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Comparative Study of the Antioxidant, Free Radical Scavenging Activity and Human LDL Oxidation Inhibition of Three Extracts From Seeds of a Cameroonian Spice, *Xylopia parviflora* (A. Rich.) Benth. (Annonaceae)

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ABSTRACT

The antioxidant activity of water, ethanol and hydroethanolic extracts of the spice *Xylopia parviflora* (A. Rich.) Benth. (Annonaceae) from Cameroon, was evaluated using different antioxidant tests such as O^{-2}, OH•, NO, $DPPH^{\cdot}$, $ABTS^{\bullet+}$ scavenging activities, FRAP, as well as metal chelation and inhibition of copper-induced *in vitro* human low density lipoprotein (LDL) and α-linoleic acid oxidation. DPPH and ABTS were determined kinetically. Compared with two other extracts, the water extract exhibited the lowest phenolic content, AE_{ABTS} and AE_{DPPH} values while the ethanol extract had the highest phenolic content and reductive potential and the hydroethanolic extract the highest AE_{ABTS} and AE_{DPPH} values ($P < 0.05$). All extracts exhibited a dose dependent inhibition of O^{-2}, OH•, NO, radicals, metal chelating activity and inhibition of α-linoleic acid and LDL oxidation ($P < 0.05$). Those various antioxidant activities were compared to standard antioxidants such as catechin and ascorbic acid. Compared with controls, each extract significantly decreased malondialdehyde and lipid hydroperoxide formation in LDL ($P < 0.05$). Contrary to other antioxidant methods where the ethanolic extract was the most potent, the hydroethanolic extract exhibited the highest inhibition of LDL oxidation, metal chelating and nitric oxide scavenging activities. Therefore, *Xylopia parviflora* could be a good source of natural antioxidant.

Keywords: ABTS, DPPH, FRAP, inhibition of lipid peroxidation, metal chelating activity, nitric oxide

INTRODUCTION

Reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals, hydroxyl radicals and non free-radical species such as hydrogen peroxide and singlet oxygen are various forms of activated oxygen. Along with reactive nitrogen species (RNS), ROS are implicated in numerous diseases such as inflammation, reperfusion damage, atherosclerosis, malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, and cancer (Alho and Leinonen 1999; Duh *et al.* 1999). In living organisms, various ROS can form in different ways. Normal aerobic respiration stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of ROS include tobacco smoke, certain pollutants, organic solvents, and pesticides (Davies 1994; Robinson *et al.* 1997; Halliwell and Gutteridge 1999). There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. Under pathological conditions, ROS and RNS are overproduced and result in lipid peroxidation and oxidative stress. ROS are formed when endogenous antioxidant defenses are inadequate. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular molecules (El-Habit *et al.* 2000).

There is a growing interest in natural antioxidants, present in medicinal and food plants that might help attenuate oxidative damage (Silva *et al.* 2007). These natural antioxidants not only protect food lipids from oxidation, but may also provide health benefits associated with preventing damages due to biological degeneration (Shahidi and Wanasundara 1992; Hu and Kitts 2005). To this effect, research has focused on the identification and isolation of compounds from natural products with high antioxidant capacities (Katsuzaki *et al.* 1993; Okamura *et al.* 1993; Parasakthy *et al.* 1996). Examples of such compounds are from spices and herbs. Spices have also been recognized to possess several medicinal properties such as efficacy as anti-diabetics (Srinivasan 2005a, 2005b), ability to stimulate digestion (Patel and Srinivasan 2004), antioxidant property and anti-inflammatory potential. There are growing epidemiological evidences suggesting positive associations between the consumption of phenolic-rich foods or beverages and the prevention of diseases. These effects have been attributed to antioxidant components such as plant phenolics, including flavonoids and phenylpropanoids among others (Scalbert and Williamson 2000).

Nkui and *Nah poh* are two traditional soups of the western province of Cameroon which contain many spices among which are seeds of *Xylopia parviflora* (A. Rich.) Benth. (Annonaceae). *X. parviflora* is a tall tree distributed in East and Central Africa, whose root decoction is taken by the coastal peoples for stomach disorders. Other medicinal uses include insertion of root pieces into nostrils for headache relief, and its bark is also used for analgesic and antispasmodic purposes (Nishiyama *et al.* 2006). In order to evaluate the possibility of using *X. parviflora* seed in food systems via exogenous addition for antioxidant protection, this study was designed to examine the non-enzymatic antioxidant capabilities of extracts from this edible plant. The possible non-enzymatic antioxidant activities include reducing power, metal ion chelating effect, scavenging acti-

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vity for radicals such as hydroxyl, superoxide anion and nitric oxide and inhibition of lipid peroxidation. It is well established that low-density lipoprotein (LDL) oxidation is strongly related to diabetic complications, atherosclerosis and cardiovascular diseases (Mertens and Holvoet 2001; Krentz 2003). Thus, use of supplements with antioxidative protection may benefit LDL stability, and prevent or alleviate LDL oxidation-associated diseases. In order to further understand the antioxidative protection of this fruit for human health, our present study also investigated the antioxidant effects of hydroethanolic, aqueous and ethanol seed extracts from this fruit on human LDL.

MATERIALS AND METHODS

Sample preparation and determination of polyphenol concentrations

Pods of *X. parviflora* were purchased from a local market in Bafoussam, Cameroon. Dried seeds removed from pods were ground and extracted in our laboratory using deionized water, hydroalcoholic solvent (50%) and ethanol (in a 1: 10 ratio). The extracts were filtered through Whatman #2 filter paper (Whatman International Limited, Kent, England) using a funnel and concentrated to about 10% of the original volume by a rotary evaporator before drying in the oven at 50°C. The amount of total phenolic content in the *X. parviflora* seed extracts was determined according to the procedure of Singleton and Rossi (1965) with some modification, using 1 mL of Folin-Ciocalteu's phenol reagent 0.2 N and 30 µL to develop a pigment whose absorbance was determined at 750 nm. The results were expressed as catechin equivalent.

Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing activity of the plant extracts was estimated based on the Ferric Reducing Ability of Plasma (FRAP) assay developed by Benzie and Strain (1996). The solutions for this assay consisted of 300 mmol/L acetate buffer pH 3.6, 10 mmol/L TPTZ (2, 4,6-tripyridyl-s-triazine) in 400 mmol/L of HCl and 20 mmol/L ferric chloride. The reagent for this assay was prepared fresh by mixing 10 parts of acetate buffer with 1 part of TPTZ solution and 1 part of ferric chloride. The assay was performed as followed: 2000 µL of freshly prepared FRAP reagent was mixed with 75 µL of sample, ethanol or hydroalcoholic solvent as appropriate for reagent blank. The absorbance was read at 593 nm using Spectronic Genesys 20 (Thermo Electron Corporation) after 30 min of incubation. The results were expressed as catechin and ascorbic acid equivalent.

Free radical scavenging activity on α,α-diphenyl-β-picrylhydrazyl (DPPH)

The antioxidant activity of *X. parviflora* seed extracts, catechin and ascorbic acid was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH method (Brand-Williams *et al.* 1995) modified by Sánchez-Moreno *et al.* (1998) in order to determine kinetic parameters. A solution (20 µL) of the sample extracts at various concentrations was added to 1000 µL of DPPH solution. The decrease in absorbance at 517 nm was determined continuously every minute with a Spectronic Genesys 20 (Thermo Electron Corp.) spectrophotometer until the reaction reached a plateau. The percentage of DPPH inhibition was calculated as follows:

DPPH Scavenging Effect (%) = $((A_0 - A_1)/ A_0)100)$

where A_0 was the absorbance of the DPPH solution control and A_1 was the absorbance in the presence of the sample DPPH assay at different time intervals until the steady-state. The parameter EC_{50}, which reflects 50% depletion of the free radical, is expressed in terms of g of dry extract/g of DDPH. It was calculated using the percentage of inhibition of all concentrations at steady state, and then the linear or logarithmic regression was applied. The time taken to reach the steady state at EC_{50} (tEC_{50}) and the antiradical efficiency ($AE = 1/EC_{50}tEC_{50}$) were also determined.

ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging activity was determined according to the method of Re *et al.* (1999) with some modifications. The $ABTS^{\bullet+}$ cation radical was produced by the reaction between 7 mM ABTS in H_2O (10 mL) and 4.9 mM potassium permanganate (10 mL) stored in the dark at room temperature for 12 h. Before usage, the $ABTS^{\bullet+}$ solution was diluted (about 1: 10 v/v) with phosphate buffer saline (0.1 M, pH 7.4, NaCl 150 mM) to get an absorbance of 2000 ± 0.025 at 734 nm. Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 20 µL solution of different concentrations of plant extracts (10–20 mg/mL). ABTS assay was expressed kinetically as described by Pérez-Jiménez and Saura-Calixto (2008) who modified the original method so as to determine kinetic parameters, the percentage of inhibition at 734 nm was calculated for each concentration relative to a blank absorbance (ethanol or water) at different time intervals until the reaction reaches a plateau. EC_{50}, tEC_{50} and AE are calculated as in the DPPH assay.

Antioxidant activity in a linoleic acid system

The antioxidant activities of extracts from *X. parviflora* were determined by the ferric thiocyanate method (Mitsuda *et al.* 1996). Each sample (25–100 µg/mL) in 0.5 mL of distilled water or ethanol and 2 mL phosphate buffer (0.04 M, pH 7.0), was mixed with linoleic acid emulsion (2.5 mL 0.04 M, pH 7.0) in a glass flask and stood, in darkness, at 37°C, to accelerate oxidation. Therefore, 50 mL of linoleic acid emulsion contained 175 µg Tween-20, 155 µL linoleic acid, and 0.04 M potassium phosphate buffer (pH 7.0). On the other hand, 5 mL control composed of 2.5 mL of linoleic acid emulsion and 2.5 mL, 0.04 M potassium phosphate buffer (pH 7.0) without the samples served as control. Aliquots of 0.1 mL were taken at several intervals during incubation. The degree of oxidation was measured according to the thiocyanate method by sequentially adding ethanol (4.7 ml, 75%), ammonium thiocyanate (0.1 mL, 30%), sample solution (0.1 mL), and ferrous chloride (0.1 mL, 0.02 M in 3.5% HCl). After the mixture had rested for 3 min, the peroxide value was determined by monitoring absorbance at 500 nm using a spectrophotometer (Spectronic Genesys 20). During the linoleic acid oxidation, peroxides are formed and that leads to oxidation of Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. The degree of oxidation was measured for every 12 h until a day, after the absorbance of the control reached its maximum. The lipid peroxidation inhibition (LPI) % was calculated as:

$$LPI\ (\%) = \left(1 - \frac{\text{Absorbance at 500 nm in the presence of sample after 48 h}}{\text{Absorbance at 500 nm in the absence of sample after 48 h}}\right).$$

All analyses were run in triplicate; the absorbance due to the extract was removed and mean values were calculated.

Superoxide anion radical scavenging activity

The superoxide radical scavenging activity was measured based on the method by Siddhuraju and Becker (2007) with some modification. The reaction mixture contained, 1 mL of each of the following solutions: 150 µM nitroblue tetrazolium (NBT), 60 µM phenazine methosulfate (PMS), 468 µM NADH, prepared in 0.1 M phosphate buffer pH 7.4 and different concentrations of the plant extracts (0–1000 µg/mL), added in that sequence. The mixture was incubated in the dark for 10 min at 25°C and the absorbance was later read at 560 nm. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. Catechin was used as the positive control and the results were expressed as percentage inhibition of the superoxide radical. All determinations were performed in triplicate and the absorbance due to the extract was removed. The scavenging activity on superoxide anion (SASA) radicals was expressed as:

SASA (%) =
(1- Absorbance at 560 nm in the presence of sample) ×100
Absorbance at 560 nm in the absence of sample

Nitric oxide radical scavenging assay

The interaction of extracts of *X. parviflora* with nitric oxide was assessed by the nitrite detection method as described by Sreejayan and Rao (1997). Nitric oxide was generated from sodium nitroprusside previously bubbled with nitrogen and measured by the Greiss reaction. 0.25 mL of sodium nitroprusside (10 mM) in phosphate-buffered saline (PBS) was mixed with 0.25 mL of different concentrations (50–300 µg/mL) of extracts dissolved in the suitable solvent system and incubated at 30°C in the dark for 180 min. The control was run as above but the sample was replaced with the same amount of water. After the incubation period, 0.25 ml of Griess reagent A (1% sulphanilamide in 5% phosphoric acid) was added, and kept at 30°C for 10 min. After incubation, 0.25 mL of Griess reagent B (0.1% *N*-1-naphthylethylene diamine dihydrochloride) was added mixed and incubated at 30°C for 20 min. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm. The same reaction mixture without the extract but with equivalent quantity of distilled water served as control. Catechin was used as reference standard. All analyses were run in triplicate and the absorbance due to the extract was removed. The percentage of inhibition was calculated as was done with superoxide anion radical scavenging activity.

Scavenging of hydroxyl radical

The hydroxyl radical scavenging activity of extracts was determined using a modification of the method of Halliwell *et al.* (1987). The reaction mixture consisted of $FeCl_3$ (300 µM). EDTA (780 µM), 2-deoxiribose (2.8 mM), ascorbic acid (300 µM), and H_2O_2 (4 mM) in potassium phosphate buffer (20 mM, pH 7.4). The final reaction volume (1 mL), which included different concentrations of extracts (250–1000 µg/mL), was incubated at 37°C for 1 hour. After incubation, 1 mL of trichloroacetic acid (2.8% w/v) and 1 mL of thiobarbituric acid (1% w/v) were added and further incubated at 100°C for 20 min. The reaction mixture was then allowed to cool at room temperature, and the absorbance read at 532 nm. The reaction mixture not containing the test sample was used as control. All determinations were performed in triplicate and the absorbance due to the extract was removed. The scavenging activity on hydroxyl radicals (HRSA) was expressed as:

HRSA (%) =
Absorbance at 532 nm in the presence of sample ×100
Absorbance at 532 nm in the absence of sample

Ferrous metal ions chelating activity

The chelating of ferrous ions by *X. parviflora* and standard molecules was estimated by the method of Dinis *et al*. (1994). Briefly, extracts (250–2000 µg/mL) in 0.5 mL were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. All determinations were performed in triplicate and the absorbance due to the extract was removed. The inhibition percentage of ferrozine–Fe^{2+} complex formation was calculated by using the formula given below:

Metal chelating effect (%) = [(Control/Sample)/Control] ×100

where Control is the absorbance of control and Sample the absorbance in the presence of the extracts or standards. The control contains $FeCl_2$ and ferrozine complex formation molecules and an equivalent amount of solvent.

In vitro copper-induced oxidation of human low-density, lipoprotein assay

1. LDL preparation and oxidation

LDL was obtained from our laboratory. The plasma was collected from a patient with hypercholesterolemia and an informed consent for study participation was obtained from that patient. LDL was isolated according to the dual precipitation procedure based on the method of Garcia Parra *et al.* (1977) as modified by Nerurkar and Taskar (1985). Briefly 3 mL of plasma was diluted to 6 mL with Tris buffer (pH 7.0 (0.05 M) in 0.15 M NaCl). The sample was centrifuged for 1 h at 20,000 × *g* under refrigerated conditions. The supernatant 1 mL was removed for chylomicrons. The subnatants (4 mL) were taken for separation of Lp (a), VLDL and LDL by progressively raising the concentration to 20, 50, 60%, saturation of ammonium sulphate followed by centrifugation. The crude LDL precipitate fraction obtained at 60% saturation was dissolved in 2 mL Tris-HCl buffer pH 7.0 (0.05 M) in 0.15 M NaCl. Then, 200 µL of a solution containing 14 mM sodium phosphotungstate and 2 mM MgCl in distilled water was added and centrifuged to obtain a precipitate of pure LDL. The precipitate was dissolved in 0.15 M NaCl solution, made alkaline with sodium carbonate (10% w/v) and dialyzed in the dark for 24 h at 4°C against three changes of 1 L each, of 0.01 M phosphate-buffered saline (PBS) 0.15 M NaCl, pH 7.4 before oxidation experiments. Protein was measured by the method of Lowry *et al*. (1951), using bovine serum albumin as standard and the final solution was adjusted to 700 µg protein/mL with 0.01 M, pH 7.4 phosphate-buffered saline (PBS). Dialyzed LDL (70 µg of protein/mL) was oxidized in PBS at 37°C for 6 h in the presence of 25 µM $CuSO_4$. The oxidation of LDL was performed in the presence and in the absence of different concentrations of extracts (0.1 to 1 µM of catechin equivalent) in a final volume of 400 µL. 10 µL of 10 mM EDTA was added to the negative control tube and refrigerated. The extract was replaced by an equivalent amount of PBS in the control.

2. Assay of lipid peroxidation product as thiobarbituric acid reactive substances (TBARS)

After incubation, 10 µL of 10 mM EDTA was added to the control and test tubes to stop the reaction. Then, 1 mL of 10% trichloroacetic and 1 mL of clear saturation solution of thiobarbituric acid (TBA) were added simultaneously and incubated at 90°C for 30 min. After centrifugation the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The percentage inhibition was calculated as follows:

Percentage inhibition =
[(Absorbance of the control+ negative)/control]

The TBARS content was also calculated using malonedialdehyde extinction coefficient (0.156×10^6 M^{-1} cm^{-1}) (Rubin *et al.* 1976).

3. Measurement of lipid hydroperoxides (lipid-OOH)

The measurement of lipid hydroperoxides was determined using the FOX2 method by Miyazawa (1989) with minor modifications (Harma *et al.* 2003). The FOX2 test system is based on the oxidation of ferrous iron to ferric iron by the various types of peroxides contained in the samples, in the presence of xylenol orange which produces a coloured ferric-xylenol orange complex whose absorbance can be measured. The FOX2 reagent was prepared by dissolving ferrous sulphate (6.75 mg) in 250 mM H_2SO_4 (10 mL) to give a final concentration of 250 µM ferrous iron in acid. This solution was then added to 90 mL HPLC-grade methanol containing 79.2 mg of butylated hydroxytoluene (BHT). Finally, 7.6 mg of xylenol orange was added, with stirring, to make the working reagent (250 µM ammonium ferrous sulphate, 100 µM xylenol orange, 25 mM H_2SO_4, and 4 mM BHT, in 90% (v/v) methanol in a final volume of 100 ml). The blank reagent contained all the components of the solution except ferrous sulphate. A sample (200 µL) of incubated control and tests were mixed with 1.8 mL

Table 1 Phenol content expressed as catechin equivalent and Ferric Reducing Antioxidant Power (FRAP) in three fractions of the fruit of *X. parviflora.*

Extract	FRAP		FOLIN
	(mg of ascorbic acid eq. /g of extract)	(mg of catechin eq. /g of extract)	(mg of catechin eq. /g of extract)
Ethanolic	310.73 ± 10.92 a	165.00 ± 5.36 a	597.97 ± 64.54 a
Water	254.59 ± 12.14 b	141.13 ± 7.99 b	273.18 ± 16.62 b
Hydroethanolic	286.39 ± 7.21 c	154.99 ± 4.29 a	536.70 ± 43.45 a

Each value is the mean (± standard deviation) of three replicate experiments.
Different letters within columns indicate significant differences at $P < 0.05$.

Table 2 Results of EC_{50}, T EC_{50} (min) of ABTS•+ assay measured at a steady state, expressed as mg or g of extract/g of free radical, for different standards and samples, as well as Antiradical Efficiency (AE) by kinetic ABTS•+ assay for the different standards and extracts tested.

Extract	ABTS scavenging activity			
	AE ($1/(EC_{50}*T\,EC_{50})$)	EC_{50} (g extract/g of ABTS)	EC_{50} (mg extract/g of ABTS)	$T\,EC_{50}$ (min)
Ethanolic	55.401 ± 6.379 a	0.001 ± 0.000 a	1.113 ± 0.095 a	16.333 ± 0.577 a
Water	3.592 ± 0.314 b	0.009 ± 0.001 b	9.440 ± 0.684 b	29.667 ± 2.082 b
Hydroethanolic	70.706 ± 1.533 a	0.001 ± 0.000 a	0.903 ± 0.015 a	15.667 ± 0.577 a
Ascorbic acid	0.20 ± 0.01 c	0.080 ± 0.002 c	80.258 ± 1.976 c	59.66 ± 1.52 b
Catechin	1.541 ± 0.160 d	0.014 ± 0.001 d	13.686 ± 0.791 d	47.667 ± 2.517 c

AE= antiradical efficiency = $[1/(EC_{50} \cdot TEC_{50})]$; TEC_{50}= time to reach the EC_{50}; EC_{50}= efficient concentration 50 (reflects 50% depletion of ABTS•+ free radical). Each value is the mean (± standard deviation) of three replicate experiments.
Different letters within columns indicate significant differences at $P < 0.05$.

Table 3 Results of EC_{50}, T EC_{50} (min) of DPPH• assay measured at a steady state, expressed as mg or g of extract/g of free radical, for different standards and samples, as well as Antiradical Efficiency (AE) by kinetic DPPH • assay for the different standards and extracts tested.

Extract	DPPH scavenging activity			
	AE ($1/(EC_{50}*T\,EC_{50})$)	EC_{50} (g extract/g of DPPH)	EC_{50} (mg extract/g of DPPH)	$T\,EC_{50}$ (min)
Ethanolic	4.158 ± 0.222 a	0.015 ± 0.000 a	15.375 ± 0.267 a	15.667 ± 0.577 a
Water	0.578 ± 0.019 b	0.029 ± 0.001 b	29.341 ± 0.772 b	59.000 ± 2.646 b
Hydroethanolic	5.250 ± 1.026 a	0.013 ± 0.002 a	12.948 ± 1.641 a	15.000 ± 1.000 a
Ascorbic acid	0.157 ± 0.014 c	0.141 ± 0.002 c	141.151 ± 1.686 c	45.333 ± 3.512 c
Catechin	0.457 ± 0.040 b	0.087 ± 0.003 d	86.750 ± 2.572 d	25.333 ± 1.528 d

AE= antiradical efficiency = $[1/(EC_{50} \cdot TEC_{50})]$; TEC_{50}= time to reach the EC_{50}; EC_{50}= efficient concentration 50 (reflects 50% depletion of DPPH· free-radical). Each value is the mean (± standard deviation) of three replicate experiments.
Different letters within columns indicate significant differences at $P < 0.05$.

FOX2 reagent. After incubation at room temperature for 30 min, the vials were centrifuged for 10 min. The absorbance of the supernatant was then determined at 560 nm. The lipid hydroperoxide content of the vials was determined as a function of the difference in absorbance between the test and blank samples using a solution of H_2O_2 as standard.

Statistical analysis

Experimental results were expressed as mean of triplicate determination ± SD. Statistical analysis of the result was performed using SPSS 10.1 for Windows (SPSS, Chicago, IL, USA). Comparison of variance was carried out using Levene test. One-way analysis of variance (ANOVA) followed up by Duncan's Multiple Range tests (or Welch followed up by C-Dunnet test when within-group variances were not equal) was performed. Correlations among the methods were established using the Pearson's correlation. Statistical significance was established for $P < 0.05$ while P values <0.01 were considered to be very significant.

RESULTS AND DISCUSSION

Many endemic species of medicinal and culinary herbs and spices have been used in the production of phytochemicals or have served as raw material in food and drug industries. The water extract (WE), ethanolic extract (ET) and hydroethanolic extract (HE) were investigated for poyphenolic concentration and their antioxidant activity by DPPH, ABTS, superoxide radical, hydroxyl radical, reducing activity, nitric oxide, lipid peroxidation assay, inhibition of LDL oxidation and metal chelating activity. In the present study, the amount of phenolic compounds was determined as the catechin equivalent. The content in phenolic compounds of *X. parviflora* extracts varied between 273.18 and 597.97 (mg catechin equivalent per g of extract) (**Table 1**). The total phenolic content of ethanolic extract was significantly ($P < 0.05$) the highest. Many of the phenolic compounds have been shown to contain high levels of antioxidant activities (Rice-Evans *et al.* 1996). Several parameters can influence phenolic yield during extraction process and these include extraction temperature, solvent type and solvent concentration (Li *et al.* 2006). A preliminary study conducted in our laboratory demonstrated that the extraction with ethanol or with ethanol and water (50/50) at room temperature yielded the highest total phenolic content and antioxidant activity according to plants. A comparison of the total phenolic content of our samples with that of several tropical spices demonstrated a much higher total phenolic content in *X. parviflora* than most of tropical spices (Runnie *et al.* 2004; Agbor *et al.* 2005; Wong *et al.* 2006; Maisuthisakul *et al.* 2007). Furthermore, the ethanol extract of our plant had a higher total phenolic content than extracts from most spices commonly consumed in the sub-Saharan, oriental and western countries and known to possess high antioxidant activities (Agbor *et al.* 2007). This suggests the potential health benefit of *X. parviflora* to be utilized as a source of nutritional phenolics.

Various mechanisms, such as free radical-scavenging (by acting as a hydrogen/electron donor or direct reaction with them), reducing capacity, metal ion-chelation (thus preventing the formation of free radicals via the Fenton reactions), inhibition of radical-producing enzymes such as cyclooxygenase and lipoxygenase or increase the expression of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase and inhibition of both lipid peroxidation and LDL oxidation (Rice-Evans *et al.* 1997), have been studied to explain how plant extracts could be used as effective antioxidants. Also, the antioxidant potential of different plant extracts and pure compounds can be measured using numerous *in vitro* assays. However, a single method is not recommended for the evaluation of the antioxidant activities of different plant products, due to their complex composition (Nuutila *et al.* 2003; Shahidi 2008). Therefore, the antioxidant effects of plant products must be evaluated by combining two or more different *in vitro* assays to get relevant data. FRAP, ABTS, DPPH methods are commonly used for determining *in vitro* antioxidant capacity; FRAP measures the ability of a sam-

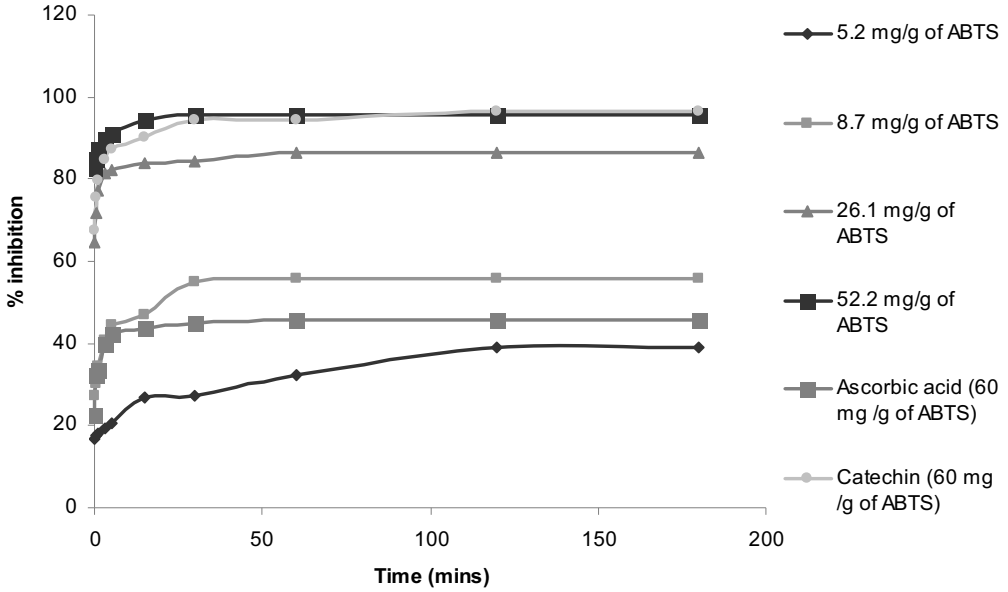

Fig. 1 Time-related changes in percentage of inhibition during incubation of $ABTS^{\bullet+}$ with different concentrations of water extract. Ascorbic acid and catechin (60 mg/g of ABTS) were used as standards. The results are the mean from three replications.

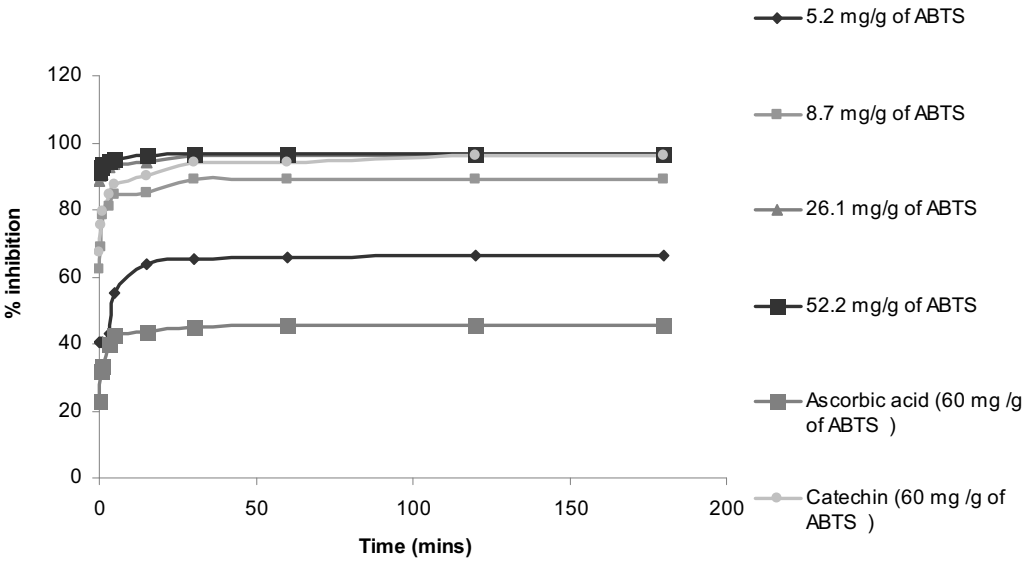

Fig. 2 Time-related changes in percentage of inhibition during incubation of $ABTS^{\bullet+}$ with different concentrations of ethanolic extract. Ascorbic acid and catechin (60 mg/g of ABTS) were used as standards. The results are the mean from three replications).

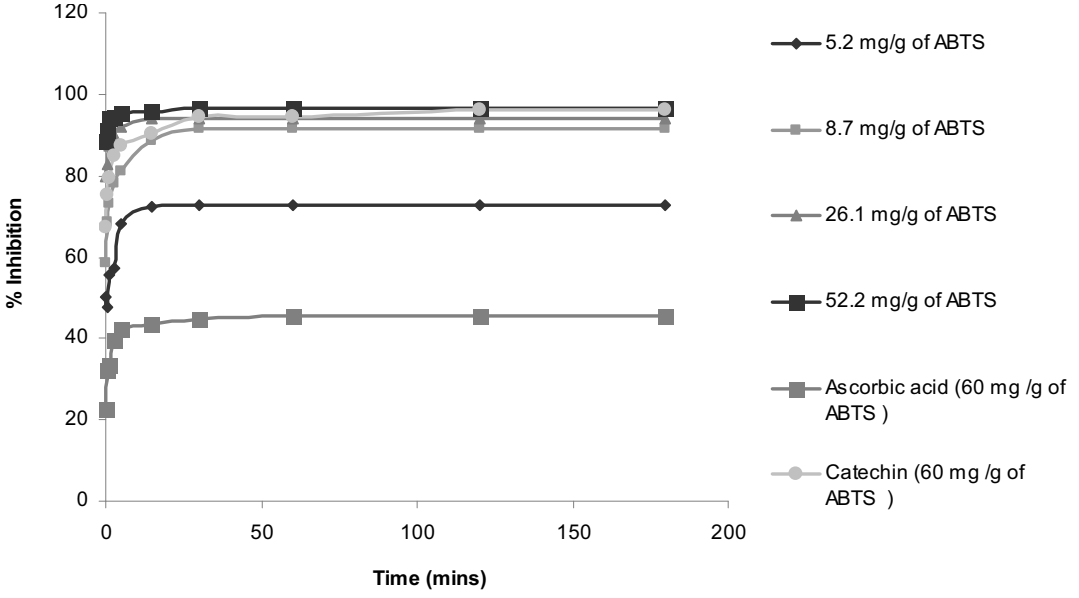

Fig. 3 Time-related changes in percentage of inhibition during incubation of $ABTS^{\bullet+}$ with different concentrations of hydroethanolic extract. Ascorbic acid and catechin (60 mg/g of ABTS) were used as standards. The results are the mean from three replications.

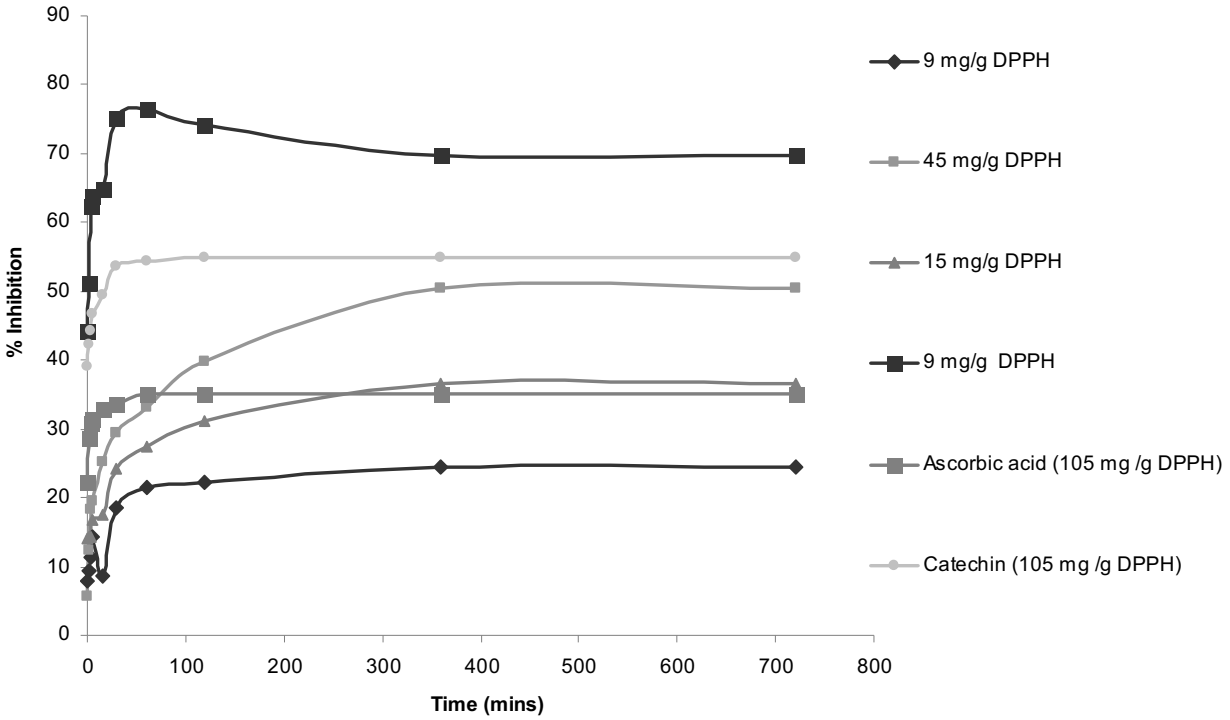

Fig. 4 Time-related changes in percentage of inhibition during incubation of DPPH with different concentrations of water extract. Ascorbic acid and catechin (105 mg/g of DPPH) were used as standards. The results are the mean from three replications.

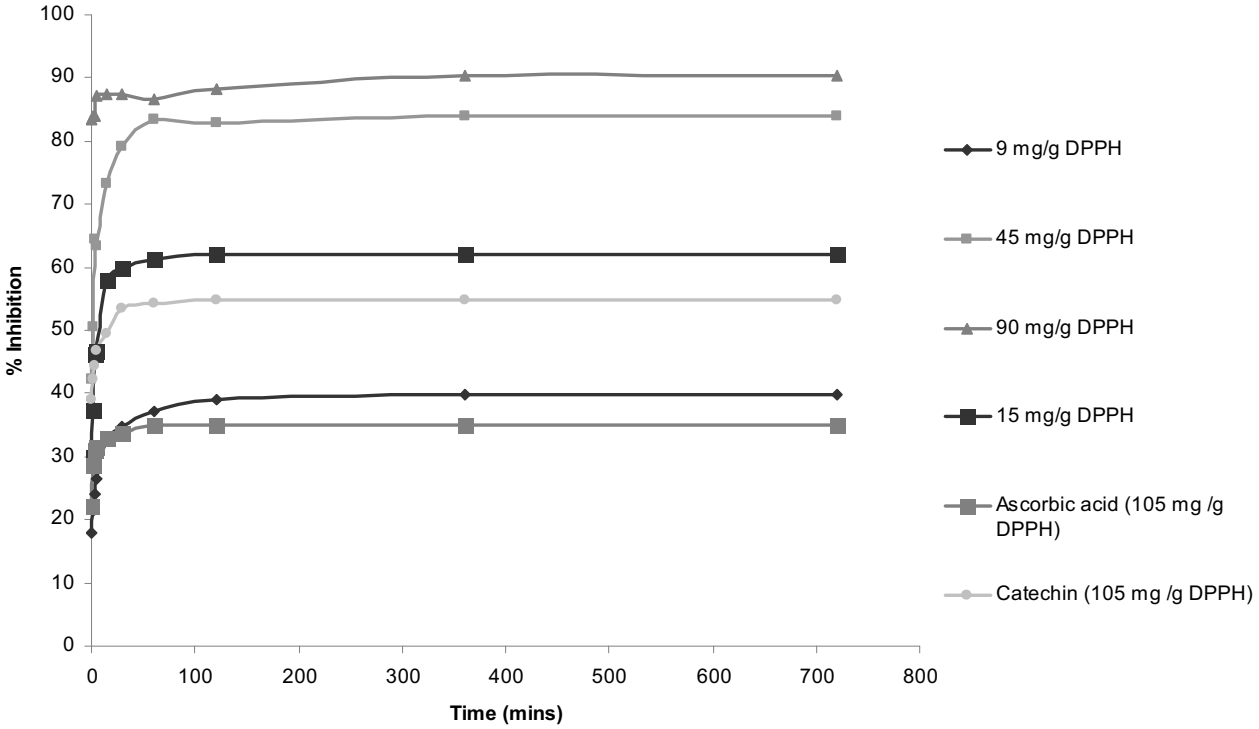

Fig. 5 Time-related changes in percentage of inhibition during incubation of DPPH with different concentrations of hydroethanolic extract. Ascorbic acid and catechin (105 mg/g of DPPH) were used as standards. The results are the mean from three replications.

ple to reduce metals, while ABTS and DPPH measure a sample's free radical scavenging. In FRAP and ABTS there is a single electron transfer (SET) reaction, whereas DPPH combines both a hydrogen atom transfer reaction (HAT) and SET (Prior *et al.* 2005).

In this study, the antioxidant capacity as measured by FRAP of the three extracts of *X. parviflora* fruits varied significantly ($P < 0.05$) (**Table 1**). Although both ET and HE values could be considered high, FRAP antioxidant activity of the ethanolic extract of *X. parviflora* was the highest. The order of FRAP activity of respective fruit samples extract was as follows: ET > HE > WE. The ethanolic extract was higher when compared with several Cameroonian spices (Agbor *et al.* 2005), supporting the antioxidative potential of this plant. The reductive ability of the samples assessed in this study suggests that the extracts were able to donate electrons, hence they should be able to donate electrons to free radicals in actual biological or food systems, making the radicals stable and unreactive). Many authors have reported that the reducing power of bioactive compounds (mainly phenolic acids and polyphenols), extracted from spices, herbs and medicinal plants, was associated with antioxidant activity, specifically scavenging of free radicals (Yen and Duh 1993; Jiménez-Escrig *et al.* 2001; Siddhuraju *et al.* 2002). For $ABTS^{\bullet+}$ and DPPH• free radical scavenging capacity, the values of the parameters EC_{50}, tEC_{50} and

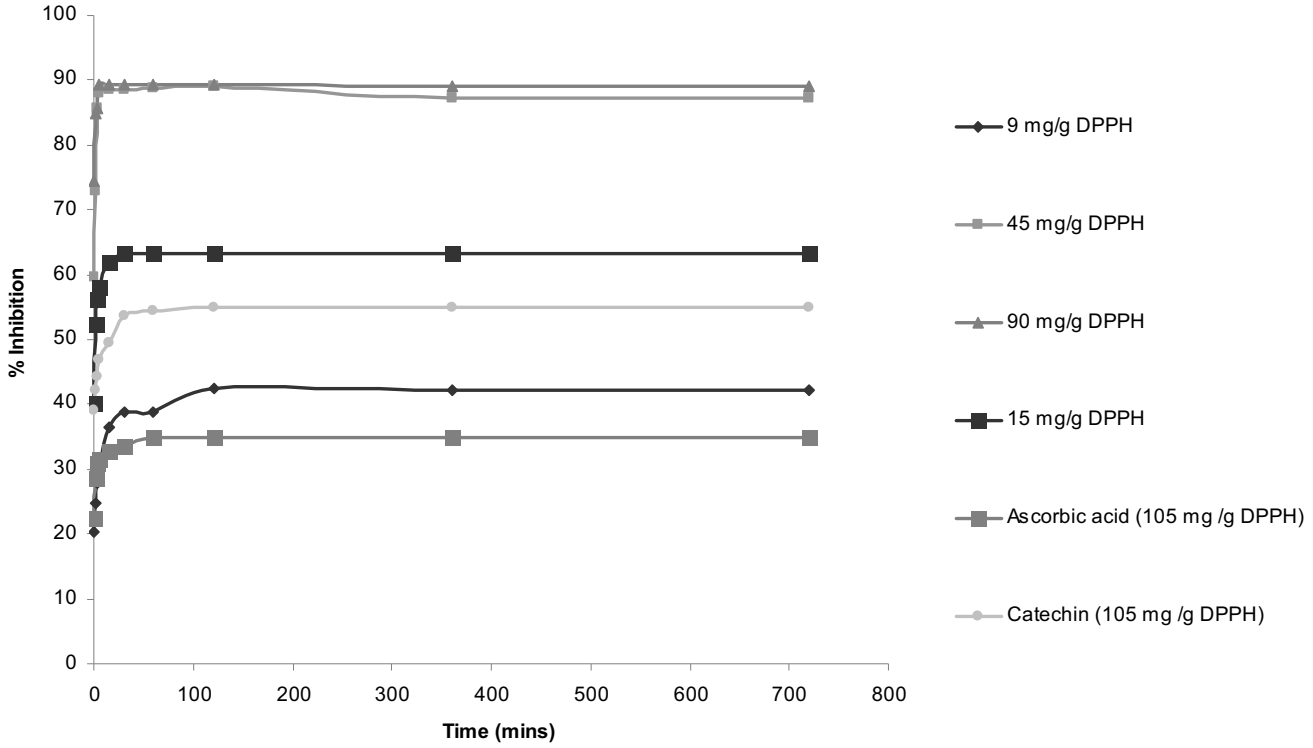

Fig. 6 Time-related changes in percentage of inhibition during incubation of DPPH with different concentrations of ethanolic extract. Ascorbic acid and catechin (105 mg/g of DPPH) were used as standards. The results are the mean from three replications.

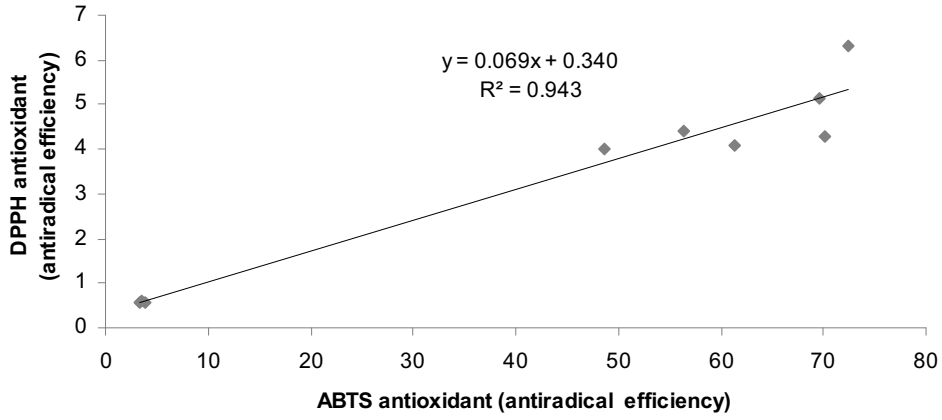

Fig. 7 Relationship between DPPH (antiradical efficiency) scavenging activity and ABTS (antiradical efficiency) scavenging activity.

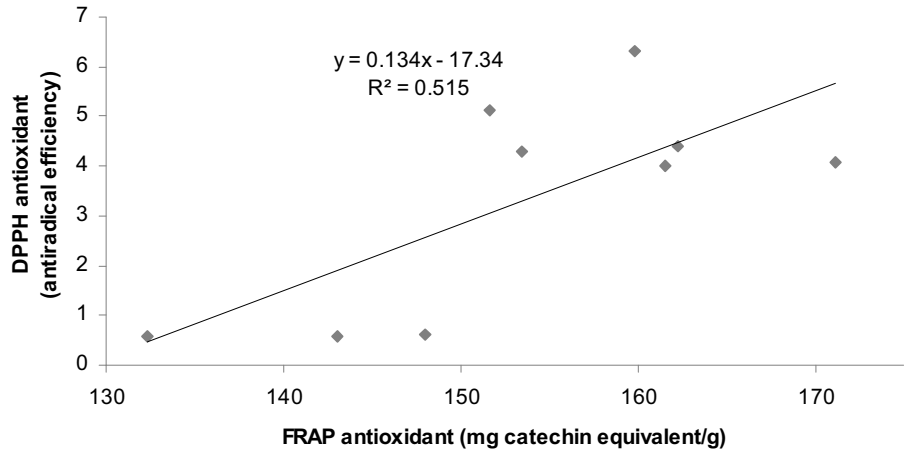

Fig. 8 Relationship between DPPH (antiradical efficiency) scavenging activity and FRAP (mg catechin equivalent/g of extract) antioxidant capacity.

antiradical efficiency (AE) are shown in **Tables 2** and **3**, respectively. Strikingly, ethanolic and hydroethanolic extracts of *X. parviflora* tested had exceptionally high scavenging activity expressed as antiradical efficiency compared to the water extract, catechin and ascorbic acid standards which presented lower AE. In this study, we ex-

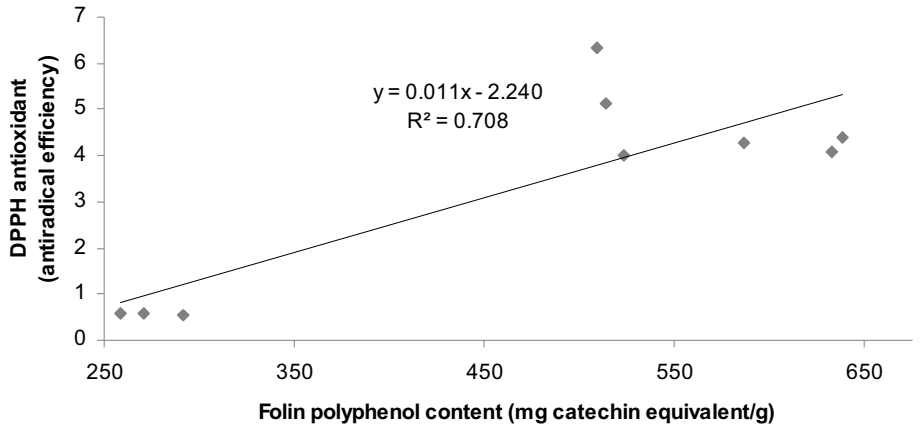

Fig. 9 Relationship between DPPH (antiradical efficiency) scavenging activity and Folin polyphenol content (mg catechin equivalent/g of extract).

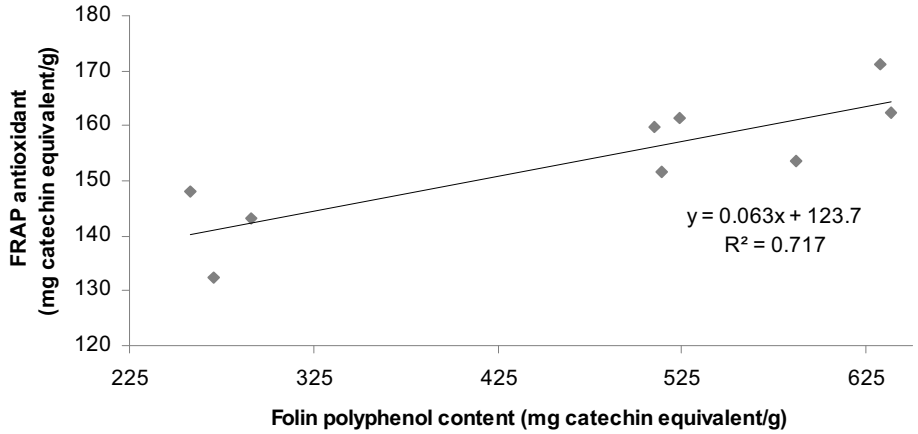

Fig. 10 Relationship between FRAP (mg catechin equivalent/g of extract) antioxidant capacity and Folin polyphenol content (mg catechin equivalent/g).

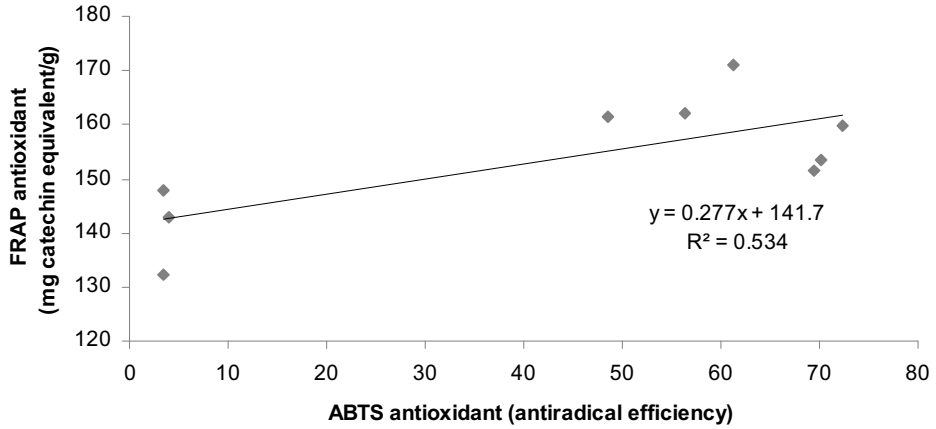

Fig. 11 Relationship between FRAP (mg catechin equivalent/g of extract) antioxidant capacity and ABTS (antiradical efficiency) scavenging activity.

pressed ABTS and DPPH antioxidant capacity results by considering kinetic parameters. **Figs. 1-6** depict the kinetic behaviors of each extract at different concentrations as measured by ABTS and DPPH. Considering all extracts, we realized that the time needed to reach the equilibrium was almost longer for lower than higher concentrations. We also established the tEC_{50} or the time taken by the EC_{50} concentration to reach equilibrium; and AE (anti-radical efficiency), that was the inverse of the product of EC_{50} and tEC_{50}. With these parameters it was advantageous to gain more comprehensive information on the sample's antioxidant capacity, taking into account not only its activity (defined by EC_{50}) but also the time it needs to act (tEC_{50}) and both simultaneously expressed by (AE). **Tables 2** and **3** show the kinetic parameters of ABTS and DPPH assays on different extracts and standards. With the exception of the water extract where the average time taken by antioxidants to react with the ABTS radical was shorter than the time taken to react with the DPPH radical, this time was almost the same for the ethanolic and hydroethanolic extracts; this may be because ET and HE had greater antioxidant activity.

The correlation between phenolic content and antioxidant capacity measured by FRAP, DPPH and ABTS on one hand and between FRAP, DPPH and ABTS on the other hand was tested (**Figs. 7-12**). Linear regression analyses of the polyphenol content and scavenging of DPPH and ABTS by extracts showed a statistically significant correlation with R^2 equal to 0.708 and 0.817 ($P < 0.05$) between AE values and estimated phenol content by Folin-Ciocalteu respectively for DPPH and ABTS. Similar correlation was ob-

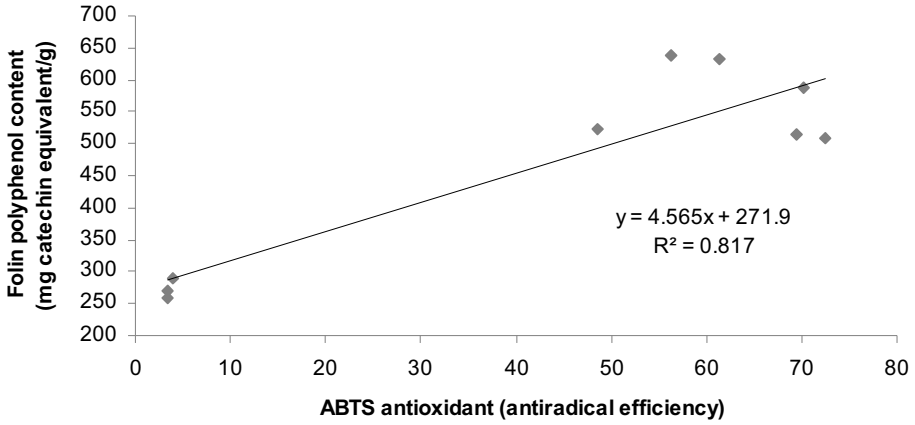

Fig. 12 Relationship between Folin polyphenol content (mg of catechin equivalent/g of extract) and ABTS (antiradical efficiency) scavenging activity.

Table 4 Inhibition of lipid peroxidation (%) of different concentrations of three extracts from *X. parviflora* and catechin.

Extract	Inhibition of lipid peroxidation (LPI (%))			
	25 µg/mL	**50 µg/mL**	**75 µg/mL**	**100 µg/mL**
Ethanolic	11.29 ± 1.03 f	39.25 ± 0.48 g	70.76 ± 1.67 h	86.52 ± 1.08 ia
Water	15.14 ± 0.79 f	41.22 ± 1.42 g	54.25 ± 1.06 h	66.44 ± 0.70 ib
Hydroethanolic	24.21 ± 0.76 f	42.61 ± 0.59 g	64.45 ± 0.76 h	78.49 ±0.84 ic
Catechin	28.12 ± 0.46 f	42.34 ± 2.74 g	61.98 ± 4.93 gh	80.27 ± 1.81 hc

The antioxidant activity of varying concentrations (25–100 µg/ml) of ethanol, hydroethanolic and water extracts of *X. parviflora* and catechin was determined using thiocyanate method. The reaction was performed in triplicates and results were expressed as % inhibition of peroxidation (± standard deviation).
Different letters within columns (a,b,c) indicate significant differences at $P < 0.05$. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

Table 5 Ferrous ion-chelating activity (%) of different amounts of three extracts from *X. parviflora*.

Extract	Metal chelating activity (%)			
	250 µg/mL	**500 µg/mL**	**1000 µg/mL**	**2000 µg/mL**
Ethanolic	23.16 ± 1.72 f	25.15 ± 0.74 f	66.67 ± 1.07 g	95.45 ± 1.18 ha
Water	6.37 ± 0.06 f	23.82 ± 0.75 g	32.40 ± 0.51 h	55.02 ± 1.30 ib
Hydroethanolic	12.64 ± 0.33 f	36.59 ± 0.34 g	54.35 ± 0.48 h	87.05 ± 0.74 ic
Catechin	20.80 ± 2.93 f	36.70 ± 2.69 g	51.09 ± 1.33 h	64.36 ± 4.50 id

The metal chelating activity of varying concentrations (250–1000 µg/ml) of ethanol, hydroethanolic and water extracts of *X. parviflora* and catechin was analysed by measuring their inhibitory effects on the absorbance of the Ferrous ion- ferrozine reaction product. Results represents mean (± standard deviation) (n = 3).
Different letters within columns (a,b,c,d) indicate significant differences at $P < 0.05$. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

served between phenol content and FRAP (R^2 = 0.717) between DPPH and ABTS (R^2 = 0.943) ($P < 0.01$), lower correlation was obtained between FRAP and DPPH (R^2 = 0.515) and between FRAP and ABTS (R^2 = 0.534) ($P < 0.05$). A linear correlation between radical scavenging activity and polyphenolic extract has been reported in an extensive range of spices, vegetables fruits and beverages (Robards *et al.* 1999; Agbor *et al.* 2005). However, in this study the ET was ranked first for polyphenol content (Folin) and FRAP assay but for ABTS and DPPH scavenging activity the HE was the highest. This suggests that HE and ET have antioxidant compounds of different structures and composition which react differently. Furthermore, FRAP, ABTS or DPPH use synthetic oxidant so it is also good to integrate methods using biologically relevant free radicals and that mimic the antioxidant activity of phenols in biological systems.

The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation, which is the primary product of oxidation. Antioxidant activity of *X. parviflora* and catechin standard was determined by the ferric thiocyanate method in the linoleic acid system. **Table 4** shows the yields and antioxidant activity of water, hydroethanolic and ethanol extracts of *X. parviflora*. All *X. parviflora* extracts and catechin exhibited effective levels of inhibitory activity towards lipid peroxidation at all concentrations. The effects of various concentrations of extracts of *X. parviflora* (25-100 µg/mL) on peroxidation in linoleic acid emulsion are shown in the table. The antioxidant activity of *X. parviflora* extracts increased with increasing concentrations. The highest concentration (100 µg/mL) of the hydroethanolic and ethanol extracts of *X. parviflora* showed higher antioxidant activities than that of the water extract and was almost equal to that of 100 µg/mL concentration of catechin. These findings suggest that *X. parviflora* seed extracts could be used in food systems to enhance lipid stability.

The chelating of ferrous ions by the extracts of *X. parviflora* was also estimated and the results are shown in **Table 5**. The production of highly ROS such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals is also catalysed by free iron through Haber–Weiss reaction, $O_2\bullet^- + H_2O_2 \rightarrow O_2 + OH^- + OH\bullet$ (Haber and Weiss 1934). Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron can stimulate lipid peroxidation by the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\bullet$), and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell 1991; Chang *et al.* 2002). Fe^{3+} ion also produces radicals from peroxides although the rate is 10-fold less than that of Fe^{2+} ion (Miller 1996). Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions (Halliwell and Gutteridge 1984). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, thereby impeding the formation of the red color imparted by the complex as well. Measurement of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelator (Yamaguchi *et al.* 2000). In this assay both extracts of *X. parviflora* and catechin standard are interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating acti-

Table 6 Percentage inhibition of nitric oxide scavenging activity in the presence of different concentrations of the extracts of *X. parviflora*

Extract	Nitric oxide scavenging activity (%)			
	50 µg/mL	100 µg/mL	200 µg/mL	300 µg/mL
Ethanolic	16.88 ± 0.58 f	40.43 ± 0.73 g	72.60 ± 0.94 h	86.79 ± 0.69 ia
Water	9.44 ± 0.88 f	35.33 ± 1.03 g	45.93 ± 0.23 h	51.40 ± 0.89 ib
Hydroethanolic	11.32 ± 0.87 f	36.99 ± 0.53 g	62.00 ± 1.86 h	76.45 ± 1.00 ic
Catechin	18.07 ± 1.57 f	28.85 ± 1.50 g	41.20 ± 1.55 h	60.25 ± 4.04 id

The radical scavenging ability of varying concentrations (50–300 µg/ml) of ethanol, hydroethanolic and water extracts of *X. parviflora* and catechin was analysed by measuring their inhibitory effects on the absorbance of the nitric oxide reaction product. The reaction was performed in triplicates and results were expressed as% inhibition of the absorbance of nitric oxide reaction product (± standard deviation).
Different letters within columns (a,b,c,d) indicate significant differences at $P < 0.05$. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

Table 7 Percentage inhibition of hydroxyl radicals scavenging activity in the presence of different concentrations of the extracts of *X. parviflora*.

Extract	Hydroxyl radical scavenging activity (HRSA) (%)			
	250 µg/mL	500 µg/mL	750 µg/mL	1000 µg/mL
Ethanolic	15.42 ± 1.04 f	30.49 ± 0.87 g	58.59 ± 0.81 h	68.87 ± 0.31 ia
Water	13.71 ± 0.73 f	33.54 ± 0.82 g	46.60 ± 0.45 h	58.81 ± 0.57 ib
Hydroethanolic	24.38 ± 0.99 f	53.23 ± 0.92 g	70.33 ± 0.66 h	90.44 ± 1.96 ic
Catechin	27.60 ± 1.87 f	53.06 ± 1.88 g	67.96 ± 1.26 h	75.41 ± 1.08 id

The radical scavenging ability of varying concentrations (250–1000 µg/ml) of ethanol, hydroethanolic and water extracts of *X. parviflora* and catechin was analyzed by measuring their inhibitory effects on the absorbance of the hydroxyl radical reaction product. The reaction was performed in triplicates and results were expressed as% inhibition of the absorbance of the hydroxyl radical reaction product (± standard deviation).
Different letters within columns (a,b,c,d) indicate significant differences at $P < 0.05$. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

Table 8 Percentage inhibition of superoxide anion scavenging activity in the presence of different concentrations of the extracts of *X. parviflora*.

Extract	Superoxide anion radical scavenging activity (SASA) (%)			
	250 µg/mL	500 µg/mL	750 µg/mL	1000 µg/mL
Ethanolic	10.46 ± 1.00 f	37.37 ± 1.19 g	75.55 ± 1.23 h	90.00 ± 0.90 ia
Water	12.73 ± 1.46 f	26.36 ± 0.98 g	45.56 ± 0.26 h	53.52 ± 0.56 ib
Hydroethanolic	20.15 ± 0.79 f	47.34 ± 0.89 g	74.75 ± 0.54 h	96.63 ± 0.52 ic
Catechin	30.65 ± 1.61 f	51.08 ± 1.24 g	63.30 ± 2.18 h	79.54 ± 1.90 id

The radical scavenging ability of varying concentrations (250–1000 µg/ml) of ethanol, hydroethanolic and water extracts of *X. parviflora* and catechin was analyzed by measuring their inhibitory effects on the absorbance of the superoxide anion reaction product. The reaction was performed in triplicates and results were expressed as% inhibition of the absorbance of superoxide anion reaction product (± standard deviation).
Different letters within columns (a,b,c,d) indicate significant differences at $P < 0.05$. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

vity and are able to capture ferrous ion before ferrozine. As shown in **Table 5**, all extracts of *X. parviflora* chelate with the iron. The absorbance of Fe^{2+}–ferrozine complex was linearly decreased dose dependently (from 0.25 to 2 mg/mL). The difference between both hydroethanolic and ethanolic extracts of *X. parviflora* and the water extract and catechin was statistically significant ($P < 0.05$). The metal scavenging effect of all extracts of *X. parviflora* and standard decreased in the order of ET > HE > WE > catechin.

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite and nitrate ions that can be measured using the Griess reagent. Scavengers of nitric oxide (NO) compete with oxygen, leading to reduced production of nitrite ions. Thus, the scavenging activity of the extract was based on their ability to prevent the formation of nitrite ions. The NO scavenging effects of extracts are presented in **Table 6**. Overall, the ethanol and hydroethanolic extracts of *X. parviflora* showed the highest NO scavenging ability compared to the water extract and catechin. All three extracts and standards had a significant ($P < 0.05$) dose-related effect on the scavenging of NO. At the highest concentration (300 µg/mL of dry extract), the ethanol extract inhibited almost 86% of NO compared to 76, 60, and 51%, respectively for the hydroethanolic, catechin and water extracts. Therefore *X. parviflora* may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation *in vivo*.

Hydroxyl radical is the most reactive of the ROS and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. It attacks almost every molecule in the body. Hydroxyl radicals react with lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine. It initiates the peroxidetion of cell membrane lipids yielding malondialdehyde, which is mutagenic and carcinogenic (Miyake and Shibamoto 1997). Indeed, the deoxyribose assay in the presence of Fe^{3+}-EDTA, H_20_2 and a reducing agent has been proposed as a simple 'test-tube' method for determining rate constants for the reaction of substrates with OH• (Omafuvbe and Kolawole 2004). If deoxyribose is incubated with H_2O_2 and an Fe^{2+}-EDTA complex (or an Fe^{3+}-EDTA complex in the presence of a reducing agent such as ascorbate or superoxide, O^{2-}), the resulting deoxyribose degradation is inhibited by any added scavenger of $OH^{\bullet}$ to an extent that depends only on the concentration of the scavenger relative to deoxyribose, and on the scavenger's second order rate constant for reaction with OH• (Purseglove *et al.* 1981). *In vitro*, *X. parviflora* extracts and catechin were able to scavenge in a concentration-dependent manner (0.25-1 mg/mL), the hydroxyl radical (**Table 7**), thus possibly capable of preventing mutagenesis and carcinogenesis. Like for the superoxide radical, the ET extract and catechin were significantly ($P < 0.05$) more effective than the HE and WE extracts, respectively. Generally, all extracts possess some antioxidant activity, with the ethanolic extract being more effective than catechin and the hydroethanolic extract in scavenging free radicals and ROS. This property of *X. parviflora* could possibly be related to its higher polyphenol content.

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide anion is an initial free radical formed from mitochondrial electron transport systems. Endogenously, superoxides could be produced in large amounts by various metabolic and physiological processes (Blaszczyk *et al.* 1994; Bedard *et al.* 2001). The formation of the superoxide radical leads to a cascade formation of other reactive oxygen species in the cell, such as hydrogen peroxide, hydroxyl radical, peroxy nitrite, or singlet oxygen in living systems (Lee *et al.* 2004). Superoxide radical decreases the activity of other antioxidant defense enzymes such as catalase and glutathione peroxidase. In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by a PMS/NADH coupling reaction reduces NBT (yellow dye) to blue coloured product called

Table 9 Inhibition of lipid hydroperoxides generation in oxidized LDL by Cu^{+2} in the presence or absence of different concentrations of extracts.

Extract	Inhibition of copper-induced LDL oxidation			
	Lipid hydroperoxides (nmole H2O2 / mg of protein)			
	0.25 µM	**0.5 µM**	**0.75 µM**	**1 µM**
Ethanolic	98.33 ± 3.21 f	92.67 ± 6.03 f	56.67 ± 2.08 g	36.01 ± 4.80 h,a
Water	192.33 ± 4.51 f	145.33 ± 4.16 g	126.33 ± 4.62 h	97.49 ± 1.99 i,b
Hydroethanolic	77.33 ± 9.71 f	54.67 ± 6.11 g	38.67 ± 3.51 h	22.07 ± 5.70 i,c
Ascorbic acid				182.23 ± 4.04 c
Catechin				98.93 ± 11.75 b
unoxidized LDL				20.18 ± 0.29 c
Oxidized LDL				287.18 ± 10.74 d

Amount of lipid hydroperoxides formation in unoxidized LDL and Oxidized LDL in the presence or absence of extracts. Values are mean (± standard deviation) of three separate observations.
Different letters within columns (a,b,c,d) indicate significant differences at $P < 0.05$ compared with unoxidized LDL. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

Table 10 Inhibition of TBARS (MDA) generation in oxidized LDL by Cu^{+2} in the presence or absence of different concentrations of extracts.

Extract	Inhibition of copper-induced LDL oxidation			
	MDA (nmole / mg protein)			
	0.25 µM	**0.5 µM**	**0.75 µM**	**1 µM**
Ethanolic	2.25 ± 0.15 f	1.85 ± 0.06 g	1.62 ± 0.05 h	1.56 ± 0.11 h,a
Water	3.79 ± 0.11 f	3.60 ± 0.07 g	2.85 ± 0.09 h	2.68 ± 0.11 h,b
Hydroethanolic	2.22 ± 0.09 f	1.96 ± 0.09 g	1.81 ± 0.12 g	1.46 ± 0.18 h,a
Ascorbic acid				2.45 ± 0.29 b
Catechin				1.06 ± 0.15 c
unoxidized LDL				0.62 ± 0.21 d
Oxidized LDL				7.11 ± 0.13 e

Amount of TBARS formation in unoxidized LDL and Oxidized LDL in the presence or absence of extracts. Values are mean (± standard deviation) of three separate observations.
Different letters within columns (a,b,c,d,e) indicate significant differences at $P < 0.05$ compared with unoxidized LDL. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

Table 11 Percentage inhibition of lipid hydroperoxides generation in oxidized LDL by Cu^{+2} in the presence or absence of different concentrations of extracts.

Extract	Inhibition of copper-induced LDL oxidation			
	Inhibition of Lipid hydroperoxides (%)			
	0.25 µM	**0.5 µM**	**0.75 µM**	**1 µM**
Ethanolic	65.76 ± 1.12 f	67.73 ± 2.10 f	80.27 ± 0.72 g	86.46 ± 1.67 h,a
Water	33.03 ± 1.57 f	49.39 ± 1.45 g	56.01 ± 1.61 h	66.05 ± 0.69 i,b
Hydroethanolic	73.07 ± 3.38 f	80.96 ± 2.13 g	86.54 ± 1.22 h	87.46 ± 1.99 i,a
Ascorbic acid				36.51 ± 1.41 c
Catechin				65.55 ± 4.09 b

Percentage of lipid hydroperoxides inhibition in unoxidized LDL and Oxidized LDL in the presence or absence of extracts. Values are mean (± standard deviation) of three separate observations.
Different letters within columns (a,b,c) indicate significant differences at $P < 0.05$. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

Table 12 Percentage inhibition of TBARS (MDA) generation in oxidized LDL by Cu^{+2} in the presence or absence of different concentrations of extracts.

Extract	Inhibition of copper-induced LDL oxidation			
	MDA (%)			
	0.25 µM	**0.5 µM**	**0.75 µM**	**1 µM**
Ethanolic	68.32 ± 2.11 f	74.04 ± 0.77 g	77.27 ± 0.69 h	78.07 ± 1.55 h,a
Water	46.72 ± 1.57 f	49.34 ± 0.94 g	59.93 ± 1.23 h	62.37 ± 1.55 h,b
Hydroethanolic	68.84 ± 1.27 f	72.49 ± 1.20 g	74.55 ± 1.76 h	79.48 ± 2.58 h,a
Ascorbic acid				65.51± 4.01 b
Catechin				85.15 ± 2.11 c

Percentage of TBARS inhibition in unoxidized LDL and Oxidized LDL in the presence or absence of extracts. Values are mean (± standard deviation) of three separate observations.
Different letters within columns (a,b,c) indicate significant differences at $P < 0.05$. Different letters within lines (f,g,h,i) indicate significant differences at $P < 0.05$.

formazon. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. In this study the effects of the water, hydroethanolic and ethanol extracts of *X. parviflora* and catechin on superoxide radical were determined by the PMS-NADH superoxide generating system and the results are shown in **Table 8**. All of the extracts had a scavenging activity on the superoxide radicals in a dose dependent manner (0.25–1 mg/mL). However, the highest scavenging ability was exhibited at the dose 1 mg/mL by the hydroethanolic extract followed by the ethanolic extract and catechin. The water extract had the lowest scavenging activity.

The antioxidant quality of the plant extracts was also determined by the ability to inhibit LDL oxidation. The lipid peroxidation products in unoxidized LDL, oxidized LDL with Cu^{2+} in the presence or absence of extracts was assayed as thiobarbituric acid reactive substances (TBARS), and as lipid hydroperoxides. The amount of lipid hydroperoxides (lipid-OOH) and TBARS formation in unoxidized LDL and Oxidized LDL in the presence or absence of extracts are presented in **Tables 9** and **10**. Even in the absence of metal ions, aerobic oxidation of LDL caused significant formation of TBARS and lipid-OOH which were greatly increased by 11.4 and 14.2 in the presence of Cu^{2+}. Addition of plant extracts and catechin in oxidation mixture containing LDL and Cu^{2+} inhibited the generation of lipid peroxidation products in a concentration dependent manner. The hydroethanolic extract gave the highest percentage of inhibition (**Tables 11** and **12**) followed by the ethanolic extract (although there was no significant difference be-

tween them), catechin, water extract and ascorbic acid at the concentration of 1 µM. The LDL particle contains large amounts of polyunsaturated fatty acids which make this lipoprotein more prone to the oxidative degradation even in the absence of proxidants. Decomposition of the peroxidized fatty acid led to the formation of lipid peroxidation products such as lipid-OOH and TBARS. Indeed, *in vitro* oxidation of LDL by metal ions (e.g., Cu^{2+} and Fe^{2+}) occurs in three phases: an initial lag phase (consumption of endogenous antioxidant) a propagation phase (rapid oxidation of unsaturated fatty acids to lipid hydroperoxides), and a decomposition phase (hydroperoxides are converted to reactive aldehydes like malondialdehyde, 4-hydroxynonenal). These aldehydes react with lysine residues in apoB-100, resulting in oxidized LDL (Esterbauer *et al.* 1992; Mertens and Holvoet 2001). The modulation by the spice *X. parviflora*, of LDL resistance to oxidative modification was tested using the classical copper-catalyzed oxidation systems. The Cu^{2+}-catalyzed LDL oxidation depends on the reduction of the metal ion probably through the reaction with endogenous lipid hydroperoxides, with lipid hydroperoxyl radicals' production (Gebicki *et al*. 1991; Thomas and Jackson 1991; Patel *et al.* 1997). The reduced copper (Cu^{+}) in its turn decomposes preexisting peroxides, producing alkoxyl radicals. Therefore, the inhibition of the Cu^{2+}-catalyzed oxidation represents the association of both chelation of metal ions and scavenging of different free radicals. Prevention of peroxidative changes in LDL lipid by extracts in the present work suggests that this spice may play a role in scavenging the free radicals from fatty hydroperoxides so as to inhibit the chain of peroxidation as well as in chelating metal ion. Since extracts effectively reduced LDL, it might be more appropriate for culinary purpose to prevent or alleviate LDL responsible for the development or progression of oxidation-associated diseases such as diabetic complications, atherosclerosis and cardiovascular diseases.

This study showed that among the ethanolic, hydroethanolic and water extracts of *X. parviflora*, the ethanol and hydroethanolic extracts possess significant highest antioxidant activities and their potency is in the order of ET > HE > WE >, for most methods. Overall, the ethanol extract is the most potent in inhibiting linoleic acid oxidation, in scavenging the superoxide anion and hydroxyl radicals, and as well as in reducing ferric ions whereas the hydroethanolic extract was the best modulator of LDL oxidation and scavenger of the DPPH, ABTS and nitric oxide radicals as well as metal chelator. In addition, the antioxidant activity of the ethanolic and hydroethanolic extracts was greater or comparable to that of catechin and ascorbic acid, standard compounds which have been reported to contain potent antioxidant activities. The presence of high levels of phenolic compounds in extracts may have contributed to the observed antioxidant activities. The results of this study show that the extract of *X. parviflora* seeds can be used as an easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. However, *in vivo* studies will be beneficial to further understand the mechanism of action of this plant as an antioxidant.

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