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

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## 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,  $\beta$ -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  $\text{CCl}_4$  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 ( $845.67 \pm 93.62$  mg/g) and total phenols ( $866.67 \pm 8.41$ ), and exhibited good antioxidant activities at higher concentrations. Doses of the extract and fractions administered at 400 mg/kg protected the  $\text{CCl}_4$ -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.

12  
13 **Keywords:** *antioxidant models, CCl<sub>4</sub> induced liver damage, serum marker enzymes*

## 1. INTRODUCTION

14  
15  
16 Free radicals and highly reactive oxygen species (ROS) are formed by normal body  
17 physiological activities, but they build up and give rise to oxidative stress when not efficiently  
18 eliminated by the endogenous systems [1, 2]. They are cytotoxic, cause abnormal enzyme  
19 activation and tissue damage. Oxygen derived free radicals such as hydrogen peroxide,  
20 superoxide anions and hydroxyl radicals give rise to oxidative stress which, in turn, could  
21 initiate serious health issues such as cardiovascular disease, cancer, Alzheimer's disease,

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

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

44 Many Nigerian ethno botanic traditions propose a rich repository of medicinal plants used by  
45 the population for treatment of oxidative stress-related diseases, including liver diseases.  
46 However, there were not enough scientific investigations to validate these claims. One of  
47 such plants from Nigerian flora is *A. senegalensis* Pers. It is a multipurpose medicinal plant  
48 which several parts are used in ethnomedicine by various peoples of tropical Africa for the  
49 management and treatment of diseases and symptoms such as malaria, cancer, dysentery,  
50 filariasis, convulsions, diarrhea, dysentery, impotency, inflammations, pains [12, 13], snake  
51 bites and sexually transmitted diseases [14]. It is also reported that decoction of the plant is  
52 used in folkloric medicine to treat kwashiorkor; marasmus, eyelid swelling and body ache  
53 [15]. In Northern Nigeria, the stem bark is used to treat hepatitis, gastroenteritis, guinea  
54 worms, toothache, pneumonia and respiratory infections [16]. Apart from the medicinal  
55 values, the leaves of *A. senegalensis* are sometimes eaten as vegetables [17] and stem  
56 bark produces an effective insecticide. The following phytoconstituents have also been  
57 reported on the plant: tannins, glycosides, flavonoids, resins, alkaloids (for example, (-)  
58 roemerine, an aporphine) saponins and carbohydrates. **Sterols**, fatty acids,  
59 sesquiterpenoids and monoterpenoids have been isolated from the leaves and fruits, while  
60 amino acids and ent-kaurenoids have been isolated from the stem bark and root bark  
61 respectively [14].  
62

## 63 2. MATERIAL AND METHODS

### 64 2.1 Plant collection

65 Stem bark of *Annona senegalensis* was collected from Nsukka, Nigeria (6°51'24"N  
66 7°23'45"E), after proper identification and authentication by Mr. Alfred Ozioko, a Plant  
67 Taxonomist at the International Centre for Ethno medicine and Drug Development  
68 (InterCEDD) Nsukka, Enugu State. A voucher sample (INTERCEDD 0314) was prepared  
69 and deposited in the herbarium of the same institution.

### 70 2.2 Experimental animals

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

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

### 77 **2.3 Extraction and fractionation**

78 The plant material was air-dried under shade (at 35 – 40 °C) and thereafter pulverized. A  
79 measured quantity (2 kg) was cold-macerated with 1:1 dichloromethane/methanol (Sigma-  
80 Aldrich analytical grade) for 48 hours. The liquid extract was concentrated using rotary  
81 evaporator (Buchi Rotavapor® R-215) under a reduced pressure to obtain the extract. A 400  
82 g of the crude extract was fixed on Silica gel (Kieselgel 60 PF<sub>254+366</sub>) and subjected to  
83 column chromatography using n-hexane, ethyl acetate and methanol (Sigma-Aldrich  
84 analytical grade) as eluents. The different fractions were obtained and air dried at room  
85 temperature.

### 86 **2.4 Phytochemical screening**

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

#### 89 **2.4.1 Determination of total phenolic and flavonoid contents**

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

96 Total flavonoid content was determined using the aluminum chloride colorimetric method  
97 according to Chang et al. [19]. Briefly, 100 µl of each extract were mixed with 1.5 ml of 95%  
98 ethanol, 100 µl of 10 % AlCl<sub>3</sub>, and 100 µl of 1 M potassium acetate and 2.8 ml of deionized  
99 water. The absorbance of the reaction mixture was measured at 415 nm. The total flavonoid  
100 content was expressed as milligram rutin equivalent per gram extract/fraction (mg RU/g  
101 extract/fraction).

### 102 **2.5 Acute Toxicity Study**

103 The Lorke [20] procedure of LD<sub>50</sub> determination was used. The experimental mice were  
104 divided into three group (n=3) and were orally administered 10, 100 and 1000, and later,  
105 1000, 1600, 2900 and 5000 mg/kg of the extract of *A. senegalensis* stem bark. The animals  
106 were constantly observed for mortality over a period of 24 hours.

### 107 **2.6 In Vitro Antioxidant Analysis**

#### 108 **2.6.1 Test for reducing power**

109 The crude extract was subjected to reducing power assay following the method of Alam *et al.*  
110 [21]. A 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of K<sub>3</sub>Fe (CN) 6 (1% w/v)  
111 were added to 1.0 mL of sample dissolved in distilled water. The resulting mixture was  
112 incubated at 50 °C for 20 min, followed by the addition of 2.5 mL of Trichloro acetic acid  
113 (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of  
114 the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of FeCl<sub>3</sub> (0.1%, w/v).  
115 The absorbance was then measured at 700 nm against blank sample.

#### 116 **2.6.2 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity**

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

#### 122 **2.6.3 Ferric reducing antioxidant power (FRAP) potential assay**

123 The total antioxidant potential of the samples was measured by ferric reducing ability of  
124 plasma (FRAP) assay as described by Benzie and Strain [23]. Different concentrations (100-  
125 1000 µmol/L) of FeSO<sub>4</sub>.7H<sub>2</sub>O were used to prepare the standard curve, and the antioxidant  
126 capacities of the samples were determined according the reaction signal given by Fe<sup>2+</sup>  
127 solution of known concentration at absorbance of 593 nm. Ascorbic acid was measured

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

130 FRAP value ( $\mu\text{M}$ ) =  $\frac{\text{Changes in absorbance from 0-4 min}}{\text{Changes in absorbance of standard from 0-4 min}}$  x FRAP value of standard (1000  
131  $\mu\text{M}$ )

132 Changes in absorbance of standard from 0-4 min

#### 133 **2.6.4 Hydrogen peroxide scavenging assay**

134 The method of Bokhari et al. [24] was followed to investigate hydrogen peroxide scavenging  
135 capacity of samples. Hydrogen peroxide (2 mM) solution was prepared in phosphate buffer  
136 (50mM, pH 7.4). Samples (100 $\mu\text{g}$ ) were pipette into flasks and their volume made up to 400  
137  $\mu\text{l}$  with 50 mM phosphate buffer (pH 7.4).  $\text{H}_2\text{O}_2$  solution (600  $\mu\text{l}$ ) was added and  
138 absorbance at 230 nm was taken 10 min after vortexing the flasks. Percent scavenging  
139 activity was determined by following formula;

140  $\text{H}_2\text{O}_2\%$  scavenging activity =  $(1 - \frac{\text{absorbance of sample}}{\text{Absorbance of control}}) \times 100$   
141

142 Ascorbic acid served as standard.

#### 143 **2.6.5 Hydroxyl radical scavenging assay**

144 The antioxidant activity was evaluated by method reported by Halliwell and Gutteridge [25].  
145 The reaction mixture comprised of 2-deoxyribose (2.8 mM, 500 $\mu\text{l}$ ) in 50 mM of phosphate  
146 buffer, 100  $\mu\text{l}$  of 0.2 M hydrogen peroxide solution, 200  $\mu\text{l}$  of 0.1M ferric chloride, 0.1M  
147 EDTA and 100 $\mu\text{l}$  of test sample. The reaction was initiated by the addition of 100  $\mu\text{l}$  of  
148 ascorbate (0.3M). the mixture was incubated at 37 °C for 60 min. TCA (2.8% w/v, 1 ml) and 1  
149 ml of thiobarbituric acid (TBA) solution in 50 mM of sodium hydroxide (1%; w/v) was added.  
150 This reaction mixture was heated for 15 min in boiling water bath and then allowed to cool.  
151 Absorbance was recorded at 532 nm.

152 Hydroxyl scavenging activity (%) =  $(1 - \frac{\text{absorbance of sample}}{\text{Absorbance of control}}) \times 100$   
153

#### 154 **2.6.6 ABTS radical cation scavenging activity**

155 Re *et al.* [26] methodology with slight modification was followed for ABTS (2, 2 azobis,  
156 3ethylbenzothiozoline-6-sulphonic acid) radical cation scavenging activity. ABTS (7 mM)  
157 solution was reacted with 2.45 mM potassium persulfate and kept overnight in dark for  
158 generation of dark colored ABTS radicals. For the assay, the solution was diluted with 50%  
159 ethanol for an initial absorbance of 0.7 at 745 nm. Activity was determined by adding 100  $\mu\text{l}$   
160 sample of different dilution with 1 ml of ABTS solution in glass cuvette. Decrease in  
161 absorbance was measured after one min and 6 min of mixing. The difference was calculated  
162 and compared with control. Percent inhibition was calculated by formula:

163 % ABTS scavenging effect =  $\frac{(\text{control absorbance} - \text{sample absorbance})}{\text{Control absorbance}} \times 100$   
164

#### 165 **2.6.7 $\beta$ -Carotene bleaching assay**

166 Elzaawely *et al.* [27] modified method was used for  $\beta$ -carotene bleaching assay. B-Carotene  
167 (2 mg) was dissolved in 10 ml of chloroform and blended with 20 mg of linoleic acid and 200  
168 mg of Tween 20 followed by removal of chloroform under nitrogen with subsequent addition  
169 of 50 ml of distilled water and vigorous shaking to prepare  $\beta$ -carotene linoleic acid  
170 emulsion. An aliquot of each sample (50  $\mu\text{l}$ ) was mixed with 1ml of the emulsion, vortexed  
171 and absorbance was determined at 470 nm immediately against the blank solution. Capped  
172 tube was then kept in a water bath at 45 °C for 2 h and the difference between the initial  
173 readings was calculated by measuring the reading after 2 h.  $\beta$ -Carotene bleaching inhibition  
174 was estimated by the following equation:

175 % bleaching inhibition =  $\frac{(A_{0t} - A_{120t})}{A_{0t} - A_{120t}} \times 100$   
176

#### 177 **2.6.8 Superoxide anion radical scavenging assay**

178 Riboflavin light NBT system assay was followed for superoxide radical scavenging activity as  
179 described by Nishikimi [28]. The reaction mixture containing 0.5 ml of phosphate buffer (50  
180 mM, pH 7.6), 0.3 ml riboflavin (50 Mm), 0.25 ml PMS (20 mM), and 0.1 ml NBT (0.5 mM),

181 prior to the addition of 1 ml sample in methanol. Florescent lamp was used for starting the  
182 reaction. Absorbance was recorded at 560 nm after incubation for 20 min under light. The  
183 percent inhibition of superoxide anion generation was calculated using the following formula:  
184 % Percent scavenging activity = (1 – Absorbance of sample/Absorbance of control) x 100

## 185 **2.7 In Vivo Anti-Oxidant Analysis**

### 186 **2.7.1 Total proteins determination (direct biuret method)**

187 The method used for the determination of total protein was that described by Tietze [29].  
188 Three test – tubes were labeled blank (BL), standard (ST) and sample (SA) respectively  
189 before the set of the experiment. To the test-tube labeled SA, 0.02ml of serum was added.  
190 To the test –tube labeled ST, 0.02ml of standard (CAL) was added and 0.02ml was added to  
191 the test tube labeled BL. Then, 1 ml of the biuret reagent was added to all the three test  
192 tubes. After the addition, it was mixed well and incubated at 30 minutes at room temperature  
193 (20-25°C). The absorbance of the sample and standard was measured against the reagent  
194 blank at 546 nm.

### 195 **2.7.2 Lipid peroxidation assay**

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

### 204 **2.7.3 Assay of Superoxide Dismutase**

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

## 211 **2.8 Hepatoprotective Assay**

212 The rats were divided into eight groups (n=5). Group 1 served as the hepatotoxic group.  
213 Group 2 received the reference drug, Silymarin (25 ml/kg body weight), and groups 3, 4 and  
214 5 received the methanol-methylene chloride (1:1) extract (100, 200 and 400 mg/kg body  
215 weight respectively). Groups 6, 7 and 8 received 400 mg/kg of the n-hexane, Ethylacetate  
216 and methanol fractions respectively. These were given to the animals once daily for 3 days.  
217 On day 3, one hour after administration of the appropriate drug for each group, carbon  
218 tetrachloride was given to the different groups, and 48 hours after carbon tetrachloride  
219 administration, blood was collected from the retro-orbital plexus to be used for the  
220 assessment of biochemical parameters.

### 221 **2.8.1 Liver function tests**

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

## 228 **2.9 Statistical analysis**

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

232

### 233 3. RESULTS AND DISCUSSION

234

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

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

244 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

245

#### 246 3.1 Acute Toxicity Test (LD<sub>50</sub>)

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

#### 249 3.2 Phytochemical Analysis

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

256

257 **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	+	-	-	+

<b>Resins</b>	+	+	+	+
<b>Steroids</b>	+	-	+	-

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

259

260

**Table 3: Total flavonoids and total phenolic contents**

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

261 Values expressed in mean ± SEM; n = 3

262

### 263 3.3 Antioxidant Assays

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

#### 269 3.3.1 *In Vitro* Antioxidant Assay

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

281 In the present study, the results showed that in all the *in vitro* antioxidant models tested, the  
 282 crude extract and the fractions from n-hexane, ethyl acetate and MeOH exhibited  
 283 appreciable levels of antioxidant activities when compared to ascorbic acid. However, the  
 284 ethyl acetate fractions exhibited the greatest activity than others and ascorbic acid in most  
 285 cases, judging from their lower IC<sub>50</sub> values (Table 4). Ethyl acetate fraction exhibited greater  
 286 activity than other extract/fractions with IC<sub>50</sub> value of 121.67µg/ml. The crude extract from  
 287 FRAP assay indicated more inhibition than other fractions. Hydrogen radical scavenging  
 288 capacity of Ethyl acetate fraction with IC<sub>50</sub> value of 75.67±6.33µg/ml indicates higher activity  
 289 than others when compared with the standard with IC<sub>50</sub> of 62.00±1.73µg/ml. Ethyl acetate

290 indicates higher activity than others in beta-carotene bleaching activity, hydrogen peroxide,  
 291 superoxide radical and ABTS scavenging activities.  
 292 Since FRAP assay is easily reproducible and linearly related to molar concentration of the  
 293 antioxidants present, it can be reported that extract/fractions of *A. senegalensis* may act as  
 294 free radical scavenger, capable of transforming reactive free radical species into stable non  
 295 radical products [23]. Hydroxyl radical scavenging capacity of an extract or compound is  
 296 directly proportional to its antioxidant activity which is depicted by the low intensity of red  
 297 colour. The sample of *A. senegalensis* when added to the reaction mixture actively  
 298 scavenged the hydroxyl radicals and prevented the degradation of 2-deoxyribose [25]. The  
 299 extract and fractions of *A. senegalensis* possessed strong ABTS scavenging activity as  
 300 noticed by their ability to generate a blue/green ABTS + chromophore from the reaction of  
 301 ABTS and potassium persulfate [26]. Because  $\beta$ -carotene is extremely sensitive to free  
 302 radical mediated oxidation of linoleic acid, it is commonly used to analyse antioxidant  
 303 potential of test samples [27]. Our results show that the tested samples inhibited  $\beta$ -carotene  
 304 oxidation which could be attributed to free hydroxyl groups found in them. Superoxide and  
 305 hydroxyl radicals are important mediators of oxidative stress that play vital role in some  
 306 clinical disorders. Any compound, natural or synthetic with antioxidant activities might  
 307 contribute towards the total/partial alleviation of such damage. Therefore, removing  
 308 superoxide and hydroxyl radical could contribute to defense of a living body against disease  
 309 [31].  
 310

311 **Table 4: IC<sub>50</sub> Values of different antioxidant Assays of *A. senegalensis* (IC<sub>50</sub>,  $\mu$ g/ml)**

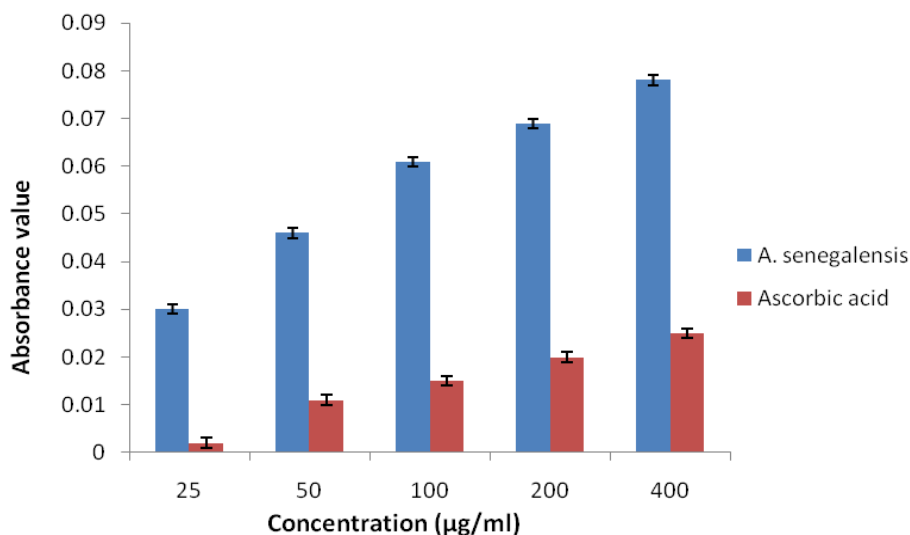
Test Method Activity	Crude Extract	n-hexane fraction	Ethyl Acetate fraction	MeOH fraction	Standard (Ascorbic acid)
<b>DPPH Scavenging activity</b>	752.67±15.60 <sup>a</sup>	355.67±4.49 <sup>b</sup>	121.67±2.40 <sup>d</sup>	293.67±16.91 <sup>c</sup>	69.67±1.86 <sup>e</sup>
<b>FRAP activity</b>	63.00±12.22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

312 Values with different letters as superscript across a row are significantly different at p < 0.05

313 Values expressed as mean  $\pm$  standard error; n = 5

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

317 **3.3.2 In Vivo Antioxidant Study**

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

327

328 **Table 5: Result of *in vivo* antioxidant study**

Ext./fraction	Dose (Mg/kg)	SOD (Mg/dl)	L.H <sub>2</sub> O <sub>2</sub> (mmol/l)	Vit. C (Mg/dl)	T. Protein (Mg/dl)
Ext.	100	38.00±4.56 <sup>a</sup>	0.32±0.04 <sup>a</sup>	14.40±2.25 <sup>b</sup>	7.66±0.54 <sup>b</sup>
"	200	30.40±3.66 <sup>b</sup>	0.26±0.02 <sup>ab</sup>	18.60±1.57 <sup>a</sup>	8.42±0.43 <sup>a</sup>
"	400	23.00±2.00 <sup>c</sup>	0.20±0.012 <sup>b</sup>	20.00±2.24 <sup>a</sup>	10.46±0.53 <sup>a</sup>
<b>FRACTIONS</b>					
MeOH	400	24.80±2.71 <sup>c</sup>	0.18±0.02 <sup>c</sup>	18.40±2.01 <sup>a</sup>	9.60±0.58 <sup>a</sup>
EtOAc	400	19.80±0.80 <sup>c</sup>	0.17±0.014 <sup>c</sup>	20.40±1.12 <sup>a</sup>	10.84±0.56 <sup>a</sup>
n-Hex	400	34.20±3.57 <sup>ab</sup>	0.27±0.03 <sup>ab</sup>	17.60±1.57 <sup>a</sup>	8.54±0.38 <sup>a</sup>
Crude Ext.	400	23.00±2.00 <sup>c</sup>	0.20±0.02 <sup>b</sup>	20.00±2.24 <sup>a</sup>	10.46±0.53 <sup>a</sup>
3% Tween 80	5ml/kg	40.00±2.30 <sup>a</sup>	0.37±0.02 <sup>a</sup>	10.40±0.51 <sup>c</sup>	6.38±1.12 <sup>b</sup>

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

330 Values expresses as mean  $\pm$  S.E. (standard error) n = 5; SOD = superoxide dismutase, L.H<sub>2</sub>O<sub>2</sub> – lipid  
 331 peroxide, T. protein = total protein.  
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### 333 3.4 Biochemical Tests (Liver Function Tests)

334 Table 6 below shows the liver function tests of *A. senegalensis*. It shows that higher blood  
 335 serum enzyme values were recorded for the animals treated with CCl<sub>4</sub> only, which signify  
 336 CCl<sub>4</sub> intoxication and liver damage in normal rats. A reduction in the blood serum enzyme by  
 337 the plant drug was also dose and solvent dependent. The 100 mg/kg extract and n-hexane  
 338 fraction had the least reductions (by 27.34% and 29.95% respectively) in the serum AST. At  
 339 200 and 400 mg/kg extract, the serum AST was reduced (by 40.34% and 45.66%  
 340 respectively) as much as the MeOH fraction (43.88%) and control (43.44%), whereas EtOAc  
 341 fractions gave significantly the best reduction (52.49%). Similarly, the MeOH and EtOAc  
 342 fractions compared favorably with the control based on the values of the serum ALP and  
 343 ALT, and total bilirubin levels. Patrick-Iwuanyanwu et al. (2010) also reported  
 344 hepatoprotective property of ethylacetate fraction of *Tapinanthus bagwensis* to be higher  
 345 than methanol and n-hexane fractions. This could be attributed to their different polarity and  
 346 degrees of extractability [37].

347 The hepatoprotective study was undertaken to demonstrate the protective ability of the  
 348 methanol-methylene chloride (1:1) extract of *A. senegalensis* on liver damage induced by  
 349 Carbon tetrachloride (CCl<sub>4</sub>) and the toxic effects of the similar doses in rats. Carbon  
 350 tetrachloride has been reported to be metabolized to CO<sub>2</sub> in the liver homogenates. It  
 351 produces free radicals, which affect the cellular permeability of hepatocytes leading to  
 352 elevated levels of serum biochemical parameters such as alanine transaminase (ALT),  
 353 aspartate transaminase (AST) and alkaline phosphatase (ALP) [38]. The levels of serum  
 354 AST, ALT and ALP were taken as indices for oxidativxe stress induced by CCl<sub>4</sub>. The serum  
 355 activities in concentrations of these enzymes decreased significantly in the animals treated  
 356 with the samples when compared to the untreated group, and this suggests that the extract  
 357 and fractions of *A. senegalensis* have hepatoprotective activities. This is in agreement with  
 358 the commonly accepted view that serum levels of AST, ALT and ALP return to normal with  
 359 the healing of hepatic parenchyma and the regeneration of hepatocytes [33]. The positive  
 360 results obtained in this study could be attributed to the presence of high concentration of  
 361 flavonoids, phenols, terpenoids, and steroids in the extracts and fractions of *A. senegalensis*.  
 362 The extracts of the stem bark of the plant contain antioxidants and hepatoprotective activity  
 363 through regulatory action on cellular permeability, stability and suppressing oxidative stress  
 364 [34, 35].

365  
 366 **Table 6: Biochemical tests (Liver function tests).**

EXTRACT	DOSES (mg/kg)	AST	ALP	ALT	T. BIL
		Mean $\pm$ SEM (% reduction)	Mean $\pm$ SEM (% reduction)	Mean $\pm$ SEM (% reduction)	Mean $\pm$ SEM (% increase)
CCl <sub>4</sub> Only	0.5 ml/kg	223.13 $\pm$ 7.53 <sup>a</sup>	491.23 $\pm$ 10.21 <sup>a</sup>	201.23 $\pm$ 8.11 <sup>a</sup>	4.54 $\pm$ 0.82 <sup>a</sup>
Ext. + CCl <sub>4</sub>	100	162.12 $\pm$ 11.01 <sup>b</sup> (27.34)	260.20 $\pm$ 12.82 <sup>b</sup> (47.07)	99.80 $\pm$ 2.31 <sup>b</sup> (50.41)	2.78 $\pm$ 0.16 <sup>b</sup> (38.77)
“	200	133.11 $\pm$ 11.90 <sup>c</sup> (40.34)	219.43 $\pm$ 8.19 <sup>c</sup> (55.33)	67.80 $\pm$ 4.43 <sup>c</sup> (66.31)	2.10 $\pm$ 0.14 <sup>c</sup> (53.74)
“	400	121.24 $\pm$ 14.18 <sup>c</sup>	186.32 $\pm$ 8.77 <sup>c</sup>	58.00 $\pm$ 3.33 <sup>c</sup>	2.08 $\pm$ 0.14 <sup>c</sup>

		(45.66)	(62.07)	(71.18)	(54.19)
<b>FRACTIONS</b>					
<b>MeOH + CCl<sub>4</sub></b>	400	125.21±12.56 <sup>c</sup>	175.12±16.55 <sup>c</sup>	63.80±7.47 <sup>c</sup>	2.08±0.54 <sup>c</sup>
		(43.88)	(64.35)	(68.29)	(54.19)
<b>EtOAc + CCl<sub>4</sub></b>	400	106.22±11.56 <sup>d</sup>	145.32±18.63 <sup>d</sup>	59.40±10.30 <sup>c</sup>	1.80±0.15 <sup>d</sup>
		(52.49)	(70.42)	(70.48)	(60.35)
<b>n-Hex + CCl<sub>4</sub></b>	400	156.30±11.43 <sup>b</sup>	187.43±10.63 <sup>c</sup>	74.40±3.84 <sup>b</sup>	2.80±0.11 <sup>b</sup>
		(29.95)	(61.84)	(63.03)	(38.33)
<b>Control</b>	25 ml/kg	126.21±10.80 <sup>c</sup>	153.25±8.80 <sup>d</sup>	51.10±5.42 <sup>d</sup>	1.56±0.01 <sup>e</sup>
<b>(Silymarin)</b>		(43.44)	(68.80)	(74.61)	(65.64)

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

368 Values expresses as mean ± S.E. (standard error) n = 5; AST = aspartate transaminase; ALP =  
369 alkaline phosphatase; ALT = alanine transaminase; T.BIL = total bilirubin.

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#### 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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