

Full Length Research Paper

The *in vitro* antioxidant property of methanolic extract of *Azelia africana* (Smith.)

D. A. Akinpelu^{1,2}, O. A. Aiyegoro¹ and A. I. Okoh^{1*}

¹Department of Biochemistry and Microbiology, Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa.

²Department of Microbiology Obafemi Awolowo University, Ile Ife, Nigeria.

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Antioxidant agents of plants origin have continued to attract interest because of the potential they hold in the maintenance of human health accompany with their minimal side effects. In this study, the antioxidant activity of the crude methanolic stem bark extract of *Azelia africana* was investigated using the scavenging activities on superoxide anions, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Hydrogen peroxide (H₂O₂), Nitric Oxide (NO) and 2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS); also the reducing power, as well as the flavonoid, proanthocyanidin and phenolic contents of the extract were determined. The extract exhibited scavenging activities in all radicals tested due to its high phenol and flavonoids contents. This study suggests that *A. Africana* is a possible source of natural radicals' scavenger and could serve as a base for future antioxidative diseases drugs.

Key words: *Azelia africana*, free radicals scavenging activity, antioxidant activity, phenolic compounds, reducing power, crude extract.

INTRODUCTION

The physiological metabolites generated in human body include types of oxygen derivatives like superoxide anion, singlet state oxygen and hydroxyl radicals along with peroxides and transition metals have degenerative effects to living cells and DNA. In human body, reactive oxygen species are produced through normal aerobic respiration and during inflammatory process. This phenomenon can be increased through radiation, alcoholism, stress and consumption of nicotine (Yildirim et al., 2001). The role of these free radicals and active oxygen is becoming increasingly recognised in the pathogenesis of many human diseases, including cancer, aging and atherosclerosis (Perry et al., 2000). Human body can be protected from these harmful compounds by enzymatic system, catalase, scavengers and antioxidants (Berger, 2006). Antioxidants are capable of preventing oxidative processes by inhibiting the initiation or propagation of an oxidative chain reaction. They are important in the prevention of many oxidative-stress related diseases

(Gerber et al., 2002).

Several studies have shown that the therapeutic effects of some medicinal plants, fruits and even vegetables which are commonly used in folklore remedies against many diseases can be attributed to the antioxidant properties of their phytoconstituents (Pietta, 1998; Pourmorad et al., 2006). Thus, antioxidant activity of plants might be due to their phenolic compounds (Duh et al., 1999). Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic oxidative enzymes and anti-inflammatory action (Frankel, 1993). Consequently, antioxidant agents of natural origin have continued to attract interests because of the potential they hold in the maintenance of human health.

Azelia africana belongs to the family Caesalpiniaceae. The English name is mahogany. The tree is widely distributed in Africa and Asia (Keay et al., 1964). It is used as food, plank and widely used as folklore remedies among many tribes in Africa. From the investigations conducted on *A. africana* by some scientists, the plant exhibited bioactive activities which included antimicrobial, anti-inflammatory, antimalaria, analgesic and trypanocidal

*Corresponding author. E-mail: aokoh@ufh.ac.za.

activities. Akah et al. (2007) studied the anti-inflammatory and analgesic properties of extract from *A. africana* on rats. The extract inhibited topical oedema as well as paw oedema induced in these experimental rats. Furthermore, Akah et al. (2007) also investigated the antimicrobial effects of the extract on *Staphylococcus aureus* and *Bacillus subtilis* which were found to be susceptible to the extract while *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi* were not susceptible to the extract. Etawodi (2005) investigated the trypanocidal activities of the leaves and stem bark extract of *A. africana* on *Trypanosoma brucei*. The protozoan was inhibited by the action of the extract. Powdered root of *A. africana* mixed with millet beer has been found to serve as treatment for hernia among some tribes in Cote d'Ivoire (Dalziel, 1937). Agbelusi et al. (2007) screened some chewing sticks aqueous extract which include *A. africana* against microbial isolates from mouth washings of some patients. Only two out of nine chewing stick aqueous extracts were found not to inhibit the growth of some of the microbial isolates and these exclude *A. africana*. In addition to the antimicrobial activities exhibited by *A. africana*, a novel xyloglucan (carbohydrate) was isolated from the seeds of this plant.

Our previous work on *A. africana* revealed that this plant is rich in polyphenolic compounds which are anti-microbial in action. Polyphenolic compounds are known to possess antioxidant activity and thus able to bolster biological resistance against the deleterious effect of reactive oxygen species. There was no information on the antioxidant activity of *A. africana* and thus the need to evaluate the possible antioxidant property of this plant.

MATERIALS AND METHODS

Plant materials

Fresh stem bark of *A. africana* was collected from Abeokuta, Ogun State, Nigeria in the month of April, 2008 and was identified by Dr. H. C. Illoh of Botany Department, Obafemi Awolowo University, Ile Ife, Nigeria. Voucher sample was prepared and deposited in the Herbarium of the Botany Department, Obafemi Awolowo University, Ile Ife, Nigeria for reference. The bark was air-dried until the constant weight of the bark was obtained. The bark was later powdered and stored in an air-tight container for further use.

Preparation of extract

Exactly 250 g of the powdered bark of *A. africana* was extracted in cold using mixture of methanol and sterile distilled water (3:2) for 4 days. The mixture was later filtered and the filtrate was first concentrated *in vacuo* using rotary evaporator to remove the organic solvent. The aqueous residue was later lyophilized to get the crude extract. The extract was brown in colour and the yield collected was 56 g.

Determination of total phenolic composition

The amount of total phenolic content of the extracts was determined

by the method described by Singleton et al. (1999). Exactly 0.5 ml of the extract was transferred to a 100 ml Erlenmeyer flask and the final volume was adjusted to 46 ml by addition of distilled water. 1 ml of Folin-Ciocalteu reactive solution was added and incubated at room temperature for 3 min. 3 ml of 2% sodium carbonate solution was added and the mixture was shaken on a shaker for 2 h at room temperature. The absorbance was measured at 760 nm. Gallic acid was used as the standard for a calibration curve. The phenolic compound content was expressed as gallic acid equivalent.

Estimation of total flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination. One milliliter (1 ml) of sample was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and remains at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm with ultraviolet (UV) visible spectrophotometer. The content was determined from extrapolation of calibration curve which was prepared by preparing gallic acid solution (0 - 0.8 mg/ml) in distilled water. The concentration of flavonoid was expressed in terms of mg/ml.

Determination of total proanthocyanidins

Total proanthocyanidins was determined based on the procedure of Sun et al. (1998). The mixture of 3 ml of vanillin-methanol (4% v/v), 1.5 ml of hydrochloric acid was added to 0.5 ml (1 mg/ml) of aqueous extract and vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Total proanthocyanidin content was expressed as gallic acid equivalent (mg/ml) from the standard curve.

Determination of reducing power

Reducing power assay was carried out as described by Yildirim et al. (2001). 0.5 ml of the extract was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The reaction mixture was incubated at 50°C for 30 min afterwards; 2.5 ml of 10% trichloroacetic acid was added to the mixture centrifuged for 10 min at 3000 rpm. 2.5 ml supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Absorbance was measured at 700 nm. Butylated hydroxyl toluene (BHT) solution was used as control. Increased absorbance of the reaction mixture indicated increased reducing power.

Antioxidant assay

The antioxidant activity of the plant extract was determined using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods (Kikuzaki et al., 1991; Ottolenghi, 1956). The FTC method was used to measure the amount of peroxide at the beginning of peroxidation while TBA method was used to measure free radicals present after peroxide oxidation.

Ferric thiocyanate method

The standard method described by Kikuzaki et al. (1991) was used for FTC determination. The absorbance of the resulting mixture (red colour) was measured at 500 nm every 24 h until the absorbance of the control reached its maximum. BHT was used as positive control. While the mixture without the plant extract was used as the negative control.

Thiobarbituric acid method

The method of Ottolenghi (1956) modified by Kikuzaki et al. (1991) was used for the determination of free radicals present after peroxide oxidation of stem bark extract. The final sample concentration of 0.02% w/v from the same samples prepared for FTC assay was used. Two milliliters of 20% trichloroacetic acid and 2 ml of 0.67% of thiobarbituric acid were added to 1 ml of sample solution followed the FTC method. The mixture was placed in a boiling water bath for 10 min and then centrifuged after cooling at 3000 rpm for 20 min. The absorbance activity of the supernatant was measured at 552 nm and recorded after the reaction has stopped.

2, 2-Diphenyl-1-Picrylhydrazyl assay

The hydrogen atom or electron donating abilities of the extract was measured from the bleaching of the purple-coloured methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical, DPPH as a reagent (Burits and Bucar, 2000; Cuender et al., 1997). One thousand microlitres of different concentrations of the extracts in ethanol were added to 4 ml of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent (I %) was calculated in following way:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

Where, A_{blank} is the absorbance of the control reaction (containing all reagents except the extract) and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of extract. Tests were carried out in triplicate.

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid Scavenging activity

The method of Re et al. (1999) was adopted for the determination of 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS+ solution and 2.4 mM potassium persulphate solution in equal amount and allowed to react for 12 h at room temperature in the dark. The resulting solution was later diluted by mixing 1 ml of freshly prepared ABTS+ solution followed by the measurement of absorbance at 734 nm after 7 min. The percentage of scavenging inhibition capacity of ABTS+ of the extract was calculated and compared with BHT.

Scavenging activity of nitric oxide

The method of Garrat (1964) was adopted to determine the Nitric Oxide (NO) radical scavenging activity of aqueous extract of *A. africana*. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. To 2 ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations (0.2 - 0.8 mg/ml). The mixture was incubated at 25°C After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent (1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid) at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v). The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm. The amount of nitric

oxide radical was calculated following this equation:

$$\% \text{ inhibition of NO} = [A_0 - A_1] / A_0 \times 100$$

Where A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place.

Scavenging activity of superoxide anion

The scavenging activity of superoxide anion was determined by the method of Yen and Chen (1995). The reaction mixture consists of 1 ml of plant extract (1 mg/ml), 1 ml of PMS (60 μ M) prepared in phosphate buffer (0.1 M pH 7.4) and 1 ml of NADH (phosphate buffer) was incubated at 25°C for 5 min, the absorbance was read at 560 nm against blank samples.

Hydrogen peroxide scavenging activity

Scavenging activity of Hydrogen peroxide (H_2O_2) by the plant extract was determined by the method of Ruch et al. (1989). Plant extract (4 ml) prepared in distilled water at various concentration was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the plant extract without H_2O_2 .

RESULTS

The total phenolic content of the stem bark extract of *A. africana* was 0.94 mg gallic acid equivalent/g of extract powder. The total flavonoid and proanthocyanidin contents of the plant extract were 0.812 and 0.004 mg gallic equivalent / g of extract powder respectively with reference to standard curve ($Y = 0.0067x + 0.0132$, $r^2 = 0.999$) (Table 1).

The *in vitro* antioxidant assay of the plant extract (Figure 1) reveals appreciable antioxidant potential compared with standard BHT and gallic acid. The percentage inhibition of lipid peroxide at the initial stage of oxidation showed antioxidant activity of 96% compared to BHT (80%) and gallic acid (96%), and the percentage inhibition of malondialdehyde by the extract showed percentage inhibition of 99% compared to both BHT (85%) and Gallic (98%). Table 2 shows the reducing power potentials of the stem bark extract of the test plant in comparison with a standard BHT at 700 nm. The reducing capacity of the extract, another considerable indicator of antioxidant activity was also found to be substantial; greater than those of the standard. The percentage inhibition of scavenging activities of the extract of the test plant for DPPH, ABTS, hydrogen peroxide, nitric oxide and superoxide anion radical were shown in Table 3. The ABTS and nitric oxide radical scavenging activity of the extract at 0.8 mg/ml (the highest concentration of the extract tested) was 82.19 and 83.92%. The plant extract showed appreciable free radical scavenging activities at the highest concentrations of 0.8 mg/ml on H_2O_2 , superoxide anion radical and DPPH with percentage inhibitions of 97.92, 92.01 and

Table 1. Gallic acid equivalent of stem bark extract of *A. africana* based on the crude methanolic extract screening.

Phytochemical compounds	Presence	Extract equivalent of gallic (mg/g)
Total phenol	+++	0.94
Total flavonoids	+++	0.812
Total proanthocyanidin	+	0.004

+++ = appreciable amount; + = trace amount.

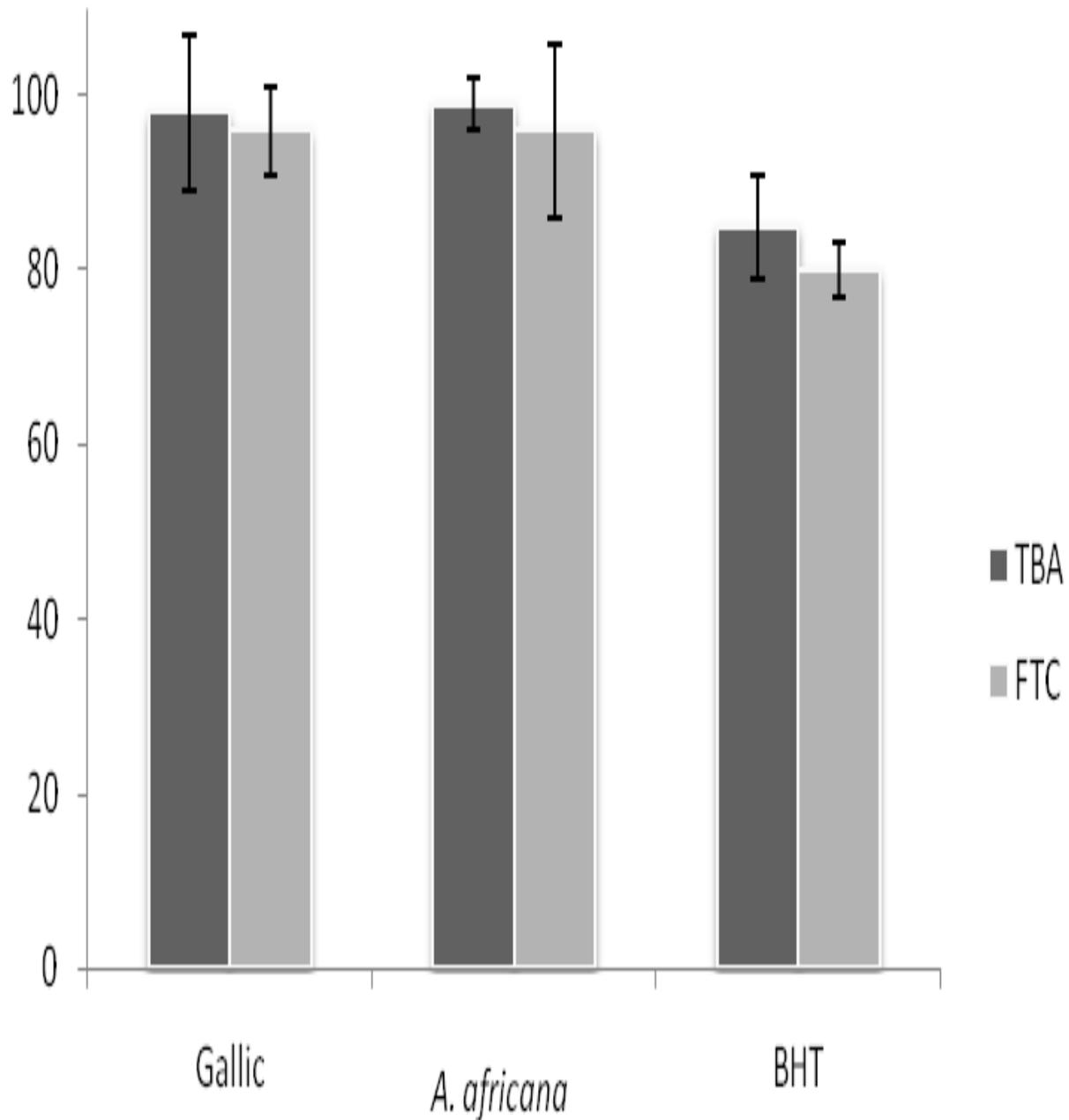


Figure 1. Antioxidant properties of plant extract compared to the standards (Gallic and BHT) as determined with the FTC (500 nm) and TBA (552 nm) methods on the 6th day.

Table 2. Reducing power activities of the stem bark extract of *A. africana* in comparison with a standard (BHT) at $\lambda = 700$ nm.

Concentrations (mg/ml)	Absorbance (700 nm)	
	Plant extract	BHT
0.2	0.210 \pm 0.00	0.110 \pm 0.10
0.4	0.770 \pm 0.00	0.510 \pm 0.00
0.6	0.87 \pm 0.00	0.790 \pm 0.00
0.8	1.90 \pm 0.00	1.050 \pm 0.00

Table 3. Radical scavenging activities of aqueous crude stem bark extract of *A. africana* and BHT as standard at different concentrations.

Extract or BHT concentration (mg/ml)	Percentage inhibition (% I) of radical scavenging of <i>A. africana</i>				
	Superoxide anion	Nitric oxide	DPPH	Hydrogen peroxide	ABTS
0.2	78.22(60.16)	42.00(40.27)	56.09(42.62)	72.12(68.61)	52.11(51.17)
0.4	87.51(73.49)	53.22(46.27)	64.23(53.00)	80.16(73.29)	68.81(63.39)
0.6	89.10(77.12)	66.32(61.87)	76.00(73.99)	89.40(76.22)	80.49(77.20)
0.8	92.01(79.96)	83.92(80.29)	89.93(82.32)	97.92(80.00)	82.19(77.95)

BHT values in bracket.

89.93% respectively (Table 3). All activities followed a concentration dependent manner and were far better than those of the standard BHT at all concentrations.

DISCUSSION

Extract of *A. africana* is endowed with phytochemicals; these phytochemicals have been known to be biologically active, aiding these activities through different mechanisms. The antioxidative effect is mainly due to phenolic components, such as phenolic acids and phenolic diterpenes (Pourmorad et al., 2006). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). Phenolic compounds are also thought to be capable of regenerating endogenous α -tocopherol, in the phosphor-lipid bilayer of lipoprotein particles, back to its active antioxidant form. They are also known to inhibit various types of oxidizing enzymes. These potential mechanisms of antioxidant action make the diverse group of phenolic compounds an interesting target in the search for health beneficial phytochemicals (McCune and Timothy, 2002). Phenol and phenolic compounds such as flavonoids have been shown to possess significant antioxidant activities and their effects on human nutrition and health are considerable (Kessler et al., 2003). Since polyphenols are responsible for the antioxidant activity; the obtained amount of total polyphenols in this extract indicated the extract to possess a high antioxidant

activity. This is further corroborated by the result of our FTC and TBA antioxidant assays. Interestingly, the reduction in peroxide level at the concentrations investigated may indicate the ability of the herb to minimize oxidative damage to some vital tissues in the body (Atawodi, 2005).

In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's blue at 700 nm. Increasing absorbance indicates an increase in reductive ability. The results (Table 2) show that there was increase in reducing power of the plant extract as the extract concentration increases. Different researchers (Yildirim et al., 2001; Thirugnanasampandan et al., 2008) have reported statistically significant correlations between reducing power and amount of total phenolics compounds in plant extract. They suggested that there may be relationship between phenolic compounds and reducing powers that is; presence of phenolic compounds might be the reason for reducing power.

Plants with antioxidant activities have been reported to possess free radical scavenging activity (Atawodi, 2005). Free radical is known as a major contributor to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases as a result of deficient natural antioxidant defense mechanism (Parr and Bolwell, 2000). The result of DPPH scavenging activity assay in this study indicates that the plant was potentially active.

This suggests that the plant extract contain compounds

that are capable to scavenge free radical which is responsible for radical's reactivity. The ability of plant extract to scavenge DPPH could also reflect its ability to inhibit the formation of ABTS+. The scavenging activity of ABTS+ radical by the plant extract was found to be wonderfully great. It is greater than those of the standard BHT at all concentration tested.

The activities are concentration dependent with the highest inhibition percentage recorded for the highest concentration (0.8 mg/ml) tested in this experiment. The results obtained in this study was contrary to findings of a previous study (Wang et al., 1998) which reported that compounds which exhibit ABTS+ scavenging activity may not possess DPPH scavenging activity. This implies that the plant extract may be useful for treating radical-related pathological damage especially at higher concentration.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that could be generated; it also has the ability to change to other harmful reactive oxygen species and free radicals within the living cells (Scok Hyun Nam and Mi Young Kang, 2004). The extract has been found to have significant superoxide radical scavenging activity, which ultimately adds to its antioxidant potential. The scavenging activity of this radical by the plant extract compared with the standard suggests that the plant is also a potent scavenger of superoxide radical like the standard gallic.

Hydrogen peroxide is a highly important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell (Gulcin et al., 2003). Scavenging of H₂O₂ by the plant extracts may be attributed to their phenolics, which donate electron to H₂O₂, thus reducing it to water. The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. NO is a reactive free radical produced by phagocytes and endothelial cells, to yield more reactive species such as peroxynitrite which can be decomposed to form OH radical.

The level of nitric oxide was significantly reduced in this study by the crude extract. Since NO plays a crucial role in the pathogenesis of inflammation (Shama et al., 2007), this may explicate the use of *A. africana* for the treatment of inflammation and for wound healing. Other investigators (Dombrowicz et al., 1994; Schinella et al, 2002; Tepe et al., 2005) have reported similar phytochemicals and their accompany antioxidant activities found in he *A. africana*, these corroborates our results from this experiment.

Conclusions

In conclusion, etiological factors of several clinical disorders could be traced to a deficient natural antioxidant defense in an individual. These disorders can be prevented or delayed by supplementing the body's natural

antioxidant defense. Plant extracts and plant-derived antioxidant compounds potentiate body's antioxidant defense, they are antioxidants of choice because of their lower toxicity and side effects over the synthetic ones. Also, they are relatively cheaper and are easily accessible. The methanolic crude stem bark extract of *A. africana* have shown to possess *in vitro* antioxidant activities which may be due to the presence of flavonoids, phenolics and proanthocyanidins. Further studies regarding the isolation and characterization of the active principles responsible for these activities is currently under progress in our research group.

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