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Original Paper

Evaluation of *in vitro* antioxidant activity of saponin-rich fraction from leaves of *Zanthoxylum zanthoxyloides*

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A.O. Olusola[1]*, A.O. Elekan[1], A.O. Olusola[1], T.O. Ogidan[1], O.E. Ekun[1], I.O. Onoagbe[2]

[1]Department of Biochemistry, Adekunle Ajasin University, Akungba Akoko, Ondo State, Nigeria
[2]Department of Biochemistry, Faculty of Life Science, University of Benin, Benin City, Edo State, Nigeria

*Corresponding author, E-mail: augustine.olusola@aaua.edu.ng

Abstract

Zanthoxylum zanthoxyloides, also known as *Fagara zanthozyloides*, is a medicinally important plant of the Rutaceae family. It contains high concentration of saponins and has several medicinal uses. The aim of this study was to evaluate *in vitro* antioxidant properties of saponin-rich extract from leaves of *Z. zanthoxyloides*. Phytochemical screening of crude methanol extract of leaves of *Z. zanthoxyloides* revealed presence of saponins, alkaloids, flavonoids and tannins. Extraction of saponins from methanolic extract was performed by a standard procedure. The resulting saponin-rich fraction was investigated for *in vitro* antioxidant activity with free radicals, superoxide anion radical, hydrogen peroxide and ferric ions. The extract exhibited potent and concentration-dependent increase in free radical scavenging activity against free radicals. Scavenging capacity of superoxide radicals and hydrogen peroxide scavenging of the extract also raised with increased extract concentration. The apparent antioxidant activity of the saponin-rich fraction of leaf extract from *Z. zanthoxyloides* suggests its potential use in prevention and treatment of diseases.

Key words: antioxidative capacity, free radical scavenging activity, saponins, *Zanthoxylum zanthoxyloides.*
Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl.

Introduction

Medicinal plants contain chemical substances that can be used for therapeutic purposes, and some of them are used as precursors for synthesis of pharmaceuticals (Sofowora 1984). Plants produce a variety of non-essential chemicals as a part of biotic defense, but numerous studies have demonstrated that many phytochemicals can protect against human diseases (Kumar et al. 2009). Historically, plants have played a significant role in health protection (Vogel 1997). Phytochemical screening has unveiled the chemicals responsible for these functions (Faraz et al. 2003), but structural analysis has provided the basis for their therapeutic potencies (Stintzing et al. 2002). This has also increased the relevance of wild plants in drug development.

One such plant considered of great importance is *Zanthoxylum zanthoxyloides*. *Z. zanthoxyloides* (Rutaceae), commonly called 'toothache bark' or 'candle wood' in English or 'Orin ata' in Yoruba, is widespread in the West Tropical Africa where it occurs in savanna and dry forests, as well as in the coastal area of Nigeria, mostly in the southern part of the country (Iwu et al. 1999). *Z. zanthoxyloides* contains various secondary metabolites, which have diverse biological activties, including antioxidative, antiinflammatory, antisickling, antibacterial, antiviral, antihepatotoxicity, antiallergic, antitumoral and antihypertensive properties (Sofowora et al. 1975; Andersson et al. 1996; Adesina 2005). The methanol extract prepared from powdered root of *Z. zanthoxyloides* has been reported to contain flavonoids, chelerythrine, berberine and phenol canthine-6-one and possesses strong antibacterial activity (Odebiyi, Sofowora 1979; Tsuchiya et al, 1996). These extracts have been used as components of antiseptic, antiparasitic and analgesic preparations for managing smallpox, syphilis and related disease conditions (Olatunji 1983).

Saponins are natural glycosides of steroid or triterpene nature, which have been shown to exhibit a variety of biological and pharmacological properties, including antioxidant, hypolipidemic potential and inhibition of erythropoesis in *Rattus novergicus* (Elekofehinti et al. 2012). However, to the best of our knowledge, there is no information available on *in vitro* antioxidant properties of saponins from leaves of *Z. zanthoxyloides*. Therefore, this study was carried out to evaluate the *in vitro* antioxidant potential of saponins from *Z. zanthoxyloides* leaves.

Materials and methods

Collection and identification of plant material

Fresh *Z. zanthoxyloides* leaves were collected in farmland towards the end of the harmattan season from Ugbe Akoko in the Akoko North-East Local Government Area of Ondo State, Nigeria. Plants were identified and certified at the herbarium of Plant Science and Biotechnology Department, Adekunle Ajasin University, Akungba Akoko. The leaves were washed with clean water, air-dried until a constant weight was obtained, and pulverized by an electric blender into powder. The powdered plant material was kept in an airtight container and preserved in a cool, dark and dry place until the extraction.

Preparation of extract

A sample (100 g) of the powdered leaf was soaked in 500 mL of methanol (95%) for 72 h, with occasional stirring using a glass rod to ensure proper mixture of the vessel content. The content was then filtered through two layers of cheesecloth. The extract (filtrate) was then concentrated under reduced pressure at 45 °C until the extraction solvent was completely removed. A green soluble crude residue was obtained.

Qualitative phytochemical analysis

Chemical tests for the screening and identification of bioactive chemical constituents were carried out on the extract using standard procedures (Trease, Evans 1989; Sofowora 1993; Harborne 1998) with minor modifications.

Isolation of saponins from crude methanolic extract

Isolation of the saponin-rich fraction was carried out using a procedure described by Abdel-Gawad et al. (1999) and Elekofehinti et al. (2013) with slight modification. The crude extract was partitioned with hexane and water (1:2, v/v). After thorough shaking, the mixture was allowed to stand overnight and the water layer was concentrated and partitioned between ethyl acetate and n-butanol (1:3, v/v). The butanol fraction was concentrated to obtain the crude saponin fraction.

Determination of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity

The free radical scavenging activity of saponin-rich fraction was measured with the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described by Braca et al. (2001) with slight modification. Aliquots (1 mL) of the extract at different concentration (0.2 to 0.8 mg mL^{-1}) were added to 1 mL 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken vigorously and incubated in the dark at room temperature for 30 min. The absorbance was read at 517 nm by a UV/Vis spectrophotometer. Ascorbic acid at the same concentration was used as a positive control. Ethanol (95%) was used as a blank. The control solution consisted of 0.1 mL of 95% ethanol and 2.9 mL of DPPH solution. Analyses were carried out in triplicate. Percentage inhibition of DPPH radical was calculated as:

$$\text{DPPH inhibition (\%)} = (Abs_{control} - Abs_{sample}) / Abs_{control} \times 100,$$

where $Abs_{control}$ is the absorbance of the blank control and Abs_{sample} is the absorbance of the test sample.

The EC_{50} (the microgram of the extract to scavenge 50% of the radicals) value was estimated from a percentage inhibition plot using a non-linear regression plot. A lower EC_{50} value indicates larger antioxidant activity.

Determination of hydrogen peroxide scavenging activity

The ability of the saponin-rich extract to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (1989) as described by Keser et al. (2012) with slight modification. A solution of hydrogen peroxide (4 mM) was prepared in 0.2 M phosphate buffer (pH 7.4). The extract at varying concentration of (0.2 to 0.8 mg mL^{-1}) in distilled water was added to 600 µL of 4 mM hydrogen peroxide solution. The absorbance of test samples was read at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The absorbance of hydrogen peroxide was taken as the control. Ascorbic acid was used as a standard antioxidant. The percentage of hydrogen peroxide scavenging effect was calculated by comparing the absorbance values of the control and test samples using:

$$H_2O_2 \text{ scavenging capacity (\%)} = (AC - AS) / AC \times 100,$$

where AC is the absorbance of the control and AS is the absorbance of the test sample.

EC_{50} values were estimated from a % inhibition versus concentration plot using a non-linear regression plot. All analyses were performed in triplicate

Determination of superoxide anion radical scavenging activity

The method described by Xie et al. (2008) was used to determine superoxide anion radical scavenging activity. Samples (1 mg mL^{-1} final concentration) were each dissolved in 50 mM Tris–base buffer, pH 8.3, containing 1 mM EDTA and 80 µL was transferred into a clear bottom microplate well; 80 µL of buffer was added to the blank well. This was followed by the addition of 40 µL 1.5 mM pyrogallol (dissolved in 10 mM HCl) into each well in the dark and the change in the rate of reaction was measured immediately at room temperature over a period of 4 min using a spectrophotometer at wavelength 420 nm. The superoxide scavenging activity was calculated using the following equation:

$$\text{Superoxide scavenging activity (\%)} = (\Delta A_b - \Delta A_s) / \Delta A_b \times 100,$$

where ΔA_b and ΔA_s are changes in absorption per min of blank and sample, respectively. Analyses were carried out in triplicate.

Determination of ferric reducing antioxidant property

The reducing power of the extract was measured according to the method of Oyaizu (1986) with slight modification. An 1 mL aliquot of different concentration (0.2 to 0.8 mg mL^{-1}) of the saponin extract (0.2 M PBS, pH 6.6) was mixed with 1 mL of 1% potassium ferric cyanide solution. The mixture was incubated at 50 °C for 30 min followed by the addition of 1 mL 10% (w/v) trichloroacetic acid. Aliquots of the incubated mixture (1 mL) were added to 1 mL of distilled water and 0.2 mL of 0.1 % (w/v) ferric chloride in test tubes. After a 10 min reaction time, the absorbance of the resulting solution was read at 700 nm. Higher absorbance suggested stronger reducing power. Ascorbic acid was used as the reference antioxidant. An aqueous solution of known Fe (II) concentrations ($FeSO_4$ $7H_2O$; 0.063 to 2.0 mM) was used for calibration. Results were expressed as mM Fe^{2+} mg^{-1} extract.

The EC_{50} of extracts was calculated from a graph of absorbance at 700 nm versus extract concentration. All the tests were performed in triplicate.

Statistical analysis

All analyses were carried out in triplicate and data were expressed as mean ± standard error of mean. The data were statistically analyzed using the Student's *t*-test and differences were considered statistically significant at $p < 0.05$ using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

Results

The DPPH radical scavenging activity of ascorbate (control) and the saponin-rich fraction of *Z. zanthoxyloides* leaves, at a concentration range of 0.2 to 0.8 mg mL^{-1} (Fig. 1). The standard antioxidant (ascorbate) as well as the saponin-rich extract showed concentration-dependent increase in DPPH scavenging activity.

Fig. 2 shows the EC_{50} values of the extract for scavenging DPPH radical, as compared to ascorbate (control). Saponin-rich extract scavenged the radical to a 50% extent at a significantly ($p < 0.05$) higher concentration when compared to ascorbate.

Evidence of the ability of the saponin-rich fraction from leaves of *Z. zanthoxyloides* to scavenge hydrogen peroxide is presented in Fig. 3, in comparison with ascorbate as a standard. Saponins had significantly ($p < 0.05$) lower H_2O_2 scavenging capacity when compared with that of ascorbate. Also, both the ascorbate and saponin-rich fractions were capable of scavenging hydrogen peroxide in a concentration-dependent manner. The EC_{50} for ascorbic acid was 25.95×10^{-2} mg mL^{-1} as compared with EC_{50} of 88.60×10^{-2} mg mL^{-1} for the saponin fraction (Fig. 4).

Superoxide radical scavenging activity of ascorbate and the saponin-rich fraction from leaves of *Z. zanthoxyloides* is presented in Fig. 5. The saponin-rich fraction had lower scavenging activity at a concentration range of 0.5 to 2.0 mg mL^{-1}, when compared to ascorbate.

Fig. 6 shows EC_{50} values of the saponin fraction in scavenging superoxide anion radical, as compared to ascorbate. Saponins scavenged the radical to a 50% inhibition at a concentration of 4.53×10^{-2} mg mL^{-1}, while ascorbate had 50% scavenging activity at concentration of 2.23×10^{-2} mg mL^{-1}. The saponin fraction had a significantly ($p < 0.05$) lower EC_{50} value than for ascorbate.

The results for ferric reducing activity of the saponin-rich fraction, compared to the ascorbic acid standard, are shown in Fig. 7. The saponin-rich fraction had significantly ($p < 0.05$) lower antioxidant activity at different concentrations when compared to ascorbate. At all concentrations used,

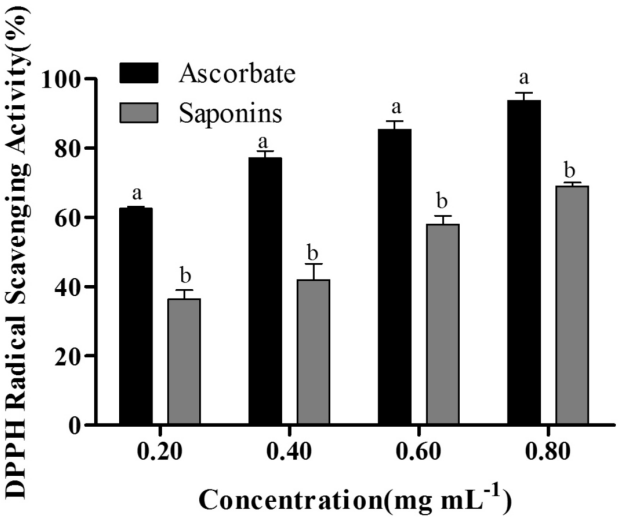

Fig. 1. DPPH radical scavenging activity of the saponin-rich fraction of leaves from *Z. zanthoxyloides*. Bars represent the mean ± standard error ($n = 3$). Comparisons between samples were made at the same concentration. Bars with the same letters do not differ significantly ($p < 0.05$).

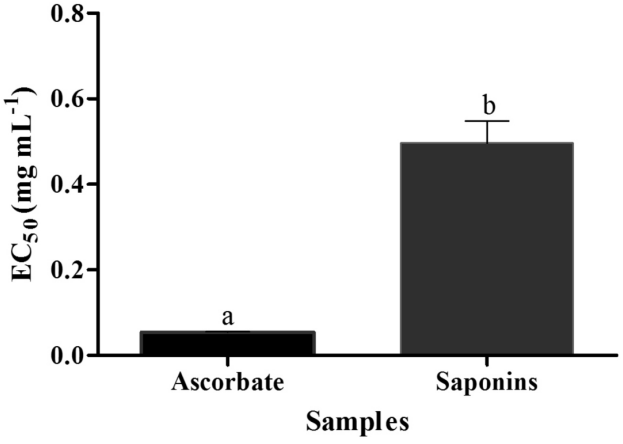

Fig. 2. EC_{50} values of saponin-rich fraction of*Z. zanthoxyloides* leaf in scavenging DPPH radical. Bars represent the mean ± standard error ($n = 3$). Bars with the same letters do not differ significantly ($p < 0.05$).

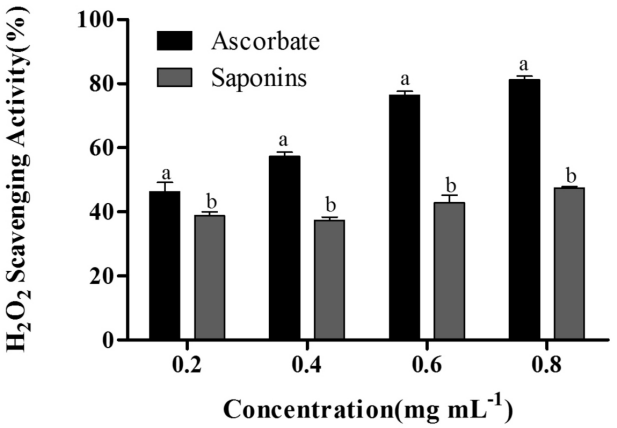

Fig. 3. Hydrogen peroxide scavenging activity of saponin-rich fraction from leaves of *Z. zanthoxyloides*. Bars represent the mean ± standard error (n = 3). Comparisons between samples were made at the same concentration. Bars with the same letters do not differ significantly ($p < 0.05$).

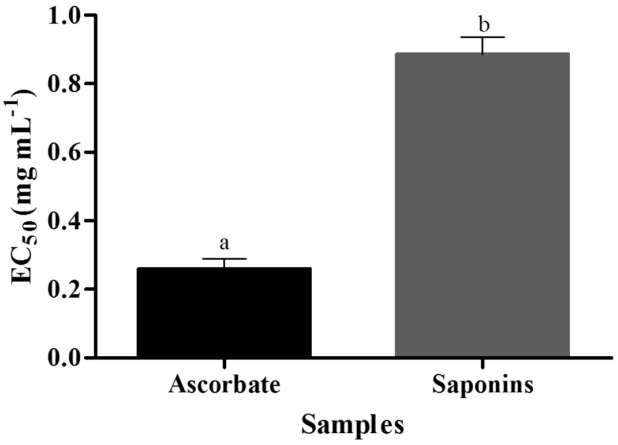

Fig. 4. EC_{50} values of saponin-rich fraction from leaves of *Z. zanthoxyloides* in scavenging hydrogen peroxide. Bars represent the mean ± standard error (n = 3). Bars with the same letters do not differ significantly ($p < 0.05$).

except 0.80 mg mL^{-1}, there was no significant difference in the activity of the saponin fraction.

Discussion

The therapeutic benefits of medicinal plants are often attributed to their antioxidant potencies (Vinay et al. 2010). Natural antioxidants found in plants and vegetables have been extensively studied for their ability to protect the organism and cells from the deleterious effects induced by oxidative stress (Kamdem et al. 2012; Elekofehinti et al. 2013; Filho et al, 2014). It is well known that the antioxidant activity of chemical compounds or plant extracts vary according to the model system used. Consequently, the use of only one assay system for the determination of antioxidant activity is not advisable (Tan, Lim 2015). In the present study, the antioxidant potential of saponin-rich

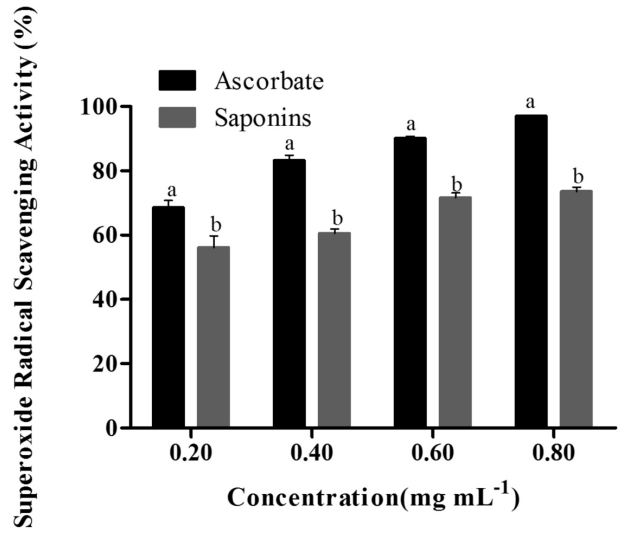

Fig. 5. Superoxide radical scavenging activities of saponin-rich fraction from leaves of *Z. zanthoxyloides*. Bars represent the mean ± standard error (n = 3). Comparisons between samples were made at the same concentration. Bars with the same letters do not differ significantly ($p < 0.05$).

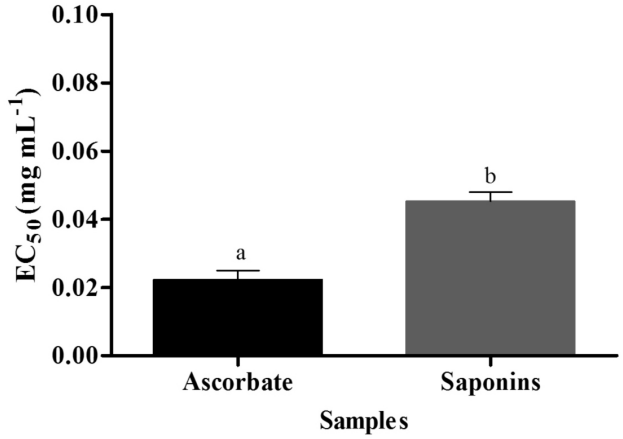

Fig. 6. EC_{50} values of saponin-rich fraction from leaves of *Z. zanthoxyloides* in scavenging superoxide radical. Bars represent the mean ± standard error (n = 3). Bars with the same letters do not differ significantly ($p < 0.05$).

fraction from leaves of *Z. zanthoxyloides* was determined by DPPH radical scavenging activity, superoxide anion radical scavenging activity, hydrogen peroxide scavenging activity and ferric reducing power activity assay. Together all these assays provided a better estimation of antioxidant properties and the obtained results revealed that antioxidant activity of saponin-rich fraction of *Z. zanthoxyloides* was concentration-dependent.

It is well accepted that the scavenging of DPPH free radical by antioxidants is due to the ability of the latter to donate hydrogen (Chen, Ho 1995). The results of the free radical scavenging activity of the saponin-rich fraction as well as its EC_{50}, in comparison to those of ascorbate (standard antioxidant) revealed that saponin-rich fraction

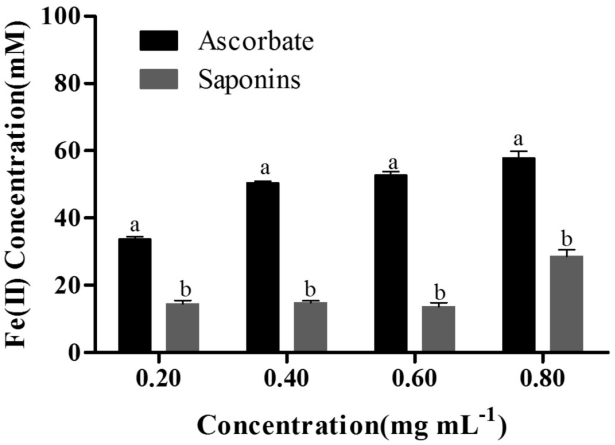

Fig. 7. Ferric reducing antioxidant properties of saponin-rich fraction from leaves of *Z. zanthoxyloides*. Bars represent the mean ± standard error (n = 3). Comparisons between samples were made at the same concentration. Bars with the same letters do not differ significantly ($p < 0.05$).

showed concentration-dependent scavenging of DPPH radical. This may be attributed to its hydrogen and/or electron donating ability, which might prevent reactive radical species from reaching functionally important molecules such as lipoproteins, polyunsaturated fatty acids, DNA, amino acids, and proteins (Halliwell et al. 1992).

These results agree with earlier reports that the scavenging effect on the DPPH radical increased sharply with increasing concentration of the samples and standards (Motalleb et al. 2005, Adekunle et al. 2012). The EC_{50} for ascorbate and the fraction of *Z. zanthoxyloides* indicated lower scavenging capacity of saponins. Although the DPPH radical scavenging ability of the saponin-rich fraction was less than that of ascorbate, it is important that the fraction had proton-donating ability and could serve as free radical inhibitor or scavenger, acting possibly as a primary antioxidant.

Hydrogen peroxide itself is not extremely reactive, but it can be toxic to cell because it may induce formation of hydroxyl radicals in cells (Halliwell 1991). Thus, the removal of H_2O_2 is very important for antioxidant defense in cell or food systems. Results presented in Fig. 5 show that the saponin-rich fraction of *Z. zanthoxyloides* exhibited significantly lower scavenging activity when compared to that of ascorbic acid, but was able to scavenge hydrogen peroxide and inhibit the formation of hydroxyl radical to some degree. The EC_{50} value of the extract was larger than that of ascorbic acid, but the scavenging capacity of the extract increased with increasing concentration.

The superoxide radical is known to be very harmful to cellular components because of high reactivity and since it can initiate free radical chain reactions (Haliwell 1991). Therefore, investigation of the free radical scavenging activity is of great importance (Kanatt et al. 2007). *Z. zanthoxyloides* leaf extract in the present study showed good ability to scavenge superoxide anions. The EC_{50} values of the leaf extract were higher than those of ascorbic acid, a standard antioxidant. This indicated lower scavenging capacity of the saponin-rich fraction. Similar to these results, saponins isolated from *Radix trichosanthis* had efficient scavenging ability for the superoxide anion radical (Chen et al. 2014).

Reducing power assay is often used to evaluate the ability of a natural antioxidant to donate an electron or hydrogen (Yildirim et al. 2000). Reducing power of a bioactive compound is associated with its antioxidant activity and may serve as a significant reflection of its potential activity (Oktay et al. 2003). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, reacting with free radicals to stabilize and block radical chain reactions (Chanda, Dave 2009). In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of the sample. The results obtained here showed an increase in absorbance with an increase in concentration, indicating that the saponin-rich extract had effective reducing power when compared to the standard ascorbate.

The saponin-rich fraction generally exhibited low ferric reducing properties, and this agrees with previous studies (Malika et al., (2015). The low ferric-reducing property of the extract, when compared with that of ascorbate, may be attributed to the structure of their aglycones and the number of attached sugar residues (Ryu et al. 2012). In a study on antioxidant activity of saponins from Vietnamese ginseng, protective action against free radical-induced tissue injury was found (Huong et al. 1998).

Free radicals induce oxidative stress *in vivo*, which may lead to oxidative modification or damage of some biological structures such as lipids, proteins, and DNA, and may give rise to degenerative diseases (Muleya 2013). An exogenous supply of antioxidants can be chosen as therapy or as a preventive measure against damaging effects of reactive oxygen species (Muleya 2013). Natural sources of antioxidants are much safer to use due to less toxicity and side effects (Akharaiyi 2011). In this respect, the results of the present study *in vitro* look encouraging, as the extract of *Z. zanthoxyloides* had some pronounced radical scavenging effects.

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