Comparative in vitro antioxidant activity of ethanol extracts of *Vernonia amygdalina* and *Annona muricata* leaves

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ABSTRACT

In this work, *in vitro* antioxidant activities of ethanol extracts of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) leaves were comparatively evaluated using reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, hydrogen peroxide (H_2O_2) and superoxide anion radical scavenging assays as well as their inhibition concentration (IC_{50}). The results showed that at maximum concentration (0.8mg/ml) on DPPH, percentage inhibition by ethanol leaf extracts of *Vernonia amygdalina* and *Annona muricata* were 81.13% and 76.77% respectively while ascorbic acid had 89.53% inhibition with IC_{50} values of 0.38, 0.41, and 0.34mg/ml respectively. The percentage inhibition of ethanol leaf extracts of *Vernonia amygdalina* and *Annona muricata* on H_2O_2 was also at the maximum concentration of 0.8mg/ml which were 78.30% and 80.14% respectively while ascorbic acid, the standard had 85.86% inhibition with IC_{50} values of 0.42, 0.41, and 0.36mg/ml respectively. Against superoxide anion radical, the maximum % inhibition was 65.36% for *Vernonia amygdalina*, 67.97% for *Annona muricata* and 83.79% for ascorbic acid, with IC_{50} values of 0.55, 0.50, and 0.37mg/ml respectively. In conclusion, *Vernonia amygdalina* and *Annona muricata* leaves possess antioxidant properties probably due to presence of bioactive compounds such as flavonoids, saponins, tannins and alkaloids which have the ability to scavenge free radicals that causes oxidative stress.

Keywords: *Annona muricata*, Antioxidant, Free radical, *in vitro*, *Vernonia amygdalina*

1. INTRODUCTION

Free radicals such as hydroxyl radicals and superoxide anion radical attack unsaturated fatty acids in biomembranes resulting in membrane lipid peroxidation (Dean and Davies, 1993) which can subsequently cause a decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane proteins, leading to cell inactivation (Kottaimuthu, 2008). Antioxidants such as flavonoids and vitamins are well known for their protecting effects in the body against reactive oxygen species (ROS) as body cells and tissues are continuously being threatened by the damage caused by free radicals and ROS which are produced during normal oxygen metabolism or elicited by exogenous damage (Omale and Okafor, 2008).

Vernonia amygdalina popularly called bitter leaf have been used traditionally in Nigeria and other cultures for tick control, as a tonic, and in the treatment of sexually transmitted diseases; feverish conditions, cough, constipation, and hypertension (Regassa, 2000; Kambizi and Afolayan, 2001). *Vernonia amygdalina* have been proven in human medicine to possess hypoglycaemic, hypolipidaemic, anti-malarial and anti-helminthic properties (Abosi and Raseroka, 2003; Akah and Okafor, 2006) as well as anti-tumorigenic properties (Izevbige *et al*., 2004). In previous studies on *Vernonia amygdalina*, we reported the presence of bioactive compounds such as flavonoids, saponins, tannins and alkaloids as well as minerals such as K^+, Ca^{2+}, Mg^{2+}, Na^+ etc and nutritional components (Usunobun and Okolie, 2015a).

Annona muricata commonly called sour sop belongs to the family Annonaceae. The bark, leaves and roots are considered sedative, anti-spasmodic, hypoglycemic, hypotensive, smooth muscle relaxant and nervine with tea made for each of these disorders (Gouemo *et al.,* 2003; Yuan *et al.,* 2003). Many bioactive compounds and phytochemicals including flavonoids have been found in *Annona muricata* (Usunobun *et al.,* 2015; Usunobun and Okolie, 2015b). Research on *Annona muricata* have also shown that a novel set of phytochemicals (Annonaceous acetogenins) found in the leaves, seeds and stem are cytotoxic against various cancer cells (Chang, 2001; Liaw *et al.,* 2002). Ethanol extract of *Vernonia amygdalina* and *Annona muricata* leaves have anti-hepatotoxic activity in (dimethylnitrosamine) DMN-induced hepatic damage in rats (Usunobun, 2014a; Usunobun, 2014b; Usunobun *et al*., 2015). This study is aimed at evaluating and comparing *in vitro* antioxidant potentials of *Vernonia amygdalina* and *Annona muricata* leaves.

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2. MATERIALS AND METHODS

2.1. Collection and identification of plant materials

Fresh leaves of *Annona muricata* were collected from its tree in Upper sakponba in Benin city while fresh leaves of *Vernonia amygdalina* were purchased from a local market in Benin City, Edo state, Nigeria. The leaves were identified by a botanist in the Department of Basic Sciences, Faculty of Basic and Applied Sciences, Benson Idahosa University, Benin city, Edo State.

2.2. Preparation of the plant materials

Fresh mature leaves of *Annona muricata* and *Vernonia amygdalina* were separated from the stalk, washed and air-dried at room temperature (24°C) and then pulverized, crushed into fine powder and weighed.

2.3. Extraction of the plant leaves

Ethanolic extracts of the plants were prepared by soaking 100g each of the dry powdered plant materials in 1000ml of absolute ethanol (Sigma) at room temperature for 48hrs. At the end of the 48hours, the extracts were filtered first through a Whatmann filter paper No. 42 (125mm) and then through cotton wool. The *Annona muricata* and *Vernonia amygdalina* ethanol extracts were concentrated *in vacuo* using a rotary evaporator with the water bath set at 60°C and 40°C respectively to one-tenth its original volume and then finally freeze dried. The dry residue (crude extract) was then stored at 4°C. Portions of the crude plant extracts were weighed and used for *in vitro* antioxidant activities.

2.4. Determination of *in vitro* antioxidant properties of extracts

2.4.1. Determination of reducing power

The reducing power of each leaf extract was evaluated according to the method described by Aiyegoro and Okoh (2010). A mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) was added to 1.0 ml of the extracts (0.2, 0.4, 0.6 and 0.8 mg/ml) prepared in distilled water. The resulting mixture was incubated for 20 min at 50°C, followed by addition of 2.5 ml of TCA (10% w/v), and then by centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1% w/v). The absorbance was measured using spectrophotometer at 700 nm against reagent blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract. IC_{50} was extrapolated from the graph of absorbance at 700 nm against extract concentration.

2.4.2. DPPH radical scavenging ability

The method of Liyana-Pathiana and Shahidi (2005) was used for the determination of DPPH free radical scavenging activity of the extract. DPPH (1 ml, 0.135 mM) prepared in methanol was mixed with 1.0 ml of aqueous extract in concentration from 0.2, 0.4, 0.6 and 0.8 mg/ml. The reaction mixture was then vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. The scavenging ability of the plant extract was calculated using the equation:

DPPH scavenging activity (%) =

$[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$,

Where: $Abs_{control}$ is the absorbance of DPPH + methanol and Abs_{sample} is the absorbance of DPPH radical + sample (or standard). The IC_{50} value which represents the concentration of the extract that caused 50% inhibition of radical formation was obtained by interpolation from linear regression analysis.

2.4.3. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging activity of the plant extract was estimated using the method of Ruch *et al* (1989). Plant extract prepared in distilled water at various concentrations was mixed with 0.6 ml of 4mM H_2O_2 solution prepared in 0.1M phosphate buffer, pH 7.4 and incubated for 10 min. at room temperature. The absorbance of the solution was measured at 230 nm against a blank solution containing the extract with or without H_2O_2. The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equation:

H_2O_2 radical scavenging activity =

$[(Abs_{control} - Abs_{sample})]/(Abs_{control}) \times 100$,

Where: $Abs_{control}$ is the absorbance of H_2O_2 radicals + methanol and Abs_{sample} is the absorbance of H_2O_2 radical + sample or standard. The IC_{50} value which represents the concentration of the extract that caused 50% H_2O_2 scavenging was obtained by extrapolation from linear regression analysis.

2.4.4. Superoxide anion radical scavenging activity

The superoxide anion scavenging ability was determined according to the method of Robak and Gryglewski (1988). Each extract and standard ascorbic acid (0.2, 0.4, 0.6 and 0.8 mg/ml) in water (1 ml) was mixed with 80 µM phenazine methosulfate (1ml), 624µM dihydronicotinamide adenine dinucleotide (1ml) and 200 µM nitro blue tetrazolium (1ml) in 0.1 M sodium phosphate buffer (pH 7.4), and then incubated at room temperature for 5 min. The absorbance was measured at 560 nm.

Scavenging capability =

$[(A_{560}$ of control $- A_{560}$ of sample$)/A_{560}$ of control$] \times 100\%$.

The IC_{50} value which represents the concentration of the extract that caused 50% scavenging of superoxide radical was obtained by extrapolation from linear regression analysis.

3. RESULTS

In reducing power activity, the antioxidant ability of *Vernonia amygdalina* and *Annona muricata* ethanol leaf extracts to reduce iron (III) to iron (II) is shown in Figure 1. Result showed that the higher the concentration, the higher the absorbance with IC_{50} value of 0.72 and 0.68mg/ml recorded for *Vernonia amygdalina* and *Annona muricata* respectively (Figure 5).

Scavenging activities of the ethanolic leaf extracts of *Vernonia amygdalina* and *Annona muricata* on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), hydrogen peroxide (H_2O_2) and superoxide anion radical are shown in Figure 2 - 4. The free radical scavenging activities were compared with the activities of ascorbic acid, a known antioxidant. The results showed that ethanolic leaf extracts of *Vernonia amygdalina* and *Annona muricata* have the capability to scavenge DPPH-radical, hydrogen peroxide (H_2O_2) radical and superoxide anion radical. Both plant extracts showed appreciable free radical scavenging activities at the maximum concentration of 0.8mg/ml on DPPH with the percentage inhibition by ethanolic leaf extracts of *Vernonia amygdalina* and *Annona muricata* being 81.13% and 76.77% respectively while ascorbic acid, the standard had 89.53% inhibition. IC_{50} value of 0.38, 0.41, and 0.34mg/ml were recorded for *Vernonia amygdalina, Annona muricata* and ascorbic acid, respectively (Figure 5). The DPPH radical scavenging activity is as follows: *Ascorbic acid >Vernonia amygdalina >Annona muricata.*

The percentage inhibition of ethanolic leaf extracts of *Vernonia amygdalina* and *Annona muricata* on hydrogen peroxide (H_2O_2) was also at the maximum concentration (0.8mg/ml) which was 78.30% and 80.14% respectively while ascorbic acid, the standard had 85.86% inhibition. IC_{50} value of 0.42, 0.41, and 0.36mg/ml were recorded for *Vernonia amygdalina, Annona muricata* and ascorbic acid, respectively (Figure 5). H_2O_2 radical scavenging activity is as follows: *Ascorbic acid >Annona muricata >Vernonia amygdalina.*

Against superoxide anion radical, the maximum % inhibition was 65.36% for *Vernonia amygdalina*, 67.97% for *Annona muricata* and 83.79% for ascorbic acid, the standard while IC_{50} value of 0.55, 0.50, and 0.37mg/ml were recorded for *Vernonia amygdalina, Annona muricata* and ascorbic acid, respectively (Figure 5). Superoxide anion radical scavenging activity is as follows: *Ascorbic acid>Annona muricata>Vernonia amygdalina.*

Results from these assays showed that when the concentration of the extracts and the standards increase, the capacity to scavenge free radicals also increases.

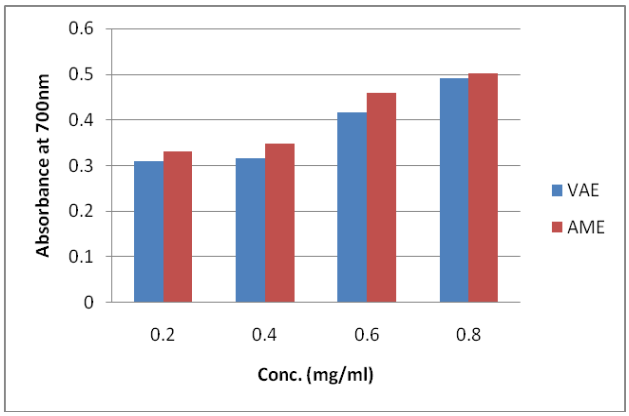

Figure 1: Reducing power of ethanol leaf extract of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) at different concentrations.

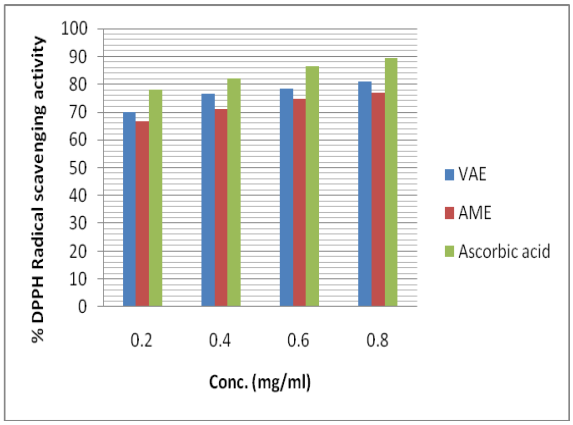

Figure 2: DPPH radical scavenging activitiesof ethano l leaf extracts of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) and Ascorbic acid as standard at different concentrations

4. DISCUSSION

Studies have indicated that electron donation capacity of bioactive compound is associated with antioxidant activity (Siddhuraju et al., 2002; Arabshahi-Deloue and Urooj, 2007). The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron which is an important mechanism of phenolic antioxidant action (Mohamed et al., 2009).

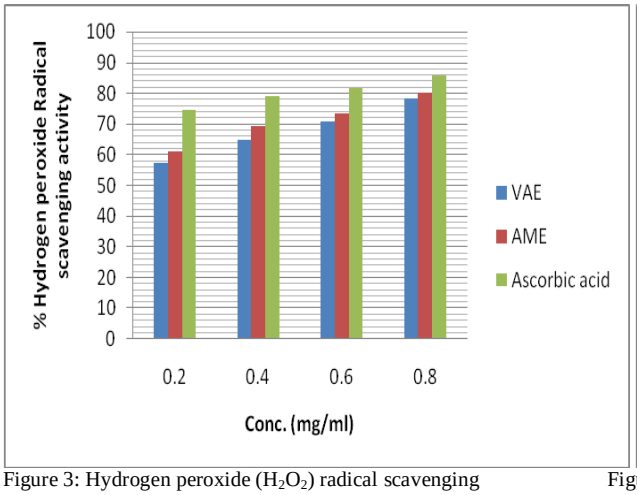

Figure 3: Hydrogen peroxide (H_2O_2) radical scavenging activity of ethanol leaf extracts of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) and Ascorbic acid as standard at different concentrations.

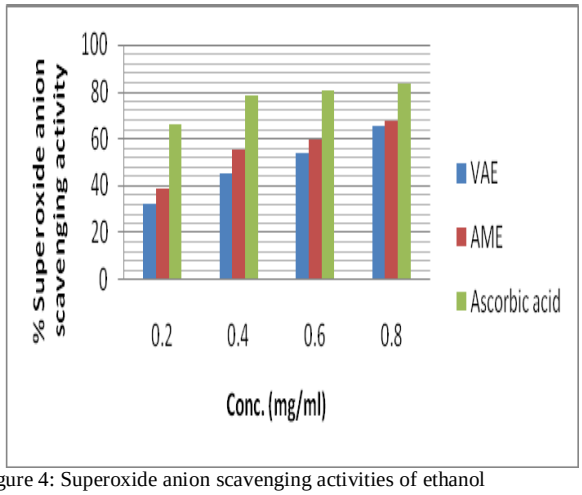

Figure 4: Superoxide anion scavenging activities of ethanol leaf extracts of *Vernonia amygdalina*(VAE) and *Annona muricata* (AME) and Ascorbic acid as. standard at different concentrations.

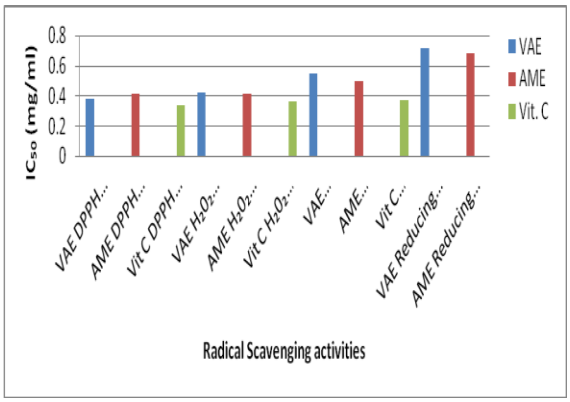

Figure 5: IC_{50} Values of ethanol leaf extracts of *Vernonia amygdalina* (VAE) and *Annona muricata* (AME) scavenging effect compared with Vitamin C.

In reducing power activities, the antioxidant ability of ethanolic leaf extracts of *Vernonia amygdalina* and *Annona muricata* showed that the higher the concentration, the higher the absorbance with *Annona muricata* having higher absorbance than *Vernonia amygdalina*. Increased absorbance of the reaction mixture indicates higher reducing power of the plant extract. The presence of antioxidants in the ethanolic leaf extracts of *Vernonia amygdalina* and *Annona muricata* caused the reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}), thereby changing the solution into various shades from green to blue, depending on the reducing power of the compounds (Ferreira *et al.*, 2007), thus proving their reducing power ability. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain via donating a hydrogen atom (Meir *et al.*, 1995). Ethanolic leaf extracts *Vernonia amygdalina* and *Annona muricata* thus contain high amount of reductones. Hence, the leaf extracts may act as electron donors and could react with free radicals to convert them into more stable products and then terminate the free radical chain reactions. This effect of both plants may suggest their ability to minimize oxidative damage to some vital organs in the body.

Scavenging of DPPH radical in this study indicates the potency of *Vernonia amygdalina* and *Annona muricata* in donating hydrogen proton to the lone pair electron of the radicals. Because the inhibition was more at a higher concentration in both extracts, it could be suggested that the plant extracts contain compounds capable of donating protons to the free radicals. In the present study, both plants extracts exhibited comparable DPPH free radical scavenging ability in a concentration-dependent manner. Between the two extracts, *Vernonia amygdalina* had better free radical scavenging ability than *annona muricata* but not more than the standard, ascorbic acid. The scavenging ability of the extracts may be a reflection of the total activities of various components present in the extracts. Superoxide anion, which is formed in almost all aerobic cells, is a major agent in the mechanism of oxygen toxicity (Xue *et al.*, 2009).

The scavenging activity of superoxide anion radical by *Vernonia amygdalina* and *Annona muricata* leaves suggests that they are potent scavengers of superoxide anion radical. However, *Annona muricata* had higher superoxide anion scavenging activity than *Vernonia amygdalina* but not more than ascorbic acid, the standard. Superoxide anion radical is one of the strongest ROS among the free radicals that are generated (Garrat, 1964).

In addition to direct attack of important biological molecules, oxygen anion ($O_2^{\bullet-}$) may also be involved in the formation of singlet oxygen and hydroxyl radicals, which may increase local oxidative stress and initiate cellular damage and pathological incidents. The probable mechanism of scavenging superoxide anions by *Vernonia amygdalina* and *Annona muricata* leaves may be due to their inhibitory effect towards generation of superoxides in the *in vitro* reaction mixture. Thus both extracts are potential scavengers of superoxide anions to prevent lipid oxidation.

Hydrogen peroxide may be toxic if converted to hydroxyl radical in the cell (Gulcin *et al.,* 2003). Hydrogen peroxide reacts with ferrous salt to form hydroxyl radical via Fenton's reaction (Lloyd *et al.*, 1997). The hydroxyl radical (OH^-) thus produced may attack the sugar of DNA base causing sugar fragmentation; base loss and DNA strand breakage (Kaneko *et al.,* 1997). In the present study, there was inhibition of hydrogen peroxide and thus hydroxyl radical by *Vernonia amygdalina* and *Annona muricata* in a concentration-dependent manner thus indicating the potency of both plant extracts in preventing the joining of nucleosides in the DNA and possible breakage. *Annona muricata* (80.14% at a concentration of 0.8mg/ml) had better scavenging potential than *Vernonia amygdalina* with 78.30%. The scavenging potential of *Vernonia amygdalina* and *Annona muricata* may be attributed to the presence of phenolic groups that could donate electrons to H_2O_2, thereby neutralizing it into water (H_2O). The antioxidant activity of both leaves may be due to the presence of natural substances mainly phenolic compounds such as flavonoids, tannins, saponins and alkaloids as we previously reported (Usunobun and Okolie, 2015a; Usunobun *et al.*, 2015; Usunobun and Okolie, 2015b).

IC_{50} is measure of inhibitory concentration and a lower IC_{50} value is a reflection of greater antioxidant activity as observed in the various IC_{50} values of *Vernonia amygdalina* and *Annona muricata* ethanol leaf extracts in this study. From the *in vitro* antioxidants results, it appeared that antioxidant activities of both *Vernonia amygdalina* and *Annona muricata* leaf extracts were relatively high in comparison with the reference standard antioxidants (ascorbic acid) probably due to the rich antioxidant compounds in the extracts.

The present study thus give proof that *Vernonia amygdalina* and *Annona muricata* leaves have the potential compound(s) to react as antioxidant which is suitable to develop drugs for the prevention of human diseases related to free radical mechanism.

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