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Comparative study on phytochemical constituents, antioxidant activity and acute toxicity of extracts of *Alstonia boonei* de Wild and *Anthocleista djalonensis*

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Abstract
The phytochemical constituents, *in vitro* antioxidant activity and acute toxicity of the aqueous and ethanol stem-bark extracts of *Alstonia boonei* and *Anthocleista djalonensis* were investigated. Phytochemical quantification test revealed that the aqueous extract of *A. djalonensis* had a significantly higher ($p<0.05$) amount of total phenol than other extracts. The ethanol extract of *A. boonei* however had significantly higher levels ($p<0.05$) of total flavonoid and proanthocyanidin. DPPH's radical scavenging activity of the aqueous extract of *A. djalonensis* (IC_{50} of 8.75±0.22) was better than that of the other extracts and close to that of ascorbic acid standard (IC_{50} 1.12±0.23). The extracts had significantly higher ($p<0.05$) ability to reduce ferric ions to the ferrous form when compared with the ascorbic acid standard. Although the extracts possessed significantly lower ($p<0.05$) reducing potential than the ascorbic acid standard, both extracts of *A. djalonensis* were able to inhibit lipid peroxidation with the aqueous counterpart proving to be more superior. The acute toxicity study involved the administration of a single oral dose of the extracts at varying concentrations. LD_{50} of both extracts of *A. djalonensis* and the ethanol extract of *A. boonei* were beyond 5000 mg/kg body weight of the extract. However, for the aqueous extract of *A. boonei* some signs of toxicity were observed. These included increase in respiratory rate for the animals that received 3000 and 5000 mg/kg and eventual death at the latter dose. These results suggest that the aqueous extract of *A. djalonensis* possess better antioxidant property than the other extracts studied.

Keywords: *Alstonia boonei*; *Anthocleista djalonensis*; Phytochemical constituents; Antioxidant capacity; Acute toxicity

INTRODUCTION

Medicinal plants have been employed in the treatment of several ailments for ages. This provided the impetus for researchers to study their use in the management of various disorders for which they are implicated (Randrianarivelojosia *et al*., 2003; Saedi *et al*., 2014).

Alstonia boonei, a large evergreen tree belonging to the family Apocynaceae is one of the widely used medicinal plants in Africa and beyond. It is reported to possess antimalarial, antipyretic, analgesic and anti-inflammatory properties (Olajide *et al.*, 2000). There are also reports of its use as diuretic, immuno-stimulant, antipsychotic and

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anxiolytic (Kucera *et al.*, 1972; Elisabetsky *et al.*, 2006; Taiwo *et al.*, 1998).

Anthocleista djalonensis belongs to the family of Loganiaceae. It is commonly referred to as the Cabbage tree. Its leaf, root and stem bark have been employed ethnomedicinally in the treatment of wound, constipation, diarrhea, dysentery and abdominal pain (Okoli and Iroegbu, 2004; Aiyeloja and Bello, 2006). Okoli and Iroegbu, (2004) reported the use of the roots of this plant in combination with those of *Nauclea latifolia* and *Uvaria afzalii* for the treatment of sexually transmitted diseases (STDs). They also showed that the cold water and ethanol extracts of the roots have remarkable bacteriostatic and bactericidal activities to gram-positive and gram-negative bacterial strains.

Radicals are atoms or groups of atoms with one or more unpaired electrons which makes them highly reactive. They can therefore steal electrons from macromolecules wreaking havoc in the process. Radicals derived from oxygen referred to as reactive oxygen species (ROS) are of most concern to biological systems (Valko *et al*., 2004). ROS are produced during normal and pathological cell metabolism. Living organisms are equipped with antioxidant defense system which helps to neutralize the damaging effects of free radicals (de Beer *et al*., 2002). However, when reactive oxygen species are produced in excess, they have the potential of overwhelming this defense thereby initiating pathophysiological processes such as diabetes, inflammation, cancer, liver injury and cardiovascular diseases (Liao and Yin, 2000).

Recently, interest in the search of naturally occurring antioxidants from plants has been rekindled. As a continuation towards the search of naturally occurring antioxidant candidate plants, this research work compared the phytochemical constituents, *in vitro* antioxidant activity as well as the acute toxicity of the aqueous and ethanol extracts of the stem-barks of *Alstonia boonei* and *Anthocleista djalonensis* using Swiss albino mice as an animal model.

EXPERIMENTAL

Collection, identification and extraction of plant materials. Plants and plant parts of *Alstonia boonei* and *Anthocleista djalonensis* were collected in July; at a forest area in Ikpoba Hill, Benin city with the help of an herbalist. They were thereafter identified and authenticated by a Botanist in the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Nigeria.

The stem-barks of both plants were thereafter washed and air dried under shade. The dried stem-barks were pulverized and then extracted with ethanol and distilled water successively at room temperature for 72 hours with stirring at interval. The extracts (ethanol and aqueous extracts) obtained were concentrated to dryness at 40^{o}C using a rotary evaporator under reduced pressure (Ayoola *et al*., 2008). The dried extracts were weighed and then stored at 4^{o}C for subsequent analysis.

Phytochemical screening Phytochemical screening was carried out on the plant samples using standard protocols as described by Harborne (1973), Sofowora (1993) and Evans, (1989). A stock solution of each extract, with a concentration of 0.1 g extract/100 mL absolute methanol, was prepared and used for the phytochemical screening.

Total phenolic content (TPC). Total phenolic content was determined according to the Folin and Ciocalteu's method (1927). Concentrations (0.2 - 1 mg/mL) of gallic acid were prepared in methanol. Then, 0.5 mL of the sample (1 mg/mL) was mixed with 2.5 mL of a ten-fold diluted Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate. The mixture was allowed to stand for 30 min

at room temperature then absorbance read at 760 nm. All determinations were performed in triplicates with gallic acid utilized as the reference control.

Total flavonoid content (TFC). Total flavonoid content was determined using the method described by Ayoola *et al*., (2008). Briefly, 2 mL of 2% $AlCl_3$ in ethanol was added to 2 mL extracts. A concentration of 1 mg/mL of the extract prepared in methanol was used. Similar concentrations of the standard control quercetin, were used. The absorbance was measured at 420 nm after 1 h.

Proanthocyanidin content. Determination of proanthocyanidin was carried out according to the method of Sun *et al.* (1998). Briefly, to 0.5 mL of 1.0 mg/mL of each extract was added 1 mL of 4 % methanol solution and 0.75 mL of concentrated hydrochloric acid. The mixture was left undisturbed for 15 minutes and the absorbance was read at 500nm. Ascorbic acid was used as standard.

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity. The capacity of the plant extracts to scavenge 1, 1–diphenyl–2–picrylhydrazyl (DPPH) free radical was determined by a slightly modified method of Brand-Williams *et al*. (1995). Briefly, 0.5 mL of 0.3 mM DPPH solution in methanol was added to 2ml of various concentrations (0.2 - 1.0 mg/mL) of the extracts. The reaction tubes were shaken and incubated for 15 min at room temperature in the dark, and the absorbance read at 517 nm. All tests were performed in triplicate. Vitamin C was used as reference control, with similar concentrations as the test samples prepared. A blank containing 0.5ml of 0.3 mM DPPH and 2ml methanol was prepared and treated as the test samples.

The radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%)

$$= [(A_0 - A_1)/ (A_0)] \times 100$$

Where A_0 = absorbance of DPPH radical + methanol;

A_1 = absorbance of DPPH radical + sample extract or standard.

The 50% inhibitory concentration (IC_{50}) value was calculated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radicals.

Ferric reducing antioxidant power (FRAP). A modified method of Benzie and Strain (1996) was adopted for the ferric reducing antioxidant power (FRAP) assay. This depends on the ability of the sample to reduce the ferric tripyridyltriazine [Fe (III)-TPTZ] complex to ferrous tripyridyltriazine [Fe (II) – TPTZ] at low pH. Fe (II)-TPTZ has an intensive blue colour which can be read at 593 nm. To 1.5 mL of freshly prepared FRAP solution (25 mL of 300 mM acetate buffer pH 3.6, 2.5 mL of 10mM 2,4,6-tripyridylstriazine (TPTZ) in 40mM HCl, and 2.5 mL of 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) solution) was mixed with 1mL of the extracts at various concentrations (0.2-1.0 mg/mL). The reaction mixtures were incubated at 37^{o}C for 30 min and increase in absorbance at 593 nm measured. $FeSO_4$ was used for calibration and values expressed as μmol $FeSO_4$ equivalents per gram of sample. Ascorbic acid served as the reference control.

Reducing power (RP). The reducing power of extract was determined according to the method described by Lai *et al.* (2001). Briefly, 1 ml of different concentrations of extracts (0.1-1.0 mg/mL) in water was mixed with 2.5 mL of 0.2 M phosphate buffer, pH 6.6 and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50^{o}C for 20 min. Thereafter, 2.5 mL of trichloroacetic acid (10%) was added to the mixture to stop the reaction. 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ was thereafter added. Higher absorbance values indicated higher reducing power. Ascorbic acid (Vitamin C) was used as standard.

Thiobarbituric acid reactive substances (TBARS). TBARS was estimated according

to the method of Ohkowa *et al*., (1979). Egg yolk homogenate (0.5 mL of 10% v/v) and 0.1 mL of extract were added to a test tube and made up to 1mL with distilled water. 0.05mL of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 mL of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.05 mL 20% TCA were added and the resulting mixture was vortex; it was then heated at 95ºC for 60 min. The generated color was measured at 532 nm. Inhibition of lipid peroxidation (%) was calculated with the formula:

$$(C\text{-}E)/C \times 100\%$$

where C is the absorbance value of the fully oxidized control and E is (Abs_{532}+TBA -$Abs_{532\text{-}TBA}$).

Acute toxicity study. A total of twenty-eight (28) male mice of the Swiss strain weighing between 20-30 g were obtained from the Nigerian Institute of Medical Research (NIMR) and used for the acute toxicity of these plant extracts. This was done according to the Organization for Economic and Cultural Development (OECD) method (OECD, 2001). The animals were allowed a one-week acclimatization period during which they were allowed access to mice chow and water *ad libitum*. Thereafter, they were randomly divided into seven (07) groups of four (04) mice per group. Groups 1 to 6 served as treatment groups and received 300, 500, 1000, 2000, 3000 and 5000 mg/kg body weight of either the aqueous or ethanol extract of *A. boonei* and *A. djalonensis* while the 7th group received 0.5 mL of normal saline and served as control.

Clinical observation. Animals were observed for signs of toxicity immediately after extract administration at 0, 2, 4, 6, 8 and 24 hrs. Thereafter, they were observed daily for a period of 14 days. Signs of toxicity that were examined included lacrimation, paw-licking, posture, diarrhea, tremors, convulsions, respiratory rate and mortality (Adebiyi and Abatan, 2013).

Statistical analysis. All analyses were replicated thrice. Where applicable, the results were expressed as mean ± SEM. The data were subjected to one-way analysis of variance (ANOVA). P values less than 0.05 ($p < 0.05$) were regarded as statistically significant. IC_{50} was calculated using the EZ-fit enzyme kinetics software version 5.03.

RESULTS

Results of the phytochemical screening of extracts of *A. djalonensis* and *A. boonei* showed that more of the phytochemicals were detected in the aqueous extract of *A. djalonensis* when compared with the other extracts studied (Table 1). Tannins were not detected in all extracts but for that of the aqueous extract of *A. djalonensis*. As for steroids, they were not detected in all extracts studied.

Total phenol content of the extracts is reported in figure 1 as mg gallic acid equivalent/g extract with reference to a standard curve ($Y = 0.802x + 0.302$; $R^2 = 0.933$). Total phenolic content was significantly higher ($p< 0.05$) for the aqueous extract of *A. djalonensis* and lowest for the aqueous extract of *A. boonei*.

Total flavonoid is reported as mg quercetin equivalent/g extract with reference to the standard curve ($Y = 0.005x + 0.390$; $R^2 = 0.940$). While proanthocyanidin is reported as mg/g ascorbic acid equivalent with reference to the standard curve ($Y = 0.003x + 0.012$; $R^2 = 0.727$). Amongst the extracts, the ethanol extract of *A. boonei* had the highest amount of total flavonoid. While the highest amount of proanthocyanidin was observed in the ethanol extracts of both plants (Figure 2).

The DPPH's radical scavenging activities of the plant extracts are presented in figure 3. The ethanol extract of *A. djalonensis* had the best ability of inhibiting the DPPH's radical at all concentrations studied. Its

aqueous counterpart however had the best ability to inhibit DPPH's radical at the lowest concentration studied. This ability however decreased with increasing concentrations. IC_{50} values of all the extracts studied were seen to be higher than the ascorbic acid standard. The aqueous extract of *A. djalonensis* however possessed the lowest IC_{50} value (8.75 ± 0.22 µg/ml) than other extracts (Table 2). The ferric reducing antioxidant potential (FRAP) of *A. boonei* and *A. djalonensis* extracts is depicted in figure 4. FRAP is reported as µmole Fe (II)/g extract by reference to the standard curve ($Y = 0.004x + 0.582$; $R^2 = 0.530$). All plant extracts were seen to have a better ability to reduce ferric ions to the ferrous form than the standard ascorbic acid with the aqueous extract of *A. djalonensis* having a significantly higher ($p< 0.05$) ability than the other plant extracts studied.

The reducing potential of the plant extracts is presented in figure 5. All extracts showed significantly lower reducing potential than the standard ascorbic acid at all concentrations studied ($p < 0.05$). The aqueous extract of both study plants however had considerably higher reducing powers than the ethanol counterparts. Inhibition of lipid peroxidation by extracts of *A. boonei* and *A. djalonensis* is presented in figure 6. Inhibition of lipid peroxidation was highest for the aqueous extract of *A. djalonensis* and lowest for the aqueous extract of *A. boonei*.

The LD_{50} of both extracts of *A. djalonensis* as well as ethanol extract of *A. boonei* were above 5000 mg/kg body weight as the mice tolerated the extracts without any signs of toxicity. For the aqueous extract however, signs of toxicity were recorded for the animals that received 3000 mg/kg and 5000 mg/kg body weight of this extract. The animals exhibited rapid respiratory rate which was followed by death of the animal that received 5000 mg/kg body weight of the extract.

Table 1: Phytochemical screening of extracts of *A. boonei* and *A. djalonensis* stem-barks

Phytochemicals	*A.b* Aq.	*A.b* Et.	*A.d* Aq.	*A.d* Et.
Flavonoids	+	-	+++	+
Tannins	-	-	+	-
Cardiac glycosides	+	+	+	+
Terpenoids	+	++	+	++
Alkaloids	+	+	+	+
Saponins	++	+	+++	+
Steroids	-	-	-	-

Key: +++ = Very high, ++ = Moderately high, + =Low, - = Not detected.
A.b = Alstonia boonei, *A.d = Anthocleista djalonensis*

Table 2: IC_{50} values of extracts of *A. boonei* and *A. djalonensis* stem-barks

Sample	IC_{50} Values
A. boonei Aq.	42.36±4.88 µg/mL^{b}
A. boonei Et.	44.16±0.63 µg/mL^{b}
A. djalonensis Aq.	8.75±0.22 µg/mL^{c}
A. djalonensis Et.	38.81±1.43 µg/mL^{d}
Ascorbic acid Std.	1.12±0.23 µg/mL^{a}

Data are presented as Mean ± S.E.M; n =3. Different lower case letters represent significant difference between means at $p< 0.05$

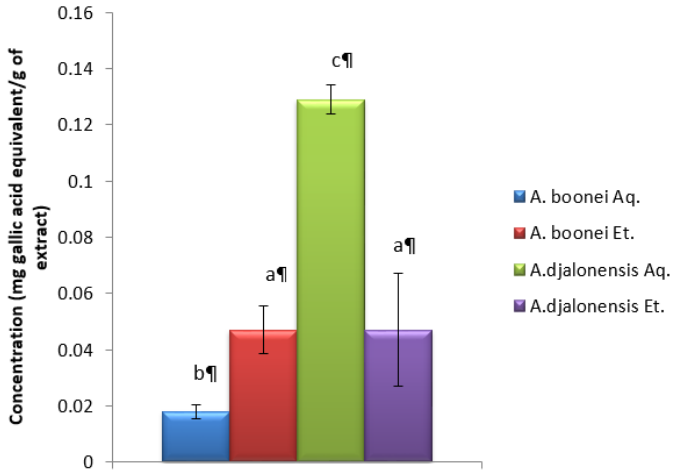

Fig 1: Total Phenolic content of extracts of *A. boonei* and *A. djalonensis*
Different lower case letters represent significant difference between means at p< 0.05.

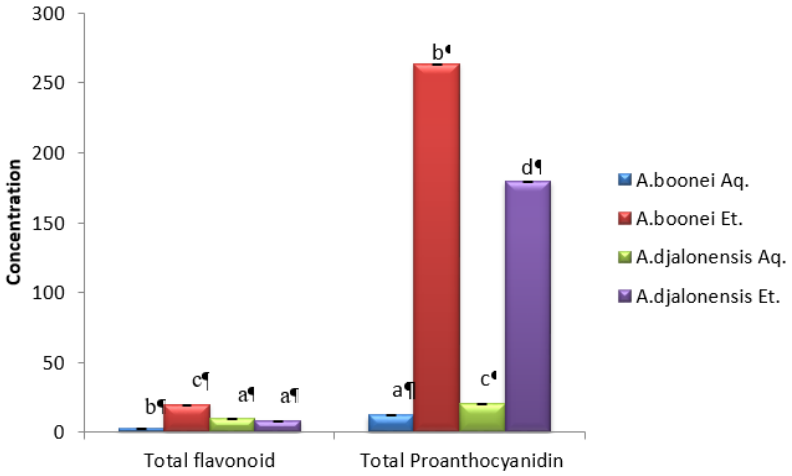

Fig 2: Total flavonoid and proanthocyanidin contents of extracts of *A. boonei* and *A. djalonensis*
Different lower case letters represent significant difference between means at p< 0.05.

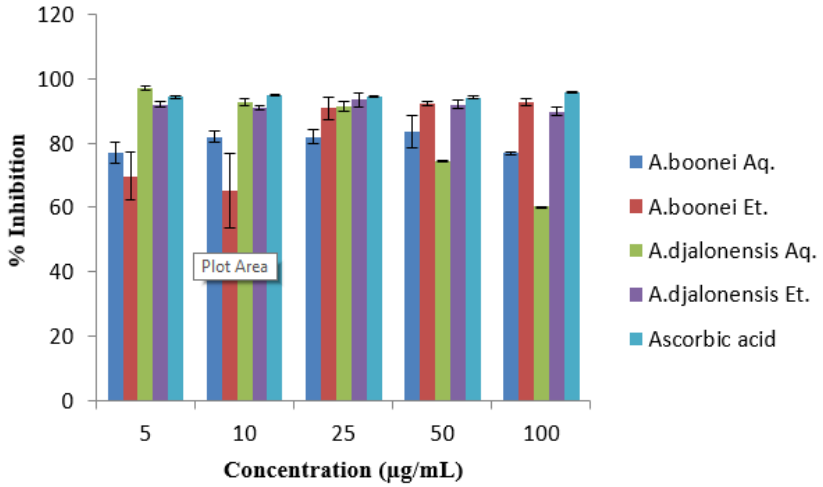

Fig 3: DPPH's radical scavenging activities of extracts of *A. boonei* and *A. djalonensis*

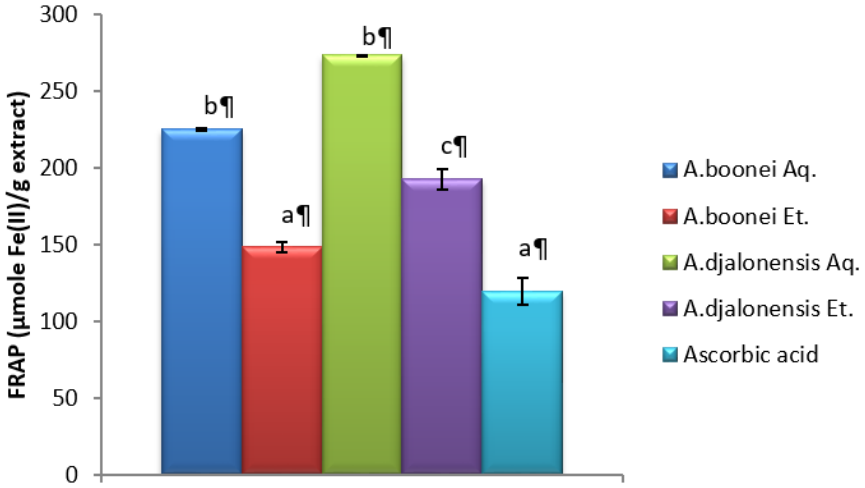

Fig 4: Ferric reducing antioxidant potential (FRAP) of extracts of *A. boonei* and *A. djalonensis* Different lower case letters represent significant difference between means at $p< 0.05$.

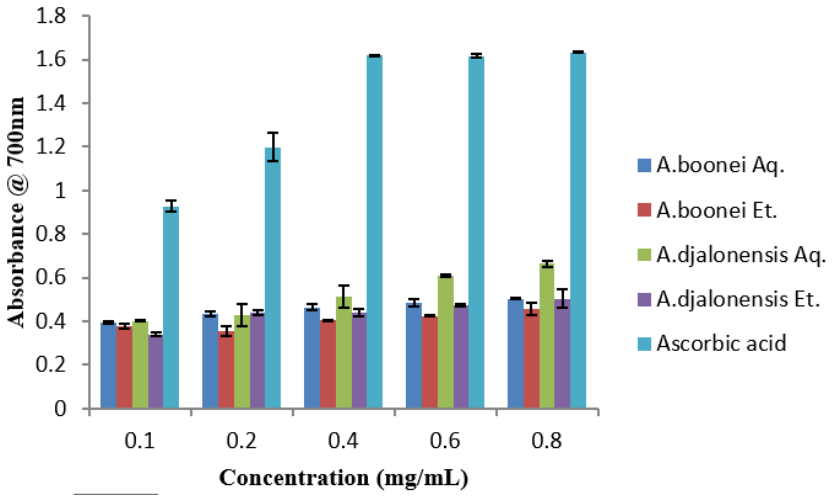

Fig 5: Reducing potential of extracts of *A. boonei* and *A. djalonensis*

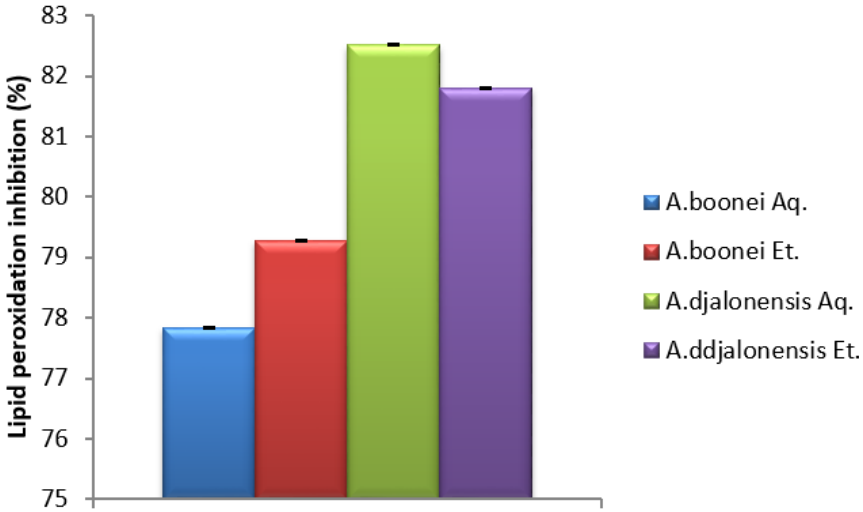

Fig 6: Percentage (%) inhibition of lipid peroxidation by extracts of *A. boonei* and *A. djalonensis*

DISCUSSION

Polyphenols are members of a large family of plant derived compounds reported to have beneficial effects on human health. They are said to possess high antioxidant capacity because of the hydroxyl (-OH) group(s) directly bonded to phenyl ring(s) making them easily donate electrons to electron seeking free radicals by so doing, neutralizing their capacity to wreak havoc to macromolecules (Ayoola *et al*., 2011). In the present study, the aqueous extract of *A. djalonensis* was found to have significantly higher ($p< 0.05$) amount of total phenolics than the other extracts.

Plant phenolics can either be of flavonoid or non-flavonoid origin. Flavonoids are the most abundant and most important plant polyphenolics having many health promoting benefits (Padmanabhan and Jangle, 2012). These attributes are conferred on them by the ortho 3',4'-dihydroxy moiety in their B-ring for electron delocalization and stability of the phenoxy radical, the 2,3-double bond in combination with the 4-keto group for electron delocalization in their C- and A-rings respectively combined with the 4-keto group in their C-ring which is necessary for their maximum scavenging potentials (de Beer *et al*., 2002). In this study, the ethanol extract of *A. boonei* showed a significantly higher amount ($P < 0.05$) of total flavonoid in contrast to the other extracts. The same trend was observed for total proanthocyanidins. Proanthocyanidins yield anthocyanins when treated with acids (de Beer *et al*., 2002). Anthocyanins are a subgroup of phenolics known as condensed tannins. Lately, they are becoming important as antioxidants (Devi *et al*., 2011).

DPPH, formally known as 1, 1-diphenyl-2-picrylhydrazyl is a cell permeable, stable free radical that is commonly used to evaluate the ability of compounds to act as free radical scavengers or hydrogen donors and to measure the antioxidant activity of tissue extracts (Kedare and Singh, 2011). The reaction of DPPH with an antioxidant or reducing compound produces the corresponding hydrazine $DPPH_2$ which absorbs maximally at 517nm. The outcome of this study revealed that the ethanol extract of *A. djalonensis* were the best inhibitors of the DPPH radical at all concentrations studied while the aqueous extract of *A. djalonensis* showed better ability to inhibit the DPPH radical at the lowest concentration studied. This ability however decreased with increasing concentrations. A similar finding was reported for the methanolic extract of *Eupatorium odoratum* by Omoregie *et al*., (2014). IC_{50} is an important variable estimated from DPPH radical scavenging assays which expresses the amount of antioxidant required to decrease the DPPH radical by 50% (Chada *et al*., 2011). IC_{50} is negatively related to antioxidant activity. The lower the IC_{50} value, the higher the antioxidant capacity of the tested sample (Chade *et al*., 2011). In this study, the IC_{50} of all the extracts studied were significantly lower than the standard ascorbic acid. But compared to other extracts, the aqueous extract of *A. djalonensis* possessed the lowest IC_{50} value which translates into a better DPPH's radical scavenging ability. However, the trend in its DPPH's radical scavenging ability suggests that this extract may lose its DPPH's radical scavenging ability at higher concentrations.

Ferric reducing antioxidant potential measures the ability of plant extracts to reduce ferric ions (Fe^{3+}) to the ferrous form (Fe^{2+}). Unlike DPPH assay which reflects only the activity of water soluble antioxidants, FRAP assay measures the total antioxidant power of biological fluids (Oikeh *et al*., 2014). All extracts studied had a significantly higher ($p< 0.05$) ability of reducing ferric ions to the ferrous form. The aqueous extract of *A. djalonensis* had a significantly higher ($p< 0.05$) ferric reducing antioxidant potential

than the other extracts studied with that of the ethanol extract of *A. boonei* being the lowest.

Oikeh *et al*., (2014) opined that the reducing potential of a compound may give a better clue as to its reductive capacity. The reducing potential measures the antioxidative capacity of extracts by assessing their ability of donating electrons to electron seeking free radicals. Although all plant extracts studied had significantly lower ($P < 0.05$) reducing potential than the standard ascorbic acid, the aqueous extract of *A. djalonensis* had the highest reducing potential compared to the other extracts.

Oxidative stress occurs when there is an imbalance in the production and neutralization of free radicals which may result in significant damage to cell structure and function (Reuter *et al*., 2010). Recently, there has been an increased interest on natural antioxidants of plant origin with strong antioxidant activities and low cytotoxicity. In this study, our results showed that the ability to reduce lipid peroxidaton was highest for the aqueous extract of *A. djalonensis* and lowest for the aqueous extract of *A. boonei*.

The first step in the toxicological evaluation of an unknown sample is the investigation of its acute toxicity (Prohp and Onoagbe, 2012). In this study, the LD_{50} of both extracts of *A. djalonensis* and the ethanol extract of *A. boonei* were established to be above 5000 mg/kg body weight of extract. Values above 5000 mg/kg body weight are said to be of no practical significance (Lorke, 1983). For the aqueous stem-bark extract of *A. boonei*, administration of 3000 mg/kg and 5000 mg/kg body weight of the extract resulted in increased respiratory rate which was followed by death of the animal that received 5000 mg/kg body weight of extract. Our result suggests that the LD_{50} of this extract is between 3000 and 5000 mg/kg body weight of the extract. This is in agreement with that of Awodele *et al*., (2010) who reported an LD_{50} of 4168.89 mg/kg body weight of the extract using the same animal model. However, other researchers (Nkono *et al*., 2014) reported an LD_{50} value greater than 5000 mg/kg body weight of extract and a subchronic toxicity dose greater than 1000 mg/kg body weight (Nkono *et al*., 2015). This group of researchers however used a different animal model. Various factors may affect the LD_{50} of a substance including species differences (Klaaseen, 2008). Accordingly, while our results suggest that the LD_{50} of this extract is between 3000 and 5000 mg/kg body weight of the extract, this may have been influenced by interspecies variation.

CONCLUSION

In conclusion, the aqueous extract of *A. djalonensis* possesses a better antioxidative capacity than the other extracts. It has a significantly higher amount of total phenol. However, the same cannot be said of its flavonoid content. Although phenolic compounds such as flavonoids, phenolic acids and tannins may be major contributors to the antioxidant capacity of plants, the optimal effectiveness of a medicinal plant may not be due to one major constituent but may be as a result of the combined action of different compounds present in the plant (Bhandarkar and Khan, 2003). Indeed, more phytochemicals are present in the aqueous extract of *A. djalonensis* than the other extracts studied. The antioxidant profile of this plant can be harnessed to treat free radical mediated diseases.

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