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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 CCl₄ 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 CCl₄-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, CCl₄ 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 PF₂₅₄₊₃₆₆) 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 % AlCl₃, 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 LD₅₀ 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 K₃Fe (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 FeCl₃ (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 FeSO₄.7H₂O were used to prepare the standard curve, and the antioxidant
126 capacities of the samples were determined according the reaction signal given by Fe²⁺
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 (μM) = $\frac{\text{Changes in absorbance from 0-4 min}}{\text{Changes in absorbance of standard from 0-4 min}}$ x FRAP value of standard (1000
131 μM)

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 μg) were pipette into flasks and their volume made up to 400
137 μl ? with 50 mM phosphate buffer (pH 7.4). H_2O_2 solution (600 μl ?) 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 μl ?) in 50 mM of phosphate
146 buffer, 100 μl ? of 0.2 M hydrogen peroxide solution, 200 μl ? of 0.1M ferric chloride, 0.1M
147 EDTA and 100 μl of test sample. The reaction was initiated by the addition of 100 μl of
148 ascorbate? (0.3M). the? mixture was incubated at 37 °C for 60 min. TCA ?What denominates this
149 abbreviation? (2.8% w/v, 1? ml) and 1? ml of thiobarbituric acid)?TBA) solution in 50 mM of
150 sodium hydroxide (1%; w/v) was added. This reaction mixture was heated for 15 min in
151 boiling water bath and then allowed to cool. 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 azo bis, 3-
156 ethylbenzothiozoline-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 μl
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 β -Carotene bleaching assay**

166 Elzaawely *et al.* [27] modified method was used for β -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 shacking to prepare β -carotene linoleic acid
170 emulsion. An aliquot of each sample (50 μl) 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. β -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?What denominates this abbreviation? (20

181 mM), and 0.1 ml NBT (0.5 mM), prior to the addition of 1 ml sample in methanol. Florescent
182 lamp was used for starting the reaction. Absorbance was recorded at 560 nm after
183 incubation for 20 min under light. The percent inhibition of superoxide anion generation was
184 calculated using the following formula:

185 % Percent **sacavenging** activity = (1 – Absorbance of sample/Absorbance of control) x 100

186 **2.7 In Vivo Anti-Oxidant Analysis**

187 **2.7.1 Total proteins determination (direct biuret method)**

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

196 **2.7.2 Lipid peroxidation assay**

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

205 **2.7.3 Assay of Superoxide Dismutase**

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

212 **2.8 Hepatoprotective Assay**

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

222 **2.8.1 Liver function tests**

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

229 **2.9 Statistical analysis**

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

233

234 3. RESULTS AND DISCUSSION

235

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

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

245 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

246

247 3.1 Acute Toxicity Test (LD₅₀)

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

250 3.2 Phytochemical Analysis

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

257

258 **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	+	-	+	-

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

260

261

Table 3: Total flavonoids and total phenolic contents

Extract/Fraction	Total flavonoid content of <i>A. senegalensis</i> (mg rutin equivalent/g extract or fraction)	Total phenolic content of <i>A. senegalensis</i> (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
Ethyl Acetate Fraction	587.33±50.83	582.00±1.73
MeOH fraction	113.67±9.28	115.33±3.84

262 Values expressed in mean ± SEM; n = 3

263

264 3.3 Antioxidant Assays

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

270 3.3.1 In Vitro Antioxidant Assay

271 Several techniques have been used to determine the antioxidant activity *in vitro* in order to
 272 allow rapid screening of substances [21]. The most commonly used is the 1, 1-diphenyl-2-
 273 picrylhydrazyl radical (DPPH) method. The DPPH test is a method that estimates the free
 274 radical scavenging activity of antioxidants through de-colourisation of the DPPH solution.
 275 The concentration and efficacy of the antioxidant is determined from the degree of colour
 276 change. A drastic decrease in the absorbance of the reaction mixture therefore signifies high
 277 free radical scavenging activity of the test sample [22]. In this study, we also analysed the
 278 antioxidant activity of the samples via several other models such as FRAP - which was
 279 based on the samples' ability to reduce Fe³⁺/Fe²⁺ [23], hydrogen peroxide scavenging assay,
 280 hydroxyl radical scavenging assay, ABTS cation radical scavenging assay, β-carotene
 281 bleaching assay and superoxide anion radical scavenging assay.

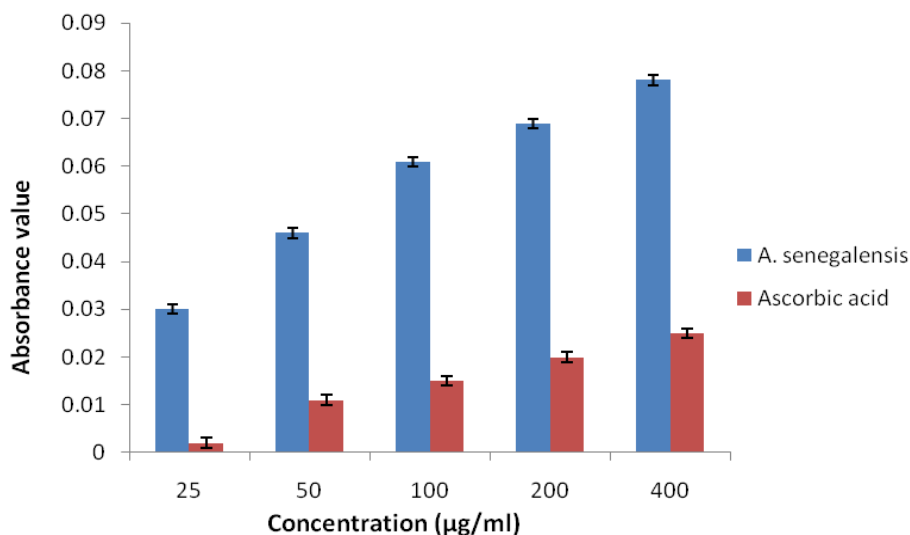
282 In the present study, the results showed that in all the *in vitro* antioxidant models tested, the
 283 crude extract and the fractions from n-hexane, ethyl acetate and MeOH exhibited
 284 appreciable levels of antioxidant activities when compared to ascorbic acid. However, the
 285 ethyl acetate fractions exhibited the greatest activity than others and ascorbic acid in most
 286 cases, judging from their lower IC₅₀ values (Table 4). Ethyl acetate fraction exhibited greater
 287 activity than other extract/fractions with IC₅₀ value of 121.67µg/ml. The crude extract from
 288 FRAP assay indicated more inhibition than other fractions. Hydrogen radical scavenging
 289 capacity of Ethyl acetate fraction with IC₅₀ value of 75.67±6.33µg/ml indicates higher activity
 290 than others when compared with the standard with IC₅₀ of 62.00±1.73µg/ml. Ethyl acetate

291 **The denomination of ethyl acetate should be unified in all text.** indicates higher activity than others in
 292 beta-carotene bleaching activity, hydrogen peroxide, superoxide radical and ABTS
 293 scavenging activities.
 294 Since FRAP assay is easily reproducible and linearly related to molar concentration of the
 295 antioxidants present, it can be reported that extract/fractions of *A. senegalensis* may act as
 296 free radical scavenger, capable of transforming reactive free radical species into stable non
 297 radical products [23]. Hydroxyl radical scavenging capacity of an extract or compound is
 298 directly proportional to its antioxidant activity which is depicted by the low intensity of red
 299 colour. The sample of *A. senegalensis* when added to the reaction mixture actively
 300 scavenged the hydroxyl radicals and prevented the degradation of 2-deoxyribose [25]. The
 301 extract and fractions of *A. senegalensis* possessed strong ABTS scavenging activity as
 302 noticed by their ability to generate a blue/green ABTS + chromophore from the reaction of
 303 ABTS and potassium persulfate [26]. Because β -carotene is extremely sensitive to free
 304 radical mediated oxidation of linoleic acid, it is commonly used to analyse antioxidant
 305 potential of test samples [27]. Our results show that the tested samples inhibited β -carotene
 306 oxidation which could be attributed to free hydroxyl groups found in them. Superoxide and
 307 hydroxyl radicals are important mediators of oxidative stress that play vital role in some
 308 clinical disorders. Any compound, natural or synthetic with antioxidant activities might
 309 contribute towards the total/partial alleviation of such damage. Therefore, removing
 310 superoxide and hydroxyl radical could contribute to defense of a living body against disease
 311 [31].
 312

313 **Table 4: IC₅₀ Values of different antioxidant Assays of *A. senegalensis* (IC₅₀, μ g/ml)**

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

314 Values with different letters as superscript across a row are significantly different at p < 0.05
 315 Values expressed as mean \pm standard error; n = 5 **Please, define more exactly what do you mind with**
 316 **superscript^{a, b, c, d} in all Tables 4 - 6?**
 317



318

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

320 **3.3.2 In Vivo Antioxidant Study**

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

330

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

Ext./fraction	Dose (Mg/kg)	SOD (Mg/dl)	L.H ₂ O 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

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

333 Values expresses as mean \pm S.E. (standard error) n = 5; SOD = superoxide dismutase, L.H₂O₂ – lipid
 334 peroxide, T. protein = total protein.
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336 3.4 Biochemical Tests (Liver Function Tests)

337 Table 6 below shows the liver function tests of *A. senegalensis*. It shows that higher blood
 338 serum enzyme values were recorded for the animals treated with CCl₄ only, which signify
 339 CCl₄ intoxication and liver damage in normal rats. A reduction in the blood serum enzyme by
 340 the plant drug was also dose and solvent dependent. The 100 mg/kg extract and n-hexane
 341 fraction had the least reductions (by 27.34% and 29.95% respectively) in the serum AST. At
 342 200 and 400 mg/kg extract, the serum AST was reduced (by 40.34% and 45.66%
 343 respectively) as much as the MeOH fraction (43.88%) and control (43.44%), whereas EtOAc
 344 fractions gave significantly the best reduction (52.49%). Similarly, the MeOH and EtOAc
 345 fractions compared favorably with the control based on the values of the serum ALP and
 346 ALT, and total bilirubin levels. Patrick-Iwuanyanwu et al. (2010) also reported
 347 hepatoprotective property of ethylacetate fraction of *Tapinanthus bagwensis* to be higher
 348 than methanol and n-hexane fractions. This could be attributed to their different polarity and
 349 degrees of extractability [37].

350 The hepatoprotective study was undertaken to demonstrate the protective ability of the
 351 methanol-methylene chloride (1:1) extract of *A. senegalensis* on liver damage induced by
 352 Carbon tetrachloride (CCl₄) and the toxic effects of the similar doses in rats. Carbon
 353 tetrachloride has been reported to be metabolized to CO₂ in the liver homogenates. It
 354 produces free radicals, which affect the cellular permeability of hepatocytes leading to
 355 elevated levels of serum biochemical parameters such as alanine transaminase (ALT),
 356 aspartate transaminase (AST) and alkaline phosphatase (ALP) [38]. The levels of serum
 357 AST, ALT and ALP were taken as indices for oxidative stress induced by CCl₄. The serum
 358 activities in concentrations of these enzymes decreased significantly in the animals treated
 359 with the samples when compared to the untreated group, and this suggests that the extract
 360 and fractions of *A. senegalensis* have hepatoprotective activities. This is in agreement with
 361 the commonly accepted view that serum levels of AST, ALT and ALP return to normal with
 362 the healing of hepatic parenchyma and the regeneration of hepatocytes [33]. The positive
 363 results obtained in this study could be attributed to the presence of high concentration of
 364 flavonoids, phenols, terpenoids, and steroids in the extracts and fractions of *A. senegalensis*.
 365 The extracts of the stem bark of the plant contain antioxidants and hepatoprotective activity
 366 through regulatory action on cellular permeability, stability and suppressing oxidative stress
 367 [34, 35].

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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 ₄ Only	0.5 ml/kg	223.13 \pm 7.53 ^a	491.23 \pm 10.21 ^a	201.23 \pm 8.11 ^a	4.54 \pm 0.82 ^a
Ext. + CCl ₄	100	162.12 \pm 11.01 ^b (27.34)	260.20 \pm 12.82 ^b (47.07)	99.80 \pm 2.31 ^b (50.41)	2.78 \pm 0.16 ^b (38.77)
“	200	133.11 \pm 11.90 ^c (40.34)	219.43 \pm 8.19 ^c (55.33)	67.80 \pm 4.43 ^c (66.31)	2.10 \pm 0.14 ^c (53.74)
“	400	121.24 \pm 14.18 ^c	186.32 \pm 8.77 ^c	58.00 \pm 3.33 ^c	2.08 \pm 0.14 ^c

		(45.66)	(62.07)	(71.18)	(54.19)
FRACTIONS					
MeOH + CCl₄	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 + CCl₄	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 + CCl₄	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)

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

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

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4. CONCLUSION

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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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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