Antioxidant Activity and Cytotoxic Studies of Anogeissus leiocarpous Root, Leaf and Stem

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Abstract

Anogeissus leiocarpus (Combretaceae), is well known for its medicinal uses in African traditional medicine for treating many human diseases and infections. Many antioxidant based drug formulations are used in the prevention and treatment of diseases whose mechanisms involve oxidative stress. Due to the carcinogenic effect of synthetic antioxidants, searching for new sources of naturally occurring antioxidants is of interest. The present study was carried out to investigate and compare the antioxidant activity and cytotoxicity of alcoholic crude extracts and their petroleum ether, chloroform and ethyl acetate fractions of the leaf, stem and root of *A. leiocarpus* growing in Sudan. The iron chelating and DPPH radical scavenging techniques were used for assessment of antioxidant activity compared to EDTA and Propylgallate as standard antioxidant agents. The brine shrimp standard method was used for the assessment of cytotoxicity of the bioactive extracts. The bioactive fractions were subjected to TLC and HPLC analysis and the results showed remarkable antioxidant activity of the plant extracts in both antioxidant techniques compared to standard antioxidant drugs, in addition to the chloroform fraction which showed the highest antioxidant activity. The cytotoxicity results showed that, there is no cytotoxic effect in all bioactive fractions. A comparison of RP/ HPLC-DAD chromatograms of the bioactive fractions confirmed the presence of different phenolic derivatives of quercetin and ellagic acid known for their antioxidant activity.

Keywords: Antioxidant activity, Cytotoxic, *Anogeissus*, *leiocarpus*, Leaf, Stem, Root.

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Introduction

Oxidative stress is the result of balance disruption between the systems generating free radicals and the systems permitting their elimination, leading to excess of highly reactive oxygen species (Tibiri, *et al*., 2010). Under pathological conditions they results in oxidative stress, and leads to oxidative modification in cellular membrane or intracellular molecules. Highly reactive oxygen species (ROS) are exacerbating factors in cellular injury and aging process (Chang *et al*., 2009; Satheesh *et al*., 2010).They cause injury by reacting with bio-molecules such as lipids, proteins, and nucleic acid and known to be involved in many human diseases (Georgetti *et al*., 2003). The antioxidants provide protection to living organisms from damage caused by uncontrolled production of ROS (Satheesh *et al*., 2010). They intervene at different levels in the oxidative process such as scavenging free radicals and lipid peroxide radicals or removing oxidative damaged bio-molecules (Tibiri, *et al*., 2010; Ersoy *et al*., 2011). Because of restrictions on synthetic antioxidants due to their carcinogenicity, the interest in finding new sources of naturally occurring antioxidants has increased to replace the synthetic ones (Tibiri, *et al*., 2010; Gallegos-Tintoré *et al*., 2010). Therefore, screening of plant materials on the basis of their antioxidant potency seems to be of central importance. Many ntioxidants are introduced to minimize actions of ROS, e.g. phenolic compounds can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes. Recently, a great interest has been given to naturally occurring antioxidants, which play important role in inhibiting both free radicals and oxidative chain-reactions within tissues and membranes (Georgetti *et al*., 2003; Chang *et al*., 2009; Tibiri, *et al*., 2010; Gallegos-Tintoré *et al*., 2010).

Combretaceae family is the family of trees or shrubs, widely distributed in tropical Africa, Asia and South America. The different species of the family are well known for their medicinal uses. *Anogeissus leiocarpus* is one of the important species of the genus *Anogeissus*, (Combretaceae). It is an evergreen tree widely distributed in Africa and well known in African traditional medicine for treating of many diseases (Angeh *et al.*, 2007; Mann *et al.*, 2008a; Eloff *et al.*, 2008; Adejumobi *et al.*, 2008; Agaie *et al.*, 2007a; Okpekon *et al.*, 2004; Chaabi, *et al.*, 2006; Mann *et al.*, 2009b). Many antioxidant metabolites were reported in the family: flavonoids, terpenoids, tannins and polyphenolic compounds such as ellagitannins and glycosidic ellagitannins, stilbenes, and other phenolic compounds (Adigun *et al.*, 2000; Mann *et al.*, 2008a; Mann *et al.*, 2009a; Yoshida *et al.*, 2010; Harbone *et al.*, 1999; Seigler, 1998; Pan *et al.*, 2008). The genus *Anogeissus*, is a rich source of various antioxidants such as, phenolics and tannins. Many species of the genus have yielded various glycosides and their derivatives (Saleemulla *et al.*, 2008; Pradeep *et al.*, 2009; Khalid *et al.*, 2002; Govindarajan *et al.*, 2005). Stilbenes, Phenolics and tannins glycosides isolated from *A. acuminate* were found responsible for its antioxidant activity (Rimando *et al.*, 2002; Moses *et al.*, 2009).

The present paper reports the results of antioxidant activity and cytotoxic properties of the plant leaf, stem and root extracts prepared with solvents of different polarities and assayed by the DPPH radical scavenging technique and EDTA and propylgallate standards. The chemical composition of thebioactive extracts fractions was investigated by the RP HPLC-DAD technique.

Materials and Methods

Plant Material Collection and Preparation

The root , leaf and stem of *A. leiocarpus* were collected separately from healthy trees in El Damazeine area in Sudan, and they were identified at the department of siliviculture, Faculty of Forestry, University of Khartoum, and the voucher specimens were deposited in the Herbarium of the Department of Biochemistry, Commission of Biotechnology and Genetic Engineering,

National Centre for Research. The bark and root were chipped using sawmill and were shade dried, ground to a coarse powder using electric grinder. The leaves were ground into powder using pestle and mortar.

Preparations of the Extracts

Plant powdered materials were macerated over night with 80%alcohol, and the extracts were fractionated by using solvents with increasing polarities: petroleum (PE), chloroform ($CHCl_{3)}$ and ethyl acetate (EtOAc). The solvents were evaporated to dryness under reduced pressure using a rotary evaporator.

Antioxidant Activity Test

Free Radical Scavenging Assay

In the free radical scavenging experiment (Mensor *et al*., 2001; Kexue *et al.*, 2006), 10 µl from the extracts (5mg/ml) were added to 90µl of 300µM DPPH solution placed in a 96-well microtiter plate. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. The mixture was incubated in the dark at room temperature for 30 min. After incubation, the absorbance of the remaining DPPH was read against a blank at 517 nm using multiplate reader spectrophotometer. Propylgallate was used as the positive control and DMSO as the negative control. All tests and analyses were carried out in triplicate. The inhibition of free-radical DPPH in percent (%) or the capacity to scavenging the DPPH radical (radical scavenging activity) was expressed as EC50 value (mg ml-1), and calculated by the following equation:

$$\text{Scavenging activity (\%)} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

Iron Chelating (Iron (III) Reduction) Assay

Iron chelating assay (Farzana *et al*. 2005; Puangpronpitag, *et al.*, 2010) is based on the reduction of Fe3+/ferricyanide complex to the ferrous (Fe^{+2}) form (Ferrozin forms a complex with Fe^{+2}) in the presence of reductants (antioxidants) in tested samples. The iron chelating ability was determined by formation of ferrous ion form (Fe^{+2}), which were monitored by measuring the formation of ferrous ion-ferrozine complex, monitored by measuring the formation of Perl's

Prussian blue at 700 nm. A decrease in absorption values can be determined by changes in the color. The decrease in absorption shows the effectiveness of chelating agent added with the exception of ferrozin. The experiment was carried out in 96 well microtiter plate with a final volume of 100 µl/well. 10 µl from the extracts (5mg/ml DMSO), were mixed with 30 µl FeSO4, then the reaction was initiated by adding 60 µl from 5mM ferrozine. The mixture was then shaken and left at room temperature for 10 min. after which the absorbance was measured at 562 nm. DMSO used as a negative control while, EDTA was used as standard antioxidant (positive control). All tests and analysis were run in triplicate. The absorbance of the mixture was measured at 562 nm against a blank (DMSO) and the ability of extracts to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating activity (\%)} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

Where A control is the absorbance of control and A sample is the absorbance of the compounds tested. The ferrous ion-chelating activity was expressed as median effective concentration (EC_{50}; mg mL^{-1}) which represented the concentration of sample at which the chelating activity was 50%.

Cytotoxicity Test

The cytotoxicity of bioactive extracts of *A. leiocarpus* were screened by using the brine shrimp (*Artemia salina*) standard method (Khan *et a*l. , 2007; Hussain *et a*l. , 2007; Batista *et a*l. , 2009; Hameed *et a*l. , 2010). A rectangular dish (22 x 32 cm) was compartmentalized into two unequal halves with plastic divider of 2 mm with several holes and filled with artificial seawater (28 g sea salt/L, Sigma). Approximately 50mg eggs (*Artemia salina* Sera, Heidelberg, Germany) were sprinkled in the larger compartment, which was darkened, while the smaller compartment was illuminated. 0.5 ml of 100, 1000 and 10,000 ppm concentrations of the extract prepared in respective solvent (methanol) [20mg of the extracts were dissolved in 2ml of and 5, 50, 500 µl, the concentrations were 10,100 and 1000µg/ml respectively] was poured in vials (3 vials / concentrations) and kept at room temperature to evaporate methanol. After 24 hours, phototropic nauplii (brine shrimp larvae) were collected by a Pasteur pipette from the lightened side, and10 shrimps were transferred to each vial. The vials were placed under the illumination at room temperature, and the volumes were made up to 5ml with sea water, and then incubated at (25-27

ºC) for 24 hours under illumination. Other vials were supplemented with solvent and reference cytotoxic drug (Etoposide) as negative and positive control respectively. The numbers of survivors were counted after 24 hours. The data were analyzed with finney computer program, and the lethal concentrations 50% (LD50) were determined.

LC-Triple Quadruple Spectrometric Analysis (LC-MS/MS)

LC-MS/MS system was equipped with:

RP-C18 HPLC column and Diode array UV detector (DAD) recorded at 320 – 380 nm for the detection of compounds.

RP-HPLC joined with a Finnigan LCQ ion trap mass spectrometer with the Electrospray Ionization (ESI) interface at negative ion mode.

Collision induced dissociation (CID) experiment was performed for fragmentation of glycoside and elucidation of compounds structures.

Results and Discussions

The results of *in vitro* antioxidant activity of *A. leiocarpus* extracts are shown in Table 1and figure 1. The effect of varying type of solvent used in the extraction, varying parts of the plant and varying techniques were the three variables examined, and the extracts dissolution solvent was used as negative control. The results showed that, all these fractions have strong radical scavenging activity (RSA) ranged from 45% to 66%, While in the iron chelating technique, the results range from 6% to 32%. The leaf ethyl acetate fraction possesed the highest antioxidant activity (66 ± 0.02), followed by root ethyl acetate (60 ± 0.01) and chloroform bark (58 ± 0.00) compared to the standard antioxidant (Propylgallate of 70± 0.00). The results showed that, the antioxidant activity of *A. leiocarpus* extracts, varied from one morphological part to another and from one fraction to another as well as the technique employed. The potent antioxidant activity results of *A. leiocarpus* was compatible with the current literature of the potent antioxidant activity of the other species in this genus, previously reported in *A. acuminata* by Rimando *et al*., 2002 and Moses *et al*., 2009 and in *A. latifolia* by Govindarajan *et al*., 2004a,b & 2005.

Table 1: Antioxidant activity of *A. leiocarpus* extracts using ferrous iron ferrozine complex and DPPH free radical scavenging (RSA) techniques

Extract /Drug	% of DPPH free radical activity (±SD) (Conc. 500µg / mL)	% of ferrous iron ferrozine complex activity (± SD) (Conc. 500µg / mL)
Leaf/ CHCL3	45 ± 0.01	17±0.10
Leaf/ EtOAc	66 ± 0.02	27±0.03
Bark/ CHCL3	58 ± 0.00	32±0.30
Bark/ EtOAc	55 ± 0.00	6 ±0.01
Root/ CHCL3	47 ± 0.02	18±0.20
Root/ EtOAc	60 ± 0.01	19±0.04
Propylgallate	70± 0.00	_
EDTA	_	80±0.30

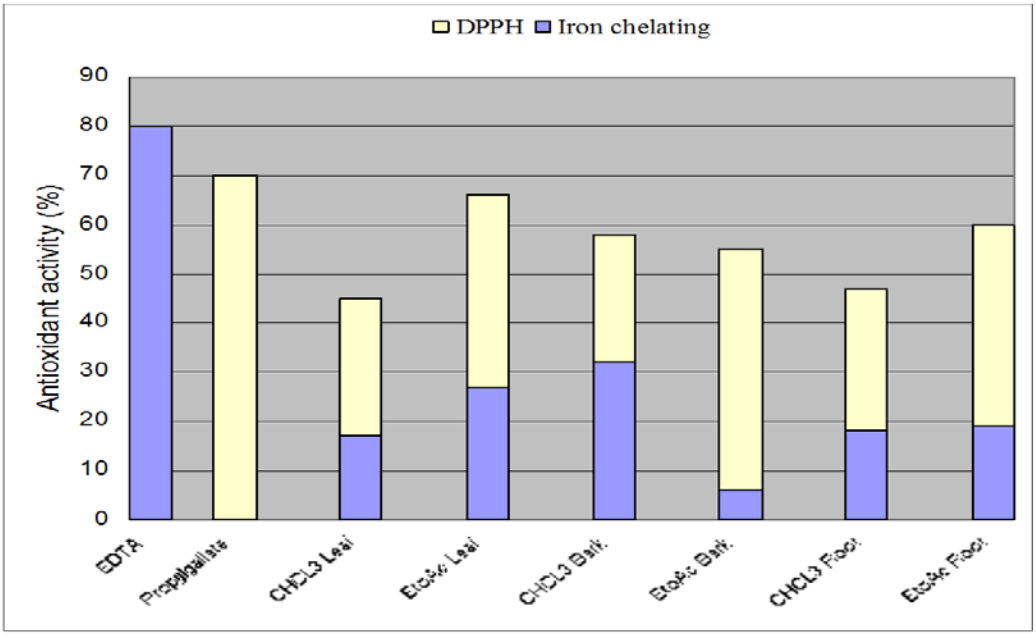
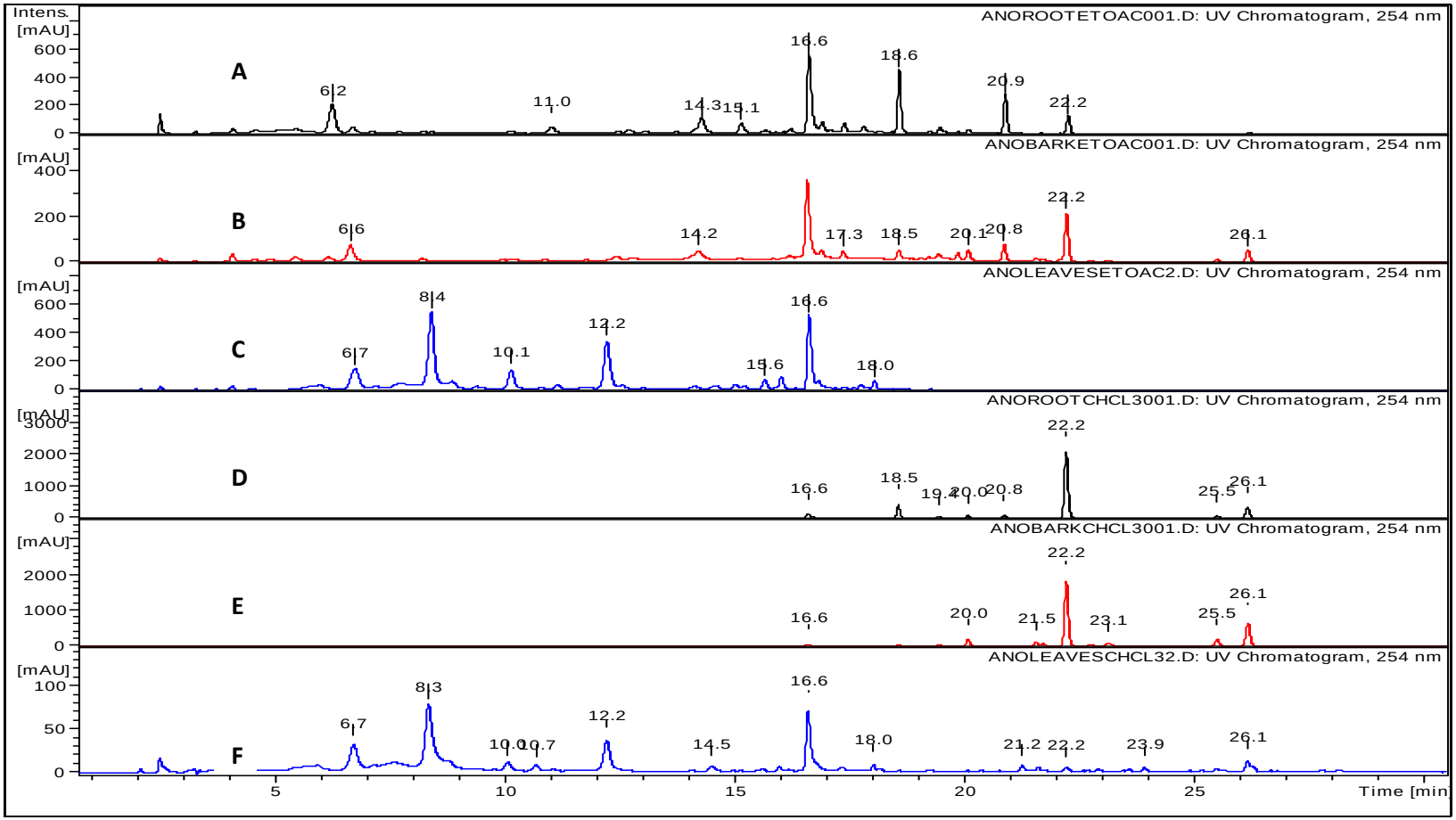


Figure 1: Antioxidant activity of *A. leiocarpus* extracts.

RP HPLC-DAD chromatogram comparative results of six bioactive fractions of the three studied parts of the plant namely, ethyl acetate root (A), ethyl acetate bark (B), ethyl acetate leave (C), chloroform root (D), chloroform bark (E) and chloroform leaf at λ_{max} 254nm and 300-380 nm are presented in Figures 2 and 3. These UV range enabled the detection of phenolic metabolites (Cuyckens & Claeys, 2004). The chromatograms clearly represented the presence of many similar phenolic compounds which were well known for their antioxidant activity (Govindarajan *et al.*, 2005).

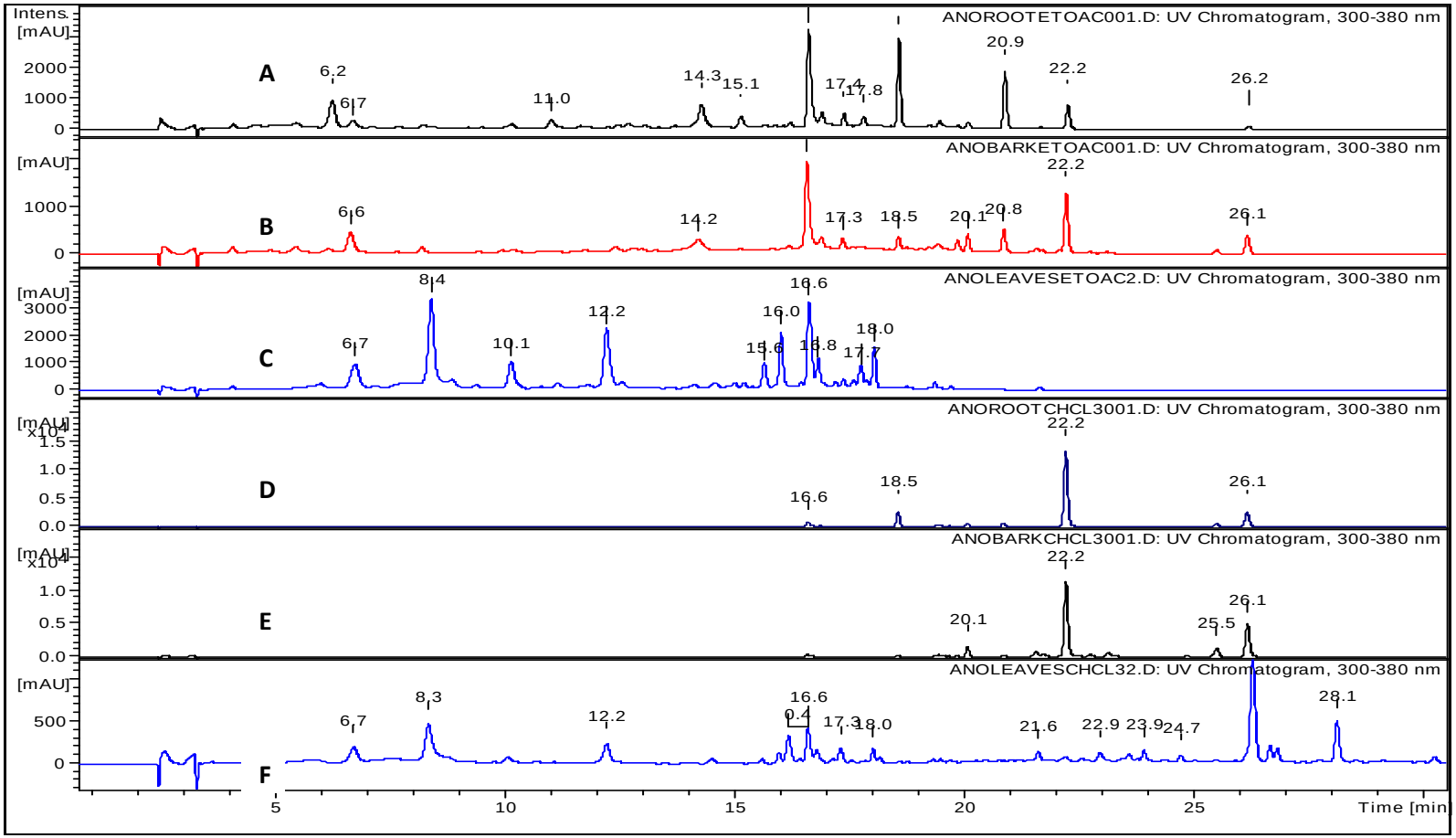
The activity result and chromatographic analysis were compatible to each other and to current literature. The chromatographic analysis of the leaf ethyl acetate fraction with highest antioxidant activity (66 ± 0.02), was shown in Table 2, the analysis showed the accumulation of ellagic acid derivatives and quercetin derivatives. Phenolic antioxidants function primarily as terminators of the free radical reactions (Georgetti *et al*., 2003), they can trap the free radicals directly or scavenge them (Chang *et al.*, 2009), Ellagic acid is a very strong anti-oxidant agent. It has a potent DPPH radical scavenging activity (Teel, 1986; Daniel *et al.*, 1990; Maas *et al.*, 1991; Devipriya *et al.*, 2007; Sturm *et al.*, 2009; Stoner & Mukhtar, 1995, Govindarajan *et al.*, 2005), so the *A. leiocarpous* extracts showed antioxidant activity with DPPH highest than the iron chelating. Quercetin was reported to exert strong anti-oxidative (Gao *et al.*, 2002; Gong & Chen, 2003; Pradeep *et al.*, 2009; Nieman *et al.*, 2010), it had the greatest activity among the different flavonoids (Buhler & Miranda, 2009). Flavonoids have been exerting beneficial effects on some diseases involving uncontrolled ROS. The capability to interact as antioxidants, iron-chelating, and free radical scavenging activity may account for the wide pharmacological profile of flavonoids (Georgetti *et al*., 2003). The results explain the traditional use of the plant in the treatment of many diseases, while, the antioxidants suggested to prevent many types of diseases (Stanner *et al*., 2004).



A. EtOAc root B. EtOAc bark C. EtOAc leaves

D. $CHCl_3$ root E. $CHCl_3$ bark D. $CHCl_3$ leaves

Figure 2: RP-HPLC-DAD chromatograms of the ethyl acetate and chloroform phases of the *A. leiocarpus* leaf, bark and root recorded at λ_{max} 254nm.



A. EtOAc root B. EtOAc bark C. EtOAc leaf

D. $CHCl_3$ root E. $CHCl_3$ bark F. $CHCl_3$ leaf

Figure 3: RP-HPLC-DAD chromatograms of the ethyl acetate and chloroform phases of the *A. leiocarpus* leaf, bark and root recorded at λ_{max} 300-380nm.

Table 2: RP-HPLC data (Peak NO. & Rt.); MS/MS data (molecular weight {*m/z*} & main fractions {*m/z*}) and assignment structures of the ethyl acetate fraction of *A. leiocarpus* leaves

Compound Peak	(R_t) (min	M-H (*m/z*)	CID M^n main fraction ions(*m/z*)	Compound Name
1	6.8	541	425, 377, 301, 275, 271, 229, 201,173	Di- Hydroxyl-Tri-Methoxy ellagic acid-7-O-β-glucoside
2	8.5	552	481, 301, 275, 271, 243	Di- Hydroxyl-Tri- Methoxy ellagic acid-7-O-β-glucoside derivative
3	15.7	615	463, 301, 300, 271, 255, 229, 193,178,151,107	Quercetin-3-O-galloyl- 7-O-β-glucoside
4	16.8	301	283, 271, 257, 240, 229, 228, 217, 202, 185, 173,139, 89	Ellagic acid
5	16.8	463	381, 301, 300, 271, 255, 229, 214,179,175, 151,107	Quercetin-7-O-β-glucopyranoside
5	18.2	447	365,301,300,283,271,255,229 ,211,179,151,107	Quercetin-7-O-β-Rhamnoside
7	22.5	329	314, 299, 299, 285, 271, 271	3, 3'-di-O-methylellagic acid
8	25.7	343	328, 313, 313, 298, 298, 285, 270	Tri-O-methylellagic acid
9	26.2	359	344, 329, 314,314,300, 285	Tetra-O-methylellagic acid

The cytotoxicity results of the bioactive leaf, bark and root fractions of the plant are shown in Table 3. All tested shrimps were resistant to the highest concentration used (1000µg/ml) and clearly demonstrated that, the extracts were devoid of any cytotoxic effect. It is noteworthy that the leaves of the plant showed no cytotoxicity in sheep according to Agaie & Onyeyili (2007a,b)

Table 3: Cytoxicity of bioactive fractions of leaves bark and root of *A. leiocarpus*

Plant part	Extract/ Treatment	Concentration	Total Shrimps/ (3vials), 10/vial	Shrimps Survive	LD50 (mg/ml)
Leaves	Chloroform	10µg/ml 100µg/ml 1000µg/ml	31 32 31	31 32 29	0 0 0
	Ethyl acetate	10µg/ml 100µg/ml 1000µg/ml	32 31 31	32 31 29	0 0 0
Barks	Chloroform	10µg/ml 100µg/ml 1000µg/ml	30 32 29	30 32 29	0 0 0
	Ethyl acetate	10µg/ml 100µg/ml 1000µg/ml	31 31 29	31 31 29	0 0 0
Roots	Chloroform	10µg/ml 100µg/ml 1000µg/ml	31 31 30	31 30 30	0 0 0
	Ethyl acetate	10µg/ml 100µg/ml 1000µg/ml	31 31 30	31 31 29	0 0 0
-	Etopoisde	10µg/ml	30	14	7.4

Conclusion

It could be concluded that *A. leiocarpus* possesses remarkable antioxidant properties. The observed activity displays a wide variation in correlation with the plant parts as well as the variation in the solvents used; thus generating different chemical compounds; in addition to the variation in the technique that has been used.

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