels of AST, ALT and ALP return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [33]. The positive results obtained in this study could be attributed to the presence of high concentration of flavonoids, phenols, terpenoids, and steroids in the extracts and fractions of *A. senegalensis*. The extracts of the stem bark of the plant contain antioxidants and hepatoprotective activity through regulatory action on cellular permeability, stability and suppressing oxidative stress [34, 35].

Table 6: Biochemical tests (Liver function tests).

		AST	**ALP**	**ALT**	**T. BIL**
EXTRACT	**DOSES (mg/kg)**	**Mean±SEM (% reduction)**	**Mean±SEM (% reduction)**	**Mean±SEM (% reduction)**	**Mean±SEM (% increase)**
CCl_4 Only	0.5 ml/kg	223.13±7.53[a]	491.23± 10.21[a]	201.23±8.11[a]	4.54±0.82[a]
Ext. + CCl_4	100	162.12±11.01[b] (27.34)	260.20±12.82[b] (47.07)	99.80±2.31[b] (50.41)	2.78±0.16[b] (38.77)
"	200	133.11±11.90[c] (40.34)	219.43±8.19[c] (55.33)	67.80±4.43[c] (66.31)	2.10±0.14[c] (53.74)
"	400	121.24 ±14.18[c]	186.32±8.77[c]	58.00±3.33[c]	2.08±0.14[c]

		(45.66)	(62.07)	(71.18)	(54.19)
FRACTIONS					
MeOH + CCl_4	400	125.21±12.56^{c}	175.12±16.55^{c}	63.80±7.47^{c}	2.08±0.54^{c}
		(43.88)	(64.35)	(68.29)	(54.19)
EtOAc + CCl_4	400	106.22±11.56^{d}	145.32±18.63^{d}	59.40±10.30^{c}	1.80±0.15^{d}
		(52.49)	(70.42)	(70.48)	(60.35)
n-Hex + CCl_4	400	156.30±11.43^{b}	187.43±10.63^{c}	74.40±3.84^{b}	2.80±0.11^{b}
		(29.95)	(61.84)	(63.03)	(38.33)
Control	25 ml/kg	126.21±10.80^{c}	153.25±8.80^{d}	51.10±5.42^{d}	1.56±0.01^{e}
(Silymarin)		(43.44)	(68.80)	(74.61)	(65.64)

Values with different letters as superscripts along a column are significantly different at $P = .05$
Values expresses as mean ± S.E. (standard error) n = 5; AST = aspartate transaminase; ALP = alkaline phosphatase; ALT = alanine transaminase; T.BIL = total bilirubin.

4. CONCLUSION

The results of the present study demonstrated that *A. senegalensis* stem bark has antioxidant properties by scavenging free radicals, decreasing lipid peroxidation and increasing the endogenous blood antioxidant enzymes levels. Also the extract/fractions (400mg/kg) have potent hepatoprotective activity against Carbon tetrachloride induced liver damage and the effects are dose dependent. The antioxidant and hepatoprotective activities are due to the presence of bioactive compounds like flavonoids, phenolics, steroids and tannins. Since *Annona senegalensis* is a very popular drug in traditional medicine, it is a promising candidate for use as an antioxidant and hepatoprotective agent.
Further investigations using state-of-the-art techniques are therefore necessary to determine the phytoconstituents responsible for these activities in order to harness them for development of new hepatoprotective drugs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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