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Evaluation of Antioxidant Activity of Leave Extract of *Borreriaverticillta* Linn (Rubiaceae)

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Abstract
Antioxidant effect of *Borreriaverticillata* used in Nigerian traditional medicine was carried out using 2,2-diphenyl-1- picrylhydrazyl radical (DPPH) and reducing power assay on the ethanol extract of the leave. The DPPH scavenging activity result indicated a concentration dependent antioxidant activity with significant (P< 0.001) difference at 750 and 1000µg/ml compared with those of the standard ascorbic acid and gallic acid. A reducing power range of 0.655± 0.001 and 1.84± 0.001nm measured at 700nm were obtained for extract concentrations range between 31.25 to 1000µg/ml. The phytochemical screening revealed the presence of alkaloids, saponins, phenols, flavonoids, tannins, glycosides, triterpenes and carbohydrates. These phytochemicals may be responsible for the observed antioxidant activity. It indicates that the ethanol extract of the leaves may serve as a promising source of antioxidant agent as well as being helpful in the treatment of ailments resulting from free radical damage.
Key words: Antioxidants, free radicals, *Borreriaverticillata*, DPPH, reducing power

1.0 Introduction

The generation of reactive oxygen species (ROS) or free radicals during metabolism and other activities beyond the oxidant capacity of a living system gives rise to oxidative stress (Mikulikova and Popov, 2001, Krishnaiah*et al*., 2007). Oxidative stress play a key role in the development of malaria, heart disease, chronic and neurodegenerative diseases, cancer, cataract AIDs and in aging process (Willcox*et al*., 2004; Sian, 2003; Pham-Huy*et al*., 2008;Aliyu*et al* .,2009). The important physiological functions of free radicals or their derivatives include: regulation of vascular tone, regulation of functions that are controlled by oxygen concentration enhancement of signal transduction from various membrane receptors including the antigen receptors of lymphocytes. Oxidative stress responses ensure the maintenance of redox homeostasis (Dröge, 2002). The balance between the advantages and detrimental effects of free radicals is logically important to life. A number of naturally occurring antioxidants have already been isolated from plants inclusive of cereals, fruits, leafy vegetables and seeds (Aliyu*et al*., 2009). Antioxidants have also reactive oxygen specie (ROS) scavenging effect and lipid peroxidation prevention (Aliyu*et al*., Khattak, 2011).The protective effect of free radicals can be explained by the antioxidant effects of phenolic compounds, flavonoids and tannins (Okwu and Okwu, 2005; Khattak, 2011).*B. verticillata* is a fine stemmed scrambling shrub that may reach a few meters of lateral extension and 1.2 meters in height as a free standing plant with tiny white flowers. *B. verticillata* occurs in agriculture areas, grass lands ruderal/disturbed shrubs/ shrubs lands and in urban areas commonly found in tropical Africa including Nigeria. The flower is used as antipyretic and analgesic (Vieira *et al*., 1999; Moreira *et al*., 2010), the roots as anti diarrhoea and for treatment of erysipelas and haemorrhoids (Lorenzi and Matos, 2002). In West India the decoction of the plant is used for diabetes and dysmenorrhoea. It is used in combination with *Cuscuta* and *Zebrinaschnizlein* for the treatment of amenorrhoea (Ayenzu, 1978; Conserva and Ferreira, 2012). *B. verticillata* is used to treat bacteria skin infections and leprosy in Senegal (Sofowora, 2008). The juice of fresh aerial part is used in Nigeria for the treatment of skin eczema. (Benjamin 1979; Ajibesin*et al*., 2008). Essential oils isolated from *B. verticillata* has been shown to inhibit the growth of *Escherichia coli*, *Staphyllococusaureus* ,*Pseudomonasaeruginosa* and *Candida albicans*(Burkill, 2000; Ushie and Adamu ,2010). Phytochemical screening of the leave extracts has shown the presence of alkaloids, anthraquinones, saponins, steroids, terpenes, flavonoids tannins and glycosides (Ushie and Adamu, 2010; Conserva and Ferreira, 2012). In continued research on the phytochemical and pharmacological properties of medicinal plant belonging to the Nigerian vegetation, the ethanol extract of *Borreriaverticillata* leaf was evaluated with a view to determine its potential for use as natural antioxidant.

2.0 Materials and methods

2.1 Drugs and chemicals

The following drugs and chemicals of analytical grade were used: Ethanol (Sigma Aldrich USA), N-hexane (Sigma Aldrich USA), Gallic acid (Fluka, U.K), Diphenyl-picryl-hydrazyl (DPPH) (Sigma Aldrich USA), Trichloro acetic acid (Sigma Aldrich USA). Potassium ferricyanide, Ferric chloride, phosphate buffer and

Ascorbic acid (all from BDH, Chemical Laboratory, England, U.K).

2.2 Equipment and Materials

U.V spectrophotometer (Jenway 6045/vis), weighing balance (Mettler, P152), stop watch, needles and syringes, spatula, pestle and mortar, test tubes, beakers.

2.3 Plant material

The whole plant *B.verticillata* was collected from Basawa in Zaria, Kaduna State Nigeria. The plant was identified and authenticated by Mallam Umar Gallah of the Herbarium section in the department of Biological Sciences, A.B.U, Zaria, Nigeria. A Voucher Specimen Number (672) was deposited at the herbarium for future reference. The leaves were then picked and dried under shade until constant weight was obtained. The dried leaves were then crushed into coarse powder using a pestle and mortar.

2.4 Extraction

The powdered leaves (200 g) were extracted with 1.2 L of N-hexane. The marc was extracted with 1.2 L of aqueous ethanol (70% ethanol and 30% water) $^{V}/_{V}$ for 24 hours at room temperature using a percolator. The solvent was removed over a water bath at temperature 45^{0} C. The extract was stored in a closed container and referred to as ethanol leaf extract of Borreriaverticillata (EEBV).

2.5 Phytochemical Screening

Preliminary phytochemical screenings were carried out on EEBV in order to confirm the presence of phytoconstituents following the methods described by Sofowora , (2008) and Evans (2009).

2.5.1 Detection of alkaloids

The extract (0.5 g) was stirred with 5ml of 1% aqueous hydrochloric acid on a water bath and then filtered. The filtrate, divided into 3 portions were treated with three drops of Wagner's reagent (iodine in potassium iodide), Dragendoff's reagent (solution of potassium bismuth iodide) and Meyers reagents (potassium mercuric iodide solution), respectively. The mixtures were observed for turbidity or precipitate (Evans, 2009).Appearance of colour brownish precipitate (with Wagner's reagent), creamy white precipitate (with Meyer's reagent) and Orange-red precipitate (with Dragendoff's reagent) would suggest the presence of alkaloids.

2.5.2 Detection of saponins

2.5.2.1 Frothing test

The leaf extract (0.5 g) of was shaken vigorously for 30 seconds with water in a test tube followed by warming on water bath. The test tube was allowed to stand in a vertical position for 30 minutes. Appearance of persistent froth for 10 - 15 minutes would suggest the presence of saponins.

2.5.2.2 Haemolysis test

Two millilitre of 1.8% of aqueous sodium chloride was taken in two test tubes. To one of the test tube the leaf extract (2 ml) was added and to the second test tube distilled water was added. Three drops of animal blood were added to each test tube and mixed gently by inverting the tubes. The contents of the two test tubes were observed for 15 – 30 minutes for haemolysis in the tubes.

2.5.3 Detection of Phenols

The leaf extract (0.5 g) was stirred in 10ml of water and filtered.The filtrate was treated with 3 drops of ferric chloride solution. Appearance of bluish black colour would indicate the presence of phenolic compounds.

2.5.4 Detection of Flavonoids

2.5.4.1 Shinoda test

To 0.2 g of the leaf extract, 5 ml of ethanol was added, stirred and filtered. To 1 ml of the filtrate, few pieces of magnesium ribbons and 3 drops of concentrated hydrochloric acid were added. Appearance of pink or red colour would suggest the presence of flavonoids.

2.5.4.2 Sodium hydroxide (Alkaline) test

To 1ml of the leaf extract 4 drops of 10% sodium hydroxide were added. Appearance of yellow colouration would suggest the presence of flavonoids.

2.5.6 Detection of tannins

2.5.6.1 Lead acetate test

To the solution of the extract in distilled water, two drops of strong lead (Pb) subacetate solution was added. Appearance of cream colour precipitate would suggest the presence of tannins.

2.5.7 Detection of glycosides

2.5.7.1 Borntrager's test

To 0.5 g of the extract in a dry test tube 5 ml of chloroform was added, shaken for 5 minutes and filtered. Five millilitre of 10% ammonium solution was added to the filtrate; the mixture was shaken and allowed to separate into two layers. The appearance of bright pink colouration in the ammonium layer would indicate the presence of free arthraquinones.

2.5.7.2 Keller-Killani test

To 5 ml of the leaf extract, 2 ml of glacial acetic acid and one drop of ferric chloride solution were added. The mixture was under layered with 1 ml of concentrated sulphuric acid. The appearance of a brown ring at the

interface and greenish supernatant would indicate the presence of deoxy-sugars characteristic of cardenolides.

2.5.7.3Salkowski's test

The plant extract (0.2 g) was dissolved in 2 ml of chloroform in a test tube, concentrated sulphuric acid was added to form a two layers. The interface was observed for a reddish brown colouration which indicates the presence of steroid ring (aglycone portion of cardiac glycosides) (Sofowora, 2008).

2.5.8 Detection of sterols and triterpenes

2.5.8.1 Lieberman-Burchard's test

To 2 ml of the extract, 2 ml of acetic anhydride was added; 1ml of concentrated sulphuric acid was added to the side of the test tube to form two layers. Colour change was observed immediately and over a period of 1 hour. Red, pink or purple colour indicates the presence of triterpenes while blue to blue green colour in the upper layer indicates the presence of steroids.

2.5.9 Detection of carbohydrates

2.5.9.1 Molisch Test

To 0.1 g of the extract 5 ml of distilled water was added and filtered. Four drops of molisch's reagent was added to the filtrate followed by concentrated sulphuric acid down the side of the test tube forming two layers, the lower layer, was observed for a reddish coloured ring at the interface, would indicate the presence of carbohydrates.

2.5.9.2 Fehling's test

To 5 ml of extract in a test tube 5 ml of Fehling's A and B solution were added and boiled on a water bath, the mixture was observed for a brick red precipitate which indicates the presence of reducing sugar.

2.6 *In vitro* Antioxidant Study

2.6.1 DPPH- Radical Scavenging Activity

The determination of DPPH radical scavenging activity of EEBV was carried out according to the method described by Mensor*et al.,* (2001) as modified by Aliyu*et al*., (2009) Different concentrations of the plant extract and standards (Gallic acid and ascorbic acid) 1000, 750, 500, 125, 50, 25 and 10 µg/ml were prepared. 1.0 ml of 0.3 mM DPPH was added to 2.5 ml of EEBV solution or standards. The mixtures were allowed to stand in a dark chamber for 30 minutes. The absorbance was measured at 518 nm using U.V spectrophotometer (Jenway 6045/vis). The percentage antioxidant activity of the plant extract was calculated using the formula,

$$\% AA = 100 - \frac{Abs\ sample - Abs\ blank}{Abs\ control} \times 100$$

Blank = Ethanol (1.0 ml) plus sample solution (2.0 ml), Negative control=DPPH solution (1.0 ml, 0.3 mM) plus methanol (2.0 ml), ascorbic acid and gallic acid were used as standards. The effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50} value) was obtained by linear regression analysis of dose response curve plotting between % antioxidant activity and concentrations.

2.6.2 Reducing power assay

This was determined according to the method of Oyaizu (1986). 1.0 ml of the extract or Standard of different concentrations (1000, 500, 250, 125, 62.5 and 31.25 µg/ml) were mixed with 2.5 ml phosphate buffer (PH 6.6, 0.2 M) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50°C for twenty minutes. Trichloro-acetic acid (2.5 ml) was added to the mixture. To 2 ml of the resulting mixture 0.5 ml of ferric chloride (0.1%) was added. The absorbance was measured at 700 nm in a UV spectrophotometer (Jenway 6045/vis). The procedure was repeated three times for each concentration. The higher absorbance of the reaction mixture indicated the reductive potential of the extract.

2.7 Statistical Analysis

The experiments were done in triplicate. All data obtained were expressed a Mean + Standard Error of Mean. Data were analysed by one way analysis of variance (ANOVA) using SPSS version 19 followed by Turkey's Post Hoc test. Values of $p<0.05$ were considered significant.

3.0 Results

3.1 Preliminary phytochemical screening

The preliminary phytochemical screening revealed the presence of alkaloids, saponins, phenol, tannins, glycosides, sterols/terpenoids, carbohydrates and flavonoids (Table.1).

Table 1 Phytochemical Constituents

Test	Constituents	Result
Dragendoff's,Wagner, Meyer's	Alkaloids	+
Frothing,Haemolysis	Saponins	+
Shinoda/ Alkaline test	Flavonoids	+
Lead acetate	Tannins	+
Borntrager test	Anthraquinones	--
Keller-Killani	Cardenolides	+
Lieberman-Buchard/ Salkowski	Steroids/Terpenoids	+
Molisch/Fehling's	Carbohydrates	+

+ = Present, - = Absent.

3.2.1 DPPH radical scavenging effect of EEBV

The result of the free radical scavenging activity on DPPH showed that EEBV was capable of scavenging free radicals and did so in a concentration dependent manner. The highest scavenging activity was observed at concentration of 1000 µg/ml (116% ± 0.42%). At low concentrations (10 and 25 µg/ml) the DPPH scavenging activity of the extract was significantly ($p< 0.001$) lower than that of ascorbic and gallic acid. However, EEBV showed significantly ($p< 0.001$) higher scavenging activity compared with ascorbic and gallic acid at concentrations 750 and 1000 µg/ml (Table 2). There was decrease in absorbance, observed with increasing concentration of the extract as well as the standards (ascorbic acid and gallic acid) (Figure 1). EC_{50} (efficient concentration) is define as the concentration of sample required to cause 50% loss in DPPH activity (Molyneux, 2004). The EC_{50} for EEBV, ascorbic acid and gallic acid were 2.98 µg/ml, 1.05 µg/ml and 1.76 µg/ml respectively (Figures 2, 3 and 4).

Table 2. Percentage antioxidant activity of EEBV on DPPH

	% Antioxidant Activity		
Concentration (µg/ml)	EEBV	Ascorbic Acid	Gallic acid
10	40.71 ± 0.45	80.11 ± 0.09*	69.36 ± 0.02*
25	65.66 ± 0.24	85.16 ± 0.17*	74.10 ± 0.02
50	76.56 ± 0.55	88.58 ± 0.26	86.58 ± 0.22
125	80.31 ± 0.47	92.49 ± 0.04	92.42 ± 0.22
250	89.56 ± 0.27	95.69 ± 0.11	93.48 ± 0.16
500	98.45 ± 0.26	97.21 ±0 .14	95.47 ± 0.19
750	109.33 ± 0.42*	98.57 ± 0.09	97.59 ± 0.12
1000	116.54 ± 0.42*	99.54 ± 0.08	99.46 ± 0.04

*p< 0.001 (n =3) significant compared with ascorbic acid and gallic acid, Turkey's post hoc test. EEBV = Ethanol leaf extract of *B.verticillata*

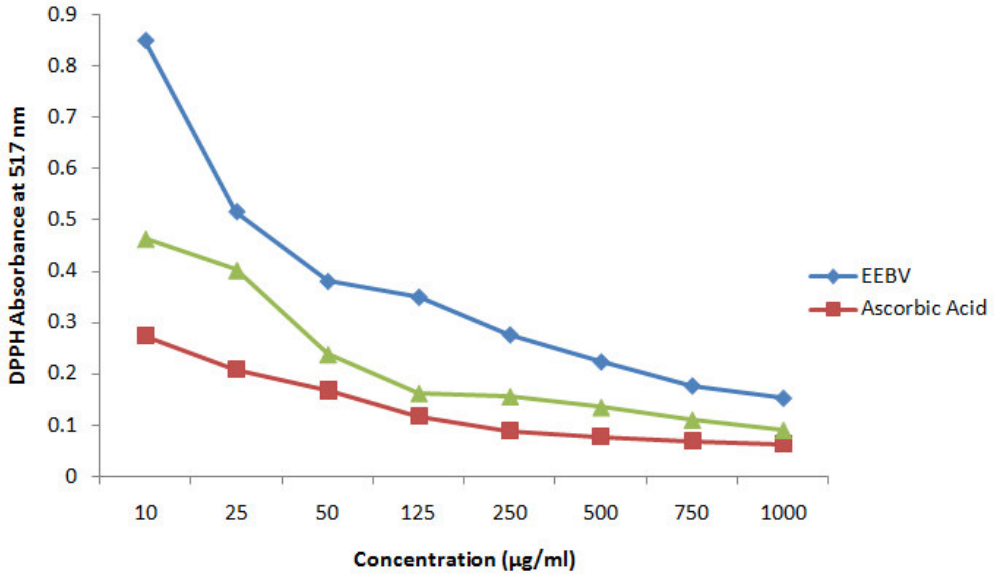

Figure 1. DPPH radical scavenging activity of EEBV,Ascorbic acid and Gallic acid

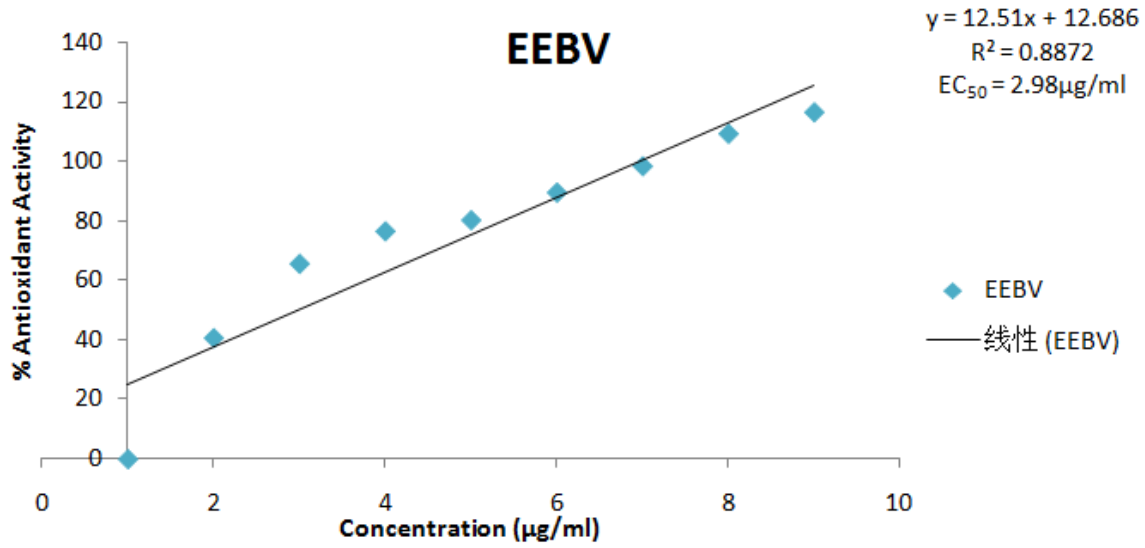

Figure 2. Linear regression of percentage antioxidant activity of EEBV

EC_{50} calculated using the regression equation, $y = ax + b$, where y = 50% antioxidant activity, x= EC_{50} , a= Regression coefficient and b= Regression constant

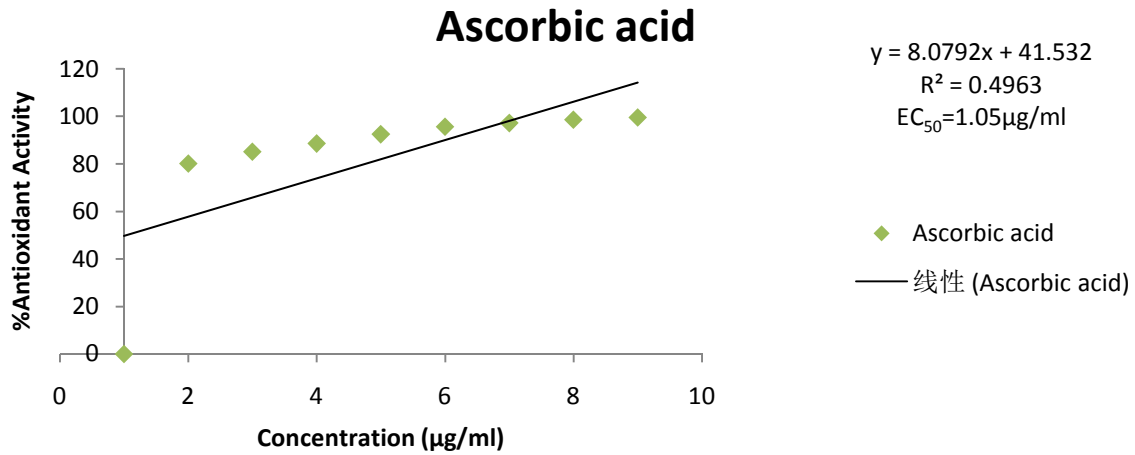

Figure 2.Linear regression of percentage antioxidant activity of ascorbic acid

EC_{50} calculated using the regression equation, $y = ax + b$, where y = 50% antioxidant activity, x= EC_{50} ,a= Regression coefficient and b= Regression constant

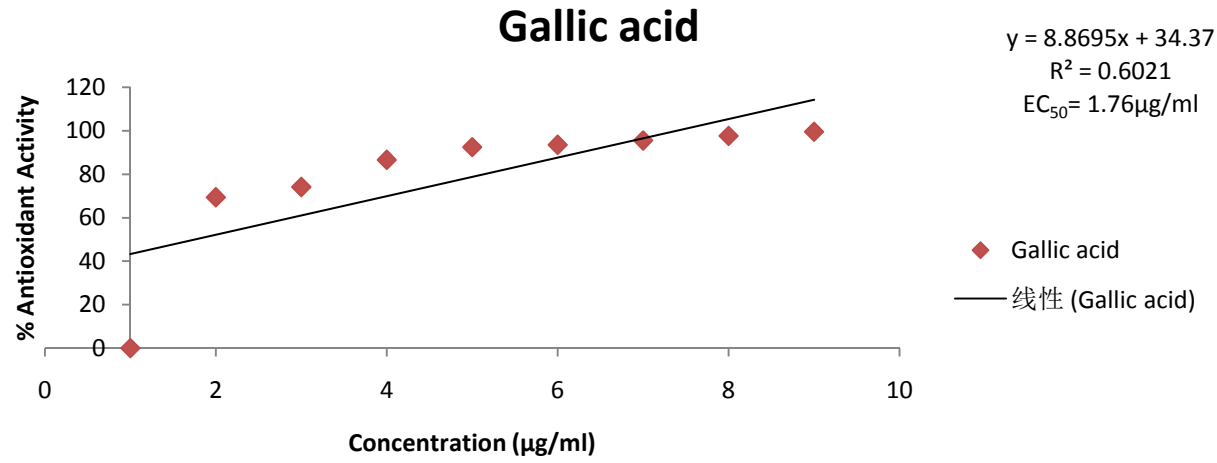

Figure 3. Linear regression of percentage antioxidant activity of gallic acid

EC_{50} calculated using the regression equation, $y = ax + b$, where y = 50% antioxidant activity, x= EC_{50} ,a= Regression coefficient and b= Regression constant

3.2.2Reducing power activity of EEBV

The reducing power activity of EEBV was found to increase with increasing concentrations. However when compared with ascorbic acid and gallic acid the reducing power of EEBV was significantly ($p< 0.05$) lower at the concentrations used (Figure 5).

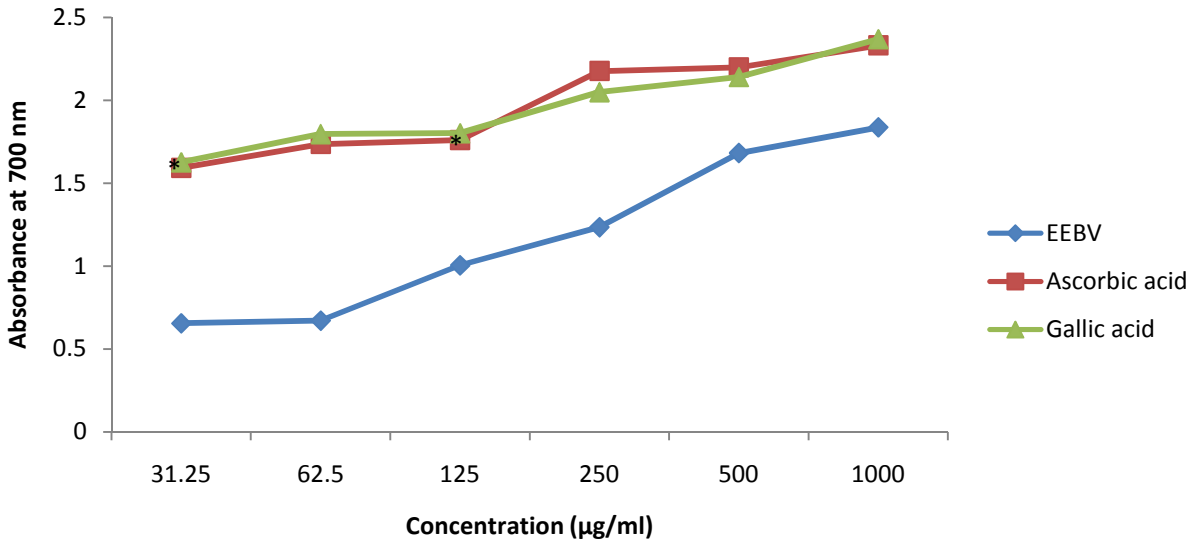

Figure 4. Reducing power pottentials of EEBV in comparison with Ascorbic acid and Gallic acid

*p< 0.05 (n =3) significant compared with ascorbic acid and gallic acid, Turkey's post hoc test. EEBV = Ethanol leaf extract of *B.verticillata*

4.0 Discussion

The preliminary phytochemical screening of *B.verticillata* showed the presence of alkaloids, saponins, Steroids, glycosides, flavonoids, tannins and phenols. This finding is consistent with those of Ushie and Adamu, (2010) and Conserva and Ferreira, (2012). These compounds have well known pharmacological activities including analgesic, anti-inflammatory and antioxidant effects (Perez, 2001; Park *et al*., 2001). The DPPH assay has been mainly used as a quick, reliable and reproducible parameter to search for *in vitro* general antioxidant activity of pure compounds and plant extracts (Kolev*aet al*., 2002; Goncalves*et al*., 2005; Ebrahimzadeh*et al*., 2008; Aliyu*et al*., 2009). DPPH is a stable nitrogen centred free radical. Its colour changes from violet to light yellow upon reduction by either the process of electron or hydrogen ion donation. Any substance capable of performing this reaction can be regarded as an antioxidant and therefore a free radical scavenger (Dehpour*et al*., 2009). The EC_{50} is the concentration of sample required to decrease the initial DPPH concentration by 50% is a parameter widely used to measure antioxidant activity (Molyneux, 2004). The higher the EC_{50} value the lower the antioxidant activity and the lower the EC_{50} the higher the antioxidant activity. EC_{50} values less than 100 µg/ml is indicative of very good free radical scavenging potential (Khattak, 2011). The R^2 values from the regression of the plot of antioxidant activity against concentration of extract and control explains the degree of the relationship between antioxidant activity and concentration. The nearer the R^2 value is to one the greater the relationship (Araoye, 2003)

The reducing power is usually used as an indicator of electron donating activity which is an important mechanism of action of phenolic antioxidants (Nabavi*et al*., 2009). In the reducing power assay the presence of antioxidants in the sample results in reduction of Fe^{3+} to Fe^{2+}. The complex is monitored by the measurement of pearl's Prussian blue formed at 700 nm. Increased absorbance at 700 nm indicated a reductive potential of the plant. The increase in absorbance observed with EEBV suggests electron donating activity and reductive potential of the extract which is further buttressed by the presence of phenols in the preliminary phytochemical screening. Flavonoids are potent water soluble antioxidants which prevent oxidative cell damage (Okwu, 2005; Akinmoladun*et al*., 2007a; Akinmoladun*et al*., 2007b).

Tannins are also known to possess antioxidant effects. Thus, the presence of these constituents may be contributory to the antioxidant activity of the plant.

5.0 Conclusion

Taking into account the phytochemical screening, the DPPH radical scavenging activity and reducing power as

indicators of antioxidant activity of EEBV, these findings revealed the potential of the extract as a source for natural antioxidants. It indicates that the plant could be an auspicious mediator in scavenging free radicals and treating diseases related to free radical reactions.

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