In Vitro Antioxidant Properties of Methanolic Leaf Extract of Vernonia Amygdalina Del

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Summary: Various methods employed in evaluating antioxidant activities of various samples gives varying results depending on the specificity of the free radical or oxidant used as a reactant. This study investigated the antioxidant/radical scavenging properties of the methanolic extract of Vernonia amygdalina (MEVA) leaves and studied the relationship between the assay methods. Antioxidant capacity of MEVA was evaluated by measuring the radical scavenging activity (RSA) of MEVA on 1,1-diphenyl-2-picrylhydrazyl radical (DPPH’), nitric oxide (NO) and hydrogen peroxide (HP), hydroxyl radical (OH’) scavenging activity (HRSA), lipid peroxidation inhibition activity (LPIA) against 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) and Trolox Equivalent Antioxidant Capacity (TEAC) of MEVA against 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS+) radicals as well as the reducing power (RP). Assay methods were subjected to regression analysis and their correlation coefficients calculated. Results were analysed using student’s t-test and ANOVA. MEVA exhibited highest percentage RSA of 85.8% on HP, followed by DPPH’ (29.6%), OH’ (26.4%) and least on NO’ (21.8%). MEVA inhibited AAPH-induced lipid peroxidation by 30.0% and ABTS-induced radical by 1489% with a marked RP of 0.242±0.01. DPPH correlated excellently with RP (r² = 0.86), TEAC (r² = 0.94) and HRSA (r² = 0.89), the four having good relationship with each other, while LPIA correlated moderately with HP (r² = 0.48 and NO (r² = 0.34). MEVA exhibited significant free radical scavenging and antioxidant activities. The assay methods correlate very well and could therefore be employed for investigating and understanding antioxidant properties and scavenging activities of plant materials.

Keywords: Antioxidants, assay methods, correlation coefficient, free radicals, radical scavenging activity, Vernonia amygdalina

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INTRODUCTION

Free radicals and reactive species are widely believed to contribute to the development of several diseases by causing oxidative stress and ultimately oxidative damage (Halliwell, 2007; Li-Bahr, 2013; Gan and Johnson, 2013; Aruoma et al., 2014; Wong and Duce, 2014; Vitetta and Linnane, 2014) which has been implicated in many pathological diseases (Lobo et al., 2010; Aprioku, 2013; Bhattacharyya et al., 2014; Urrutia et al., 2014).

Many years back, Halliwell (2001), alongside with other scientists (Liu et al., 2002a; Liu et al.,2003) came up with the fact that if oxidative damage could be found to be responsible for the ever increasing incidence of various pathological conditions, then timely actions that could decrease or prevent its occurrence would be therapeutically beneficial. It was then suggested that successful antioxidant treatment should be employed in the delay or prevention of onset of diseases induced by oxidative damage (Halliwell 2002a; 2002b; Galli et al., 2002; Steinberg and Witzum, 2002). Since then, knowledge regarding the chemical nature and mechanism of action of antioxidants, especially endogenous antioxidant and their important roles in disease prevention and treatment has been rapidly increasing (Neerghheen et al., 2006; Halliwell, 2011; 2012; 2013; Choudhari et al., 2014). In view of this, much attention has been focused on the protective biochemical functions of naturally occurring antioxidants in biological systems, and the mechanisms of their actions.

The phenolic compounds, which are widely distributed in plants, were considered to play a very important role as dietary antioxidant component for the prevention of oxidative damage in living systems (Perron et al., 2008; Perron and Brumaghim, 2009; Albarracin et al., 2012; Aboul-Enein et al., 2013; Gao et al., 2014). Flavonoids are a large group of polyphenolic compounds abundantly present in
human diet (Gonzalez et al., 2011), first identified as plant pigment but later recognised as very potent antioxidant and immunomodulators (Middleton et al., 2000; Krifa et al., 2013). Flavonoids have been referred to as ‘nature’s biological modifiers because of strong experimental evidence of their inherent ability to modify the body’s reaction to allergens, viruses and carcinogens. They show anti-allergic, anti-inflammatory, antimicrobial and anti-cancer activities (Yamamoto and Gaynor, 2001) which are attributable to the phenolic hydroxyl groups attached to the flavonoid structure (Divakaran et al., 2013; Pereira et al., 2013). In recent times, flavonoids have attracted tremendous interest as possible therapeutics against free radical mediated diseases (Middleton et al., 2000; Ishizawa, 2011; Grassi et al., 2013; Kamel et al., 2014; Peng et al., 2014; Liang et al., 2014).

Vernonia amygdalina (Astereaceae) is a shrub or small tree of between 1 and 5m in height which grows throughout tropical Africa. The plant commonly known as bitter leaf due to the bitterness of its leaves is well cultivated and is common market merchandise in some African countries like Nigeria, Cameroon, Ethiopia and Zimbabwe. All parts of the plant have been found to be pharmacologically useful. In Nigeria, leaves of the plant are used as a green vegetable or spice in the popular bitter-leaf soup. The leaves could also be macerated and the water extract taken as appetizer or digestive tonic (Singha, 1972; Igile et al., 1995; Adesanoye and Farombi, 2010; Momoh et al., 2012). Many herbalists and naturopathic Doctors have recommended the aqueous extract for the treatment of ailments like diabetes, dysentery, gastrointestinal problems, nausea and appetite-induced abrosia. The root and the leaves are also used in ethnomedicine to treat fever, hiccups, kidney problems and stomach discomfort (Burkill, 1985; Hamowia and Saffaf, 1994; Ojako and Nwanjo, 2006; Adesanoye et al., 2013; Yedjou et al., 2013). Various phytoconstituents have been discovered and isolated from Vernonia amygdalina (Farombi and Owueye, 2011; Ijeh and Ejike, 2011; Toyang and Verpoorte , 2013). This study was designed to evaluate the antioxidant and radical scavenging activities of methanolic extract of Vernonia amygdalina (MEVA) in vitro and to study the correlation between the methods employed.

MATERIALS AND METHODS

Plant material
The leaves of Vernonia amygdalina were obtained from gardens of Forestry Research Institute of Nigeria (FRIN), Ibadan and were authenticated at the herbarium of the Institute. The powdered leaves were packed into soxhlet extractor and defatted with n-hexane. The defatted leaves was extracted in absolute methanol and the methanolic fraction evaporated in water bath at 40 - 50 °C to obtain a concentrated extract from which stock solutions were prepared.

Reducing power of MEVA
Reducing power of MEVA was determined according to the method of Oyiazu, (1986). Graded amounts of the extract (10-800 µg) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of 10% TCA was added to the mixture which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% FeCl₃ and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Scavenging effect of MEVA on Hydrogen peroxide
The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Ruch et al., (1989). Extracts doses in 4 ml distilled water (20-400µg) were added to hydrogen peroxide solution (0.6 ml). Absorption of hydrogen peroxide was determined 10 minutes later against a blank solution containing extract in phosphate buffer without hydrogen peroxide. Hydrogen peroxide concentration was determined spectrophotometrically from absorbance at 230 nm using the molar absorptivity of 81M⁻¹cm⁻¹ (Beers and Sizer, 1952).

Determination of the effect of MEVA on DPPH radical
Effect of MEVA on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was estimated according to the method of Hatano et al.,(1988). MEVA (25-500 µg) in 4 ml of distilled water was added to a methanolic solution of DPPH (1 mM, 1 ml). The mixture was shaken and left to stand at room temperature for 30 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. Catechin (50 µg) was used as standard. The radical scavenging activity (RSA) was calculated as percentage of DPPH discoulouration using the equation: % RSA = 100 x (1-Af/Ad) where Af is the absorbance of the solution with extract, and Ad is the absorbance of the DPPH solution without extract.

Trolox equivalent antioxidant capacity (TEAC) of MEVA
TEAC of MEVA was carried out using an improved 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammmonium salt (ABTS) decoulourization assay (Re et al., 1999) as described by Neergheen et al., (2006). The ABTS’ radical was generated by a reaction between ABTS (0.5 mM) and 1 mM potassium persulfate each in 100 ml of 0.1 M phosphate buffer. To 3 ml of the ABTS’ solution, 0.5 ml of the extract was added and the decay in absorbance was followed for 6 minutes at 734 nm. Trolox was used as a

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reference standard and TEAC values were expressed as µmol trolox equivalent.

**Effect of MEVA on 2,2′-azobis (2-aminopropane) hydrochloride (AAPH)-induced lipid peroxidation**
The effect of MEVA on AAPH-induced lipid peroxidation was carried out according to the method described by Neergheen et al., (2006). An aliquot of 200 µl of post-mitochondrial fraction (PMF) of liver homogenate was diluted in 0.1 M potassium phosphate buffer, pH 7.5 (1 in 10 dilution). Then, 400 µl of extract (100-1000 µg) was added followed by 200 µl of AAPH (20 mM) to initiate peroxidation. The mixture was incubated at 37°C for 1 hour with the solution gently shaken at 10 minutes interval. After incubation, 1.6 ml TCA-TBA-HCl stock solution (15% w/v TCA, 0.375% w/v TBA, 0.25 N HCl) was added to the solution and heated in a boiling water bath for 15 minutes. After cooling, the precipitate was removed by centrifugation and the absorbance of the resulting supernatant measured at 532 nm. Results were expressed as percentage inhibition of peroxidation with catechin used as standard.

**Deoxyribose assay – hydroxyl radical scavenging activity of MEVA**
The hydroxyl radical (OH) scavenging potential of MEVA was determined using the deoxyribose assay (Halliwell et al., 1987; Aruoma, 1994a; 1994b) as described by Neergheen et al., 2006. About 200-1000 µg of extract in 100 µl of distilled water was added to a solution containing 200 µl KH$_2$PO$_4$ – KOH (100 mM), 200 µl deoxyribose (15 mM), 200 µl FeCl$_3$ (500 µM) and 100 µl EDTA (1 mM) in a test tube and allowed to mix. Then, 100 µl H$_2$O$_2$ (10 mM) and 100 µl ascorbic acid (1 mM) were added to initiate the reaction. The reaction mixture was incubated at 37°C for 1 hour after which 1 ml of 1% α/β, TBA was added to each mixture followed by the addition of 1ml of 2.8% w/v TCA. The solution was heated in a water bath at 80°C for 20 minutes to develop the pink coloured MDA-(TBA)$_2$ adduct. After cooling, the solution was centrifuged and the absorbance of the supernatant measured at 532 nm against distilled water as blank. Results were expressed as percentage inhibition of deoxyribose degradation. % inhibition = (Abs control – Abs test/ Abs control) x100.

**Scavenging of nitric oxide radical by MEVA**
The scavenging effect of MEVA on nitric oxide (NO) radical was measured according to the method of Marcocci et al., 1994. About 10-400 µg of MEVA was added in the test tubes to 1 ml of sodium nitroprusside solution (25 mM) and the tubes incubated at 37°C for 2 hours. An aliquot (0.5 ml) of the incubation solution was removed and diluted with 0.3 ml of Griess reagent (1% sulphanilamide in 5% H$_3$PO$_4$ and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed was immediately read at 570 nm against distilled water as blank with catechin (50 µg) used as standard. Results were expressed as percentage radical scavenging activity (%RSA) = (1 – ΔAbs of sample/ΔAbs of control) x 100.

**RESULTS**
The antioxidant ability of methanolic extract of Vernonia amygdalina (MEVA) to inhibit, scavenge and quench free radicals and reactive oxygen species or ameliorate their effects was examined in this study. Effect of MEVA on oxidative stress biomarkers was investigated using different method.

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**Table 1: Radical Scavenging activity (RSA) of methanolic extract of Vernonia amygdalina (MEVA) on DPPH’ radical**

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>Absorbance</th>
<th>% RSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.699± 0.001</td>
<td>-</td>
</tr>
<tr>
<td>MEVA 40</td>
<td>1.371±0.060*</td>
<td>19.3</td>
</tr>
<tr>
<td>100</td>
<td>1.299±0.004*</td>
<td>23.5</td>
</tr>
<tr>
<td>250</td>
<td>1.284±0.016*</td>
<td>24.4</td>
</tr>
<tr>
<td>500</td>
<td>1.263±0.003*</td>
<td>25.7</td>
</tr>
<tr>
<td>750</td>
<td>1.241±0.032*</td>
<td>27.0</td>
</tr>
<tr>
<td>1000</td>
<td>1.196±0.037*</td>
<td>29.6</td>
</tr>
<tr>
<td>1500</td>
<td>1.206±0.018*</td>
<td>29.0</td>
</tr>
<tr>
<td>2000</td>
<td>1.212±0.013*</td>
<td>28.7</td>
</tr>
<tr>
<td>Catechin 50</td>
<td>1.216±0.029*</td>
<td>25.8</td>
</tr>
<tr>
<td>100</td>
<td>1.246±0.006*</td>
<td>26.7</td>
</tr>
</tbody>
</table>

Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates. *p<0.05 when compared with Control.

**Figure 1: Reducing power of methanolic extract of Vernonia amygdalina (MEVA) using the Fe$^{3+}$/ferricyanide method.**

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10 – 1250 µg. Table 1 shows the radical scavenging activity (RSA) of MEVA against DPPH radical exhibiting maximum RSA of 29.6% on DPPH radical (p<0.05) at 1000 µg. Possible inhibitory effect of MEVA on hydrogen peroxide (HP) is displayed in Table 2. MEVA showed highest percentage inhibition of 85.8% on HP at 20 µg (0.102±0.007) thereafter reducing to 24.2% (0.545± 0.021) at 500 µg. At 50 µg, MEVA (84.8%) compares very well with catechin (83.9%), showing similar scavenging activity. The antioxidant capacity of MEVA against ABTS radical was investigated in the TEAC (trolex equivalent antioxidant capacity) system as presented in Figure 2.

Table 2: Radical scavenging activity (RSA) of methanolic extract of Vernonia amygdalina (MEVA) on Hydrogen peroxides.

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>Absorbance</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.719±0.007</td>
<td>-</td>
</tr>
<tr>
<td>MEVA 20</td>
<td>0.102±0.007*</td>
<td>85.8</td>
</tr>
<tr>
<td>50</td>
<td>0.109±0.008*</td>
<td>84.8</td>
</tr>
<tr>
<td>100</td>
<td>0.154±0.003*</td>
<td>78.6</td>
</tr>
<tr>
<td>200</td>
<td>0.337±0.006*</td>
<td>67.0</td>
</tr>
<tr>
<td>300</td>
<td>0.330±0.006*</td>
<td>54.1</td>
</tr>
<tr>
<td>400</td>
<td>0.447±0.004*</td>
<td>37.8</td>
</tr>
<tr>
<td>500</td>
<td>0.545±0.021*</td>
<td>24.2</td>
</tr>
<tr>
<td>Catechin 50</td>
<td>0.116±0.005*</td>
<td>83.9</td>
</tr>
<tr>
<td>100</td>
<td>0.161±0.006*</td>
<td>77.6</td>
</tr>
</tbody>
</table>

Absorbance values expressed as mean ± SEM of three replicates. *p<0.05 when compared with control.

Figure 2: Trolox equivalent antioxidant capacity (TEAC) of methanolic extract of Vernonia amygdalina (MEVA). Absorbance values expressed as mean ± standard error of mean (SEM) of three replicates. A=Control, B=20µg MEVA, C=50µg MEVA, D=100µg MEVA, E=200µg MEVA, F=400µg MEVA, G=600µg MEVA, H=1000µg MEVA, I=50µg catechin, J=100µg catechin.

Table 3: Scavenging effect of methanolic extract of Vernonia amygdalina (MEVA) on AAPH-induced lipid peroxidation.

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>Absorbance</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.087±0.004</td>
<td>-</td>
</tr>
<tr>
<td>MEVA 50</td>
<td>0.078± 0.004</td>
<td>10.3</td>
</tr>
<tr>
<td>100</td>
<td>0.062±0.003*</td>
<td>28.7</td>
</tr>
<tr>
<td>200</td>
<td>0.061±0.005*</td>
<td>30.0</td>
</tr>
<tr>
<td>400</td>
<td>0.073± 0.0001*</td>
<td>16.1</td>
</tr>
<tr>
<td>800</td>
<td>0.076±0.001*</td>
<td>12.6</td>
</tr>
<tr>
<td>1000</td>
<td>0.081±0.001</td>
<td>6.9</td>
</tr>
<tr>
<td>Catechin 50</td>
<td>0.078±0.004</td>
<td>10.3</td>
</tr>
<tr>
<td>100</td>
<td>0.058±0.004*</td>
<td>33.3</td>
</tr>
</tbody>
</table>

Absorbance values expressed as mean ± SEM of three replicates. *p<0.05 when compared with control.

Similarly, MEVA showed greater ability at 100 µg by 14.0% when compared with control as shown in Table 5. Catechin showed greater activity at 100 µg by 33.3% when compared with MEVA (28.7%) at the same concentration. MEVA significantly (p<0.05) inhibited OH•-induced deoxyribose degradation from 50 – 1000 µg with the highest potency at 600 µg by 26.4% when compared with control as shown in Table 4. Similarly, MEVA exhibited scavenging effect on nitric oxide radical (NO•) at relatively low concentration (Table 5).
Table 6: Correlation Coefficient between Assay methods

<table>
<thead>
<tr>
<th>Assay methods</th>
<th>Correlation coefficients (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH/RP</td>
<td>0.86</td>
</tr>
<tr>
<td>DPPH/LPIA</td>
<td>-0.25</td>
</tr>
<tr>
<td>DPPH/TEAC</td>
<td>0.94</td>
</tr>
<tr>
<td>DPPH/HP</td>
<td>-0.89</td>
</tr>
<tr>
<td>DPPH/HRSA</td>
<td>0.89</td>
</tr>
<tr>
<td>DPPH/NO</td>
<td>-0.93</td>
</tr>
<tr>
<td>RP/LPIA</td>
<td>-0.57</td>
</tr>
<tr>
<td>RP/TEAC</td>
<td>0.97</td>
</tr>
<tr>
<td>RP/HP</td>
<td>-0.99</td>
</tr>
<tr>
<td>RP/HRSA</td>
<td>0.71</td>
</tr>
<tr>
<td>RP/NO</td>
<td>-0.98</td>
</tr>
<tr>
<td>LPIA/TEAC</td>
<td>-0.50</td>
</tr>
<tr>
<td>LPIA/HP</td>
<td>0.63</td>
</tr>
<tr>
<td>LPIA/HRSA</td>
<td>-0.28</td>
</tr>
<tr>
<td>LPIA/NO</td>
<td>0.59</td>
</tr>
<tr>
<td>TEAC/HP</td>
<td>-0.98</td>
</tr>
<tr>
<td>TEAC/HRSA</td>
<td>0.85</td>
</tr>
<tr>
<td>TEAC/NO</td>
<td>-0.98</td>
</tr>
<tr>
<td>HP/HRSA</td>
<td>-0.76</td>
</tr>
<tr>
<td>HP/NO</td>
<td>0.99</td>
</tr>
<tr>
<td>HRSA/NO</td>
<td>-0.88</td>
</tr>
</tbody>
</table>

- Negative correlation coefficients

Table 7: Summary of the relationships between Assay methods

<table>
<thead>
<tr>
<th>Assay methods</th>
<th>Relations</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH:</td>
<td>RP, TEAC, HRSA</td>
</tr>
<tr>
<td>RP:</td>
<td>DPPH, TEAC, HRSA</td>
</tr>
<tr>
<td>TEAC:</td>
<td>DPPH, RP, HRSA</td>
</tr>
<tr>
<td>HRSA:</td>
<td>DPPH, RP, TEAC</td>
</tr>
<tr>
<td>LPIA:</td>
<td>HP, NO</td>
</tr>
<tr>
<td>HP:</td>
<td>NO, LPIA</td>
</tr>
<tr>
<td>NO:</td>
<td>LPIA, HP</td>
</tr>
</tbody>
</table>

Very strong linear relationships (R²) were observed between DPPH, RP, TEAC and HRSA while medium to low correlation was observed between LPIA, NO and HP. The level of correlation coefficients between the assays methods are shown in Table 6 while Table 7 summarised the relationship between the various methods.

**DISCUSSION**

Phytochemicals are diverse and complex in nature. As such, the antioxidant activities of plant extracts cannot be evaluated by only a single method. Different antioxidant components are resident in the antioxidant defence system of the body and the antioxidant capacity of these antioxidant components depends to a great extent on which free radical or oxidants are produced in the system (Choi et al., 2002; Akinmoladun et al., 2010; Jan et al., 2013; Tenore and Ciampaglia, 2013). Therefore, various methods used in evaluating the antioxidant activity of various samples can give varying results depending on the specificity of the free radical used as a reactant (Frankel and Meyer, 2001; Prakash, 2001; Aruoma, 2003; Akinmoladun et al., 2010; Patel et al., 2011; Ramasarma, 2012; Shabbir et al., 2013).

In the present study, MEVA was able to reduce the Fe³⁺/ferri cyanide complex to the ferrous form, Fe²⁺, which was measured as the formation of Perl's Prussian blue colour at 700nm (Chung et al., 2002). MEVA at 250 µg (0.059±0.004) when compared with the reference antioxidant, catechin at 50 µg (0.051±0.003) indicates that the reducing power of MEVA is less than that of catechin, but nevertheless potent enough to function as a good electron and hydrogen-atom donor which can react with free radicals to convert them to more stable products and terminate radical chain reaction. Several studies have also reported the reducing power activity of several plants extracts (Rice-Evans et al., 1996; Amarowicz et al., 2004; Perez-Perez et al., 2006; Manian et al., 2008; Sen et al., 2013; Rahman et al., 2014), and this has been linked to the polyphenolic constituents of these extracts. The assay method used for measuring RP correlates excellently with other methods employed in this study, especially, DPPH, TEAC and HRSA at correlation coefficients (r²) of 0.86, 0.97 and 0.71 respectively and as such is an excellent method for measuring the reductive ability of antioxidants. Amarowicz et al., (2004) in their findings also confirmed that there is a good relationship between RP and DPPH (Sen et al., 2013; Das et al., 2014).

DPPH radical has been used extensively as a free radical to test the reductive ability of extracts or compounds as free radical scavengers or hydrogen donors and to evaluate the antioxidant activity of plant extracts and foods (Porto et al., 2000; Manian et al., 2008). Antioxidants react with DPPH* by providing electron or hydrogen atom, thus reducing it to 1,1-diphenyl-2-hydrazine (DPPH-H) or a substituted analogous hydrazine. The deep violet colour of DPPH at maximum absorption of 517 nm is changed to light yellow, colourless or bleached product, resulting in decrease in absorption (Miliauskas et al., 2004; Koksal et al., 2009). MEVA exhibiting significant scavenging effect on DPPH radical between 40 – 1000 µg (19.3% - 29.6%) compares well with catechin though showing less scavenging activity. Likewise, the assay method of DPPH scavenging activity correlates excellently with RP, TEAC and HRSA with correlation coefficients (r²) of 0.86, 0.94 and 0.89 respectively. The scavenging effect of plant extracts on DPPH has been shown to be related to the phenolic concentration of the extracts (Manian et al., 2008; Akinmoladun et al., 2010; Sen et al., 2013; Das et al., 2014), which is believed to contribute to their electron transfer / hydrogen donating ability. It could therefore be suggested that MEVA contains flavonoids with hydroxyl groups that could stabilize free radicals or scavenge their activities and that the method employed is reliable.
The activity of hydrogen peroxide (HP) as an active oxygen species has been reported to come mainly from its potential to produce the highly reactive hydroxyl radical through the fenton reaction: 

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$$

(Namiki, 1990; Farombi et al., 2002; Koksal et al., 2009; Jeong et al., 2010; Ogasawara et al., 2014). Therefore, inhibition of HP formation will prevent further generation of radicals. In the present study, MEVA showed highest percentage inhibition of 85.8% on HP at 20 µg (0.102±0.007) thereafter reducing to 24.2% (0.545±0.021) at 500 µg. At 50 µg, MEVA (84.8%) compares very well with catechin (83.9%), showing similar scavenging activity. The regression analysis of HP versus other methods in the study revealed a very good relationship between HP scavenging activity and nitric oxide scavenging activity (NO) with a correlation coefficient of 0.99, and a moderate relationship with lipid peroxidation inhibition activity with correlation coefficient of 0.35. Although HP on its own is not very reactive, but it could still be toxic both in vitro and in vivo; it could induce cell death in vitro and attack many cellular energy-producing systems in vivo. For instance, it has been reported that HP could deactivate the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Hyslop et al., 1998; Koksal et al., 2009; Song et al., 2014).

ABTS’ radical was generated by oxidation of ABTS with potassium persulfate (Re et al., 1999). MEVA increased TEAC values in a dose dependant manner from 50–600 µg with 339.4±155.3 – 4320.0±33.6 trolox equivalent and shows almost equal potency with catechin at 50 µg. ABTS’, a more reactive radical (more reactive than DPPH’) is stabilized by antioxidants that can supply electrons, unlike H atom in DPPH’. MEVA was thus able to scavenge ABTS’ radical-induced peroxodisulfate formation by electron transfer. The regression analysis for the assay methods employed in this study reveals that measurement of ABTS’ scavenging activity as Trolox equivalent antioxidant capacity (TEAC) compares very well with other methods in showing strong linear correlation with DPPH, RP and HRSA with correlation coefficients (r²) of 0.94, 0.97 and 0.85 respectively. This indicates that TEAC is a reliable method for measuring the total antioxidant activities of substances as reported by previous works (Koksal et al., 2009; Piljac-Zegarac et al., 2009; Chohan et al., 2012). Likewise, the report of Sasikumar et al., (2010) supported the relationship between TEAC and DPPH.

Lipid peroxidation is one of the consequent actions of ROS and free radicals. In this study, lipid peroxidation was induced with AAPH, a peroxy radical initiator. Thermal decomposition of AAPH produces peroxy radical which can attack polyunsaturated lipids initiating lipid peroxidation (Neergheen et al., 2006). MEVA was able to significantly (p<0.05) inhibit AAPH-induced lipid peroxidation by 28.7%, 30.0%, 16.1% and 12.6% at 100, 200, 400 and 800 µg respectively when compared with control. Catechin showed greater ability at 100 µg by 33.3% when compared with MEVA (28.7%) at the same concentration.

The decreasing lipid peroxidation product measured as thiobarbituric reactive substances (TBARS) showed the ability of MEVA to inhibit AAPH-induced lipid peroxidation by 10.3% – 30.0%. LPIA (Lipid peroxidation inhibitory activity of MEVA) correlates fairly well with HP and NO; the other methods employed in this study, with correlation coefficients (r²) of 0.63 and 0.59 respectively and as such, LPIA is another reliable method of measuring the scavenging and inhibitory ability of antioxidants. In the report of Akinmoladun et al., (2010), very low to fair correlations were observed between LPIA and other methods, which is similar to what was observed in this study.

Furthermore, the antioxidant activity of MEVA was demonstrated against hydroxyl radical (OH’). OH’ has been reported to be the most reactive radical known; it attacks and damages almost every molecule it makes contact with (Halliwell, 1989; Aruoma, 1999; Manian et al., 2008; Ramasarma, 2012). OH’ rapidly reacts with any molecule, forming another radical species, thereby giving rise to chain peroxide formation. The deoxyribose method employed in this study is a simple assay to determine the rate constants of hydroxyl radical formation as reported by Halliwell et al., (1987). OH’ radical is generated when the mixture of FeCl₂-EDTA, H₂O₂ and ascorbate is incubated with deoxyribose in phosphate buffer (pH 7.4). OH’ attack on deoxyribose results ultimately in chain peroxidation which could be measured as TBARS. MEVA exhibited 17.8 – 26.4% inhibition of deoxyribose oxidation by OH’. The ability of MEVA to inhibit OH’ by preventing the oxidation of deoxyribose in the current in vitro model could be related to prevention of propagation of the process of lipid peroxidation in vivo as has been shown in our studies with CCl₄ (Adesanoye and Farombi, 2010). In this vein, Farombi et al., (2002) reported that the overall antioxidant effect of flavonoids on lipid peroxidation is the result of scavenging hydroxyl radicals and superoxide anions at the stage of initiation and termination of peroxy radicals according to Hussain et al., (1987). In addition, MEVA showed better inhibition potential of 17.8% against 9.9% for catechin at 50 µg. The regression analysis in this study revealed a very high relationship between hydroxyl radical scavenging activity (HRSA) and other methods which is in line with some previous works (Perez-Perez et al., 2006; Sasikumar et al., 2010). HRSA correlated excellently...
with DPPH, RP and TEAC, with correlation coefficients ($r^2$) of 0.89, 0.71 and 0.85 respectively. The relationship between these four methods is excellent as has been observed and reported by various scientists (Halliwell, 1989; Amarowicz et al., 2004; Manian et al., 2008; Piljac-Zegarac et al., 2009; Akinmoladun et al., 2010).

Similarly, MEVA exhibited scavenging effect on nitric oxide radical (NO•) but at relatively low concentration. NO• shows dual function as both an antioxidant and pro-oxidant depending on the relative ratios of the reactants (Aruoma, 1996; Groβ et al., 2013; Meenakshi and Agarwal, 2013). Antioxidant effects of NO• occurs when NO• reacts with alkoxy and peroxy radical intermediates during lipid peroxidation thereby stabilizing the inhibition of LDL oxidation while the pro-oxidant reaction occurs when NO• reacts with O$_2^•$ to yield peroxynitrite (ONOO$^-$) (Zielonka et al., 2010). This dual effect could explain why the scavenging effect on NO• decreased with increasing concentration of the extract and became pro-oxidant at concentrations greater than 100 µg. MEVA exhibited similar scavenging effect with catechin. At 50 µg, MEVA elicited 16.9% inhibition while catechin showed 16.2% inhibition potential. The ability of MEVA to scavenge NO• and especially its deleterious metabolite, (ONOO$^-$) will be highly beneficial in biological system as ONOO$^-$ and some other NO• metabolites have been implicated in various pathological conditions such as malaria, cardiovascular diseases, inflammation, cancer and diabetes (Aruoma, 1996; Groβ et al., 2013; Meenakshi and Agarwal, 2013). NO correlated with LPIA and HP with correlation coefficients ($r^2$) of 0.59 and 0.99 respectively.

**CONCLUSION**

In conclusion, this study demonstrated that MEVA is a potent antioxidant and scavenger of reactive species in vitro. The observation concerning the diversity and complexity of the phytochemicals in plant extracts with different mechanisms of reaction for specific antioxidant species applies very well to the present study. The antioxidant capacity observed by all the methods assayed in this work appears to be reliable for measuring antioxidant and scavenging capacity of substances. From various experimental results (Dasgupta and De, 2004; Perez-Perez et al., 2006; Akinmoladun et al., 2010; Rao et al., 2010; Sasikumar et al., 2010; Patel et al., 2011; Ramasarma, 2012; Shabbir et al., 2013), a linear correlation with high correlation coefficients has been observed between total phenolic/flavonoid content and various antioxidant and free radical scavenging activities of various plants indicating that the various activities are based on the flavonoid and phenolic contents of the plant materials. It could therefore be inferred that the activities of MEVA in this study is due to its phenolic content, especially the flavonoids. Therefore, MEVA could be recommended as a potential antioxidant in ameliorating oxidative and free radical-induced pathologies.

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