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EVALUATION OF ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF FRUIT EXTRACT FROM *ZANTHOXYLUM ALATUM*: A COMMONLY USED SPICE FROM PAKISTAN

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Abstract

Zanthoxylum alatum Roxb., is a medicinal plant which abundantly grows in the hilly areas of Pakistan. The dried fruit of the plant is used as condiment and has excellent spice value. In the present study, the antioxidant activity of the *Z. alatum* whole fruit was evaluated by several *In vitro* systems e.g., lipid peroxidation, 2,2-diphenlyl-1-picrylhydrazyl (DPPH), hydroxyl radicals scavenging activities, phosphomolybdenum assay and metal chelation activity. The ethanolic extracts of fruit showed inhibition against Thiobarbituric acid reactive substances (TBARS), induced by different pro-oxidants (10 μM $FeSO_4$ and 5 μM Sodium nitroprusside) in rat liver, brain and kidney homogenates. The extracts also caused the scavenging of DPPH (IC_{50} = 4.56 ± 1.3 mg/ mL) and hydroxyl radicals, and exhibited Fe^{2+} chelating activity. The ability of the ethanolic extract to prevent the Fe^{2+}/H_2O_2 induced deoxyribose decomposition was also determined. The extract showed its potential to prevent the decomposition of deoxyribose induced by iron and hydrogen peroxide at low concentration. The results suggest that the fruit of *Z. alatum* may act as an antioxidant agent which may be associated with its potential use as a functional food.

Introduction

It was suggested recently that generation of free radicals play a major role in the progression of a wide range of pathological disturbances such as brain dysfunction and oxidative stress. Free radicals, together with other derivatives of oxygen are inevitable by products of biological redox reactions. The potential toxicity of synthetic antioxidants (butylated hydroxyanisole-BHA, butylated hydroxytoluene-BHT, tertiary butylhydroquinone, esters of 3,4,5-trihydroxybenzoic acid, etc.) has aroused an increased interest and scientists have focused on isolation and characterization of natural antioxidants from natural sources such as herbs, spices, seeds, cereals, fruits and vegetables by extraction, fractionation and purification (Dillard & German, 2000; Wang & Linn, 2000). Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases such as cancer, atherosclerosis, gastric ulcer and other conditions (Smith *et al*., 1992; Akhter *et al*., 2009). Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious

consequences of oxidative stress. Spices and herbs contain polyphenols and flavonoids that act as free radical scavengers (Sabir & Rocha, 2008).

The genus *Zanthoxylum* L., comprises of over 200 trees, shrubs and lianes and is included in the tribe Zanthoxyleae of the Rutaceae (Watreman, 1975). Species of *Zanthoxylum* are primarily pan-tropical in distribution. Different species of *Zanthoxylum* L., have been reported from Pakistan (Majid *et al*., 2004). Among these, *Zanthoxylum alatum* Roxb., is a medicinal shrub which is locally known as "Timber" and is found in moist and hilly areas of Pakistan (Majid *et al*., 2004). The powdered seed of the plant has excellent spice value and are taken as aromatic tonic, stomachic and for fever, dyspepsia, cholera etc. (Rout *et al*., 2008). The fruits, branches and thorns are considered to be carminative and stomachic, are used as a remedy for toothache Halliwell & Gutteridge, 1984). In Pakistan the dry fruit of *Z. alatum* is mixed with salt, *Mentha avensis* and *Carum capticum* for blanks, dyspepsia and headache (Majid *et al*., 2004). While, in India the fruit and bark of this plant is used in skin diseases, abdominal pain, anorexia, warm infestation and ataxia in Ayurvedic practice (Chaudiere & Ferrari, 1999). The phytochemical analysis of *Z. alatum* fruit has shown the presence of 33% monoterpene hydrocarbons. The main constituents are 1,8-cineole (15.7%), linalool (18.8%) and undecan-2-one (17.0%) (Weyerstahl *et al*., 1999). Two new flavonoids, zanthoxyl flavone and geranioloxyalatum flavone, have been isolated from the seeds of *Z. alatum* (Crowell *et al*., 1992).

The antioxidant activity of other species of *Zanthoxylum* such as *Z. piperatum* which is a Japanese pepper has been already reported (Eiji *et al*., 2007). However, the literature data on the antioxidant activity of *Z. alatum* is not available and its tentative mechanism(s) are still unknown. The present study was therefore aimed to analyze the antioxidant activity of ethanolic extracts of *Z. alatum* fruit, these being non-toxic and environmentally friendly solvents and suitable for its use in different formulations.

Materials and Methods

i. Materials: Thiobarbituric acid (TBA), malonaldehyde-bis-dimethyl acetal (MDA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, rutin, and phenanthroline were purchased from Sigma (St. Louis, MO, USA). Sodium nitroprusside (SNP) was obtained from Merck (Darmstadt, Germany) and Iron (II) sulphate from Reagen (Rio de Janeiro, RJ, Brazil).

ii. Preparation of plant extract: The mature fruits of the *Zanthoxylum alatum* were collected in June, 2007 from District Rawalakot Azad Kashmir Pakistan, identified by a Taxonomist at the University of Azad Jammu and Kashmir AK Pakistan.

The whole fruit (25 g) was dried in an oven at 40-50°C, powdered in a blender and was soaked in and extracted with 95% ethanol (250 mL) with constant stirring for 48 h. The extract was filtered using Whatman filter paper and the filtrate was evaporated to dryness at 50°C under reduced pressure in a rotary evaporator. The yield of the extract was found to be 8.73%. Serial dilutions (with 95% ethanol) of these were made to obtain the desired concentration of plant for the experiment. The extract was kept in dark at 4°C until further analyses.

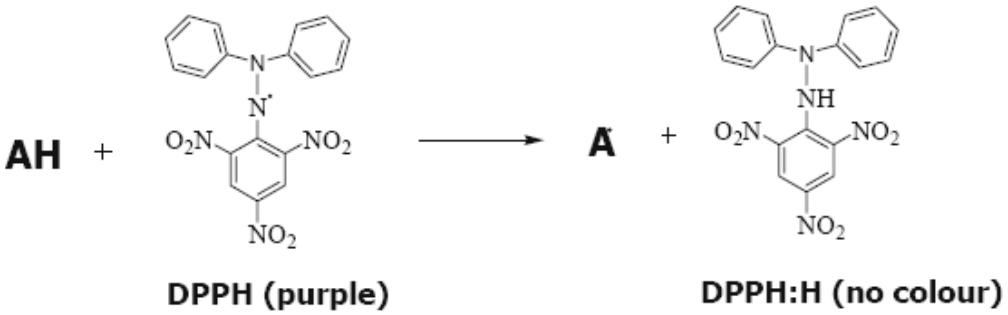
iii. Animals: All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by University Federal Santa

Maria Ethical Council (UFSM 10067). Locally bred male albino-Wistar rats with an average weight of 200±20 g, fed on standard diet and allowed water *ad libitum* were used for *In vitro* studies. The animals were group-housed (two rats per cage) in an environmentally controlled room (ambient temperature 24±2°C and relative humidity 55±5 %) on a 12:12 h light/ dark cycle (lights on at 7:00 a.m.) before experimentation.

iv. *In vitro* assays

i. Production of thiobarbituric acid reactive species (TBARS) from animal tissues: Production of TBARS was determined using a modified method as described by Ohkawa *et al*., (1979). The rats were anesthetized with ether and sacrificed by decapitation. The tissues (liver, brain and kidney) were quickly removed and placed on ice. One gram tissues were homogenized in cold 100 mM Tris-HCl buffer pH 7.4 (1 : 10 w/v) with ten up and down strokes at approximately 1200 rev/min. in a Teflon glass homogenizer. The homogenates were centrifuged for ten minutes at 1400 g to yield a pellet that was discarded and a low-speed supernatant (S1) were used for the assay. The homogenates (100 µL) were incubated with or without 50 µL of the various freshly prepared oxidants (iron sulphate and sodium nitroprusside) and different concentrations of the plant extracts together with an appropriate volume of deionized water to give a total volume of 300 µL at 37°C for 1 h. The color reaction was carried out by adding 200, 250 and 500 µL each of the 8.1% Sodium dodecyl sulphate (SDS), acetic acid (pH 3.4) and 0.6% TBA, respectively. The reaction mixtures, including those of serial dilutions of 0.03 mM standard MDA, were incubated at 97°C for 1 h. The absorbance was read after cooling the tubes at a wavelength of 532 nm in a spectrophotometer.

ii. DPPH radical scavenging assay: Scavenging of the stable DPPH radical was assayed *In vitro* (Hatano *et al*., 1988; Fig.1). The extract was added to a 0.5 mL solution of DPPH (0.125 mM in 95% ethanol). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm in a spectrophotometer. Percent inhibition was calculated from the control. Ascorbic acid was used as a standard compound in DPPH assay.



AH = *antioxidant compound*
DPPH = *1,1-diphenyl-2-picrylhydrazyl*

Fig. 1. The *In Vitro* chemical representation of DPPH *(1,1-diphenyl-2-picrylhydrazyl)* free radical scavenging assay.

iii. Antioxidant potential assay: The antioxidant potential of the extracts was assessed by the phosphomolybdenum reduction assay (Prieto *et al*., 1999). The reagent solution contained ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulfuric acid (600 mM) mixed with the extracts diluted in methanol at the concentrations of 5, 10, 25, 50, 100 µg/mL. The samples were incubated for 60 min at 90°C and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. For reference, the appropriate solution of ascorbic acid was used and the reducing capacity of the extracts was expressed as the ascorbic acid equivalents.

iv. Assay of hydroxyl radical (OH) scavenging activity: The assay was based on benzoic acid hydroxylation method, as described earlier (Chung *et al*., 1997). In a screw-capped tube, 0.2 mL Sodium benzoate (10 mM) and 0.2 mL of $FeSO_4.7H_2O$ (10 mM) and EDTA (10 mM) were added. Then, the sample solution and phosphate buffer (pH 7.4, 0.1 mM) were added to give a total volume of 1.8 mL. Finally, 0.2 mL of an H_2O_2 solution (10 mM) was added. The reaction mixture was incubated at 37°C for 2 h. After this, the fluorescence was measured at 407 nm emission (Em) with excitation (Ex) at 305 nm. OH scavenging % = [(1 - (F.I.s - F.I.o)/(F.I.c- F.I.o)] 100 where, F.I.o is fluorescence intensity at Ex 305 and Em 407 nm with no treatment, F.I.c is fluorescence intensity at Ex 305 and Em 407 nm of treated control, F.I.s is fluorescence intensity at Ex 305 and Em 407 nm of treated sample.

v. Degradation of deoxyribose (Fenton's reaction): The ability of the ethanolic extract of the pepper to prevent Fe^{2+}/ H_2O_2-induced decomposition of deoxyribose was carried out using the method of Halliwell & Gutteridge (1984). Briefly, freshly prepared ethanolic extract (20-150 µL) was added to a reaction mixture containing 20 mM deoxyribose (100 µL), 500 mM phosphate buffer (300 µL), 20 mM hydrogen peroxide (50 µL) and 500 µM $FeSO_4$ (50 µL), and the volume was made up to 800 µL with distilled water. The reaction mixture was incubated at 37°C for 30 minutes and the reaction was then stopped by the addition of 0.5 mL of 2.8% TCA (trichloroacetic acid), this was followed by the addition of 0.4 mL of 0.6% TBA solution.

The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm in a spectrophotometer.

vi. Iron chelation assay: The ability of the ethanolic extract to chelate Fe (II) was determined using a modified method as described before (Puntel *et al*., 2005). Briefly 150 µL of freshly prepared 2 mM $FeSO_4$.$7H_2O$ was added to a reaction mixture containing 168 µL of 0.1 M Tris-HCl (pH 7.4), 218 µL saline and the aqueous extract of the plant (10-100 µL). The reaction mixture was incubated for 5 min, before the addition of 13 µL of 0.25% 1,10-Phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer.

vii. Determination of total phenolic content: Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to the method of Singleton *et al*., (1999). Briefly, the plant extract (0.1 mL) wasmixed with 0.75 mL of FC reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at 22°C, then 0.06% Na_2CO_3 solution was added. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. The mean of three readings was used and the total phenol content was expressed in milligram of gallic acid equivalents/g extract.

viii. Determination of total flavonoid content: The total falavonoid content was determined with aluminium chloride ($AlCl_3$) according to a known method (Kosalec *et al*., 2004) using quercetin as a standard. The plant extract (0.1 mL) was added to 0.3 mL distilled water followed by $NaNO_2$ (0.03 mL, 5%). After 5 min at 25°C, $AlCl_3$ (0.03 mL, 10%) was added. After a further 5 min, the reaction mixture was treated with 0.2 mL of 1 mM NaOH. Finally the reaction mixture was diluted to 1 mL with water and the absorbance was measured at 510 nm. The total flavonoid content was expressed in milligram of quercetin equivalents/g extract.

ix. Thin layer chromatography (TLC) of plant extracts: Ethanolic extract of fruit was characterized by thin layer chromatography (Silica gel coated TLC plates, Merck; mobile phase, n-butanol: ethyl acetate: water 2: 4: 4). Chromatograms were evaluated under UV light at 254 and 365 nm to detect the presence of flavonoids. The presence of flavonoids was further confirmed by spraying the plates with 2.25 mM DPPH in ethanol. Rutin and quercetin were used as standard flavonoids.

v. Statistical analysis: The results were expressed as mean ± standard deviation (n = 12). The data were analyzed statistically by one way ANOVA and different group means were compared by Duncan's multiple range test (DMRT); $p<0.05$ was considered significant in all cases. The software package Statistica was used for analysis of data.

Results

Lipid peroxidation in rat liver homogenate was induced with iron and sodium nitroprusside and the effect of *Z. alatum* ethanolic extracts was determined. There was a statistically ($p<0.05$) significant increase about 70% and 64% in the formation of TBARS in iron sulphate (10 μM) and SNP (5 μM) induced liver homogenates when compared to the basal or normal (Table 1). *Zanthoxylum alatum* significantly reduced ($p<0.05$) the accumulation of lipid peroxides in a dose dependent manner for iron and SNP. However, the plant afforded greater protection against SNP (56.7%) induced lipid peroxidation compared to iron (45.2%) at the highest tested concentration of the extract (Table 1).

Table 2 shows the interaction (inhibition) of the plant extracts with Fe (II) and SNP induced lipid peroxidation in rat brain. The result revealed that incubation of the brain tissue in the presence of 10 μM Fe (II) and 5 μM SNP caused 86% and 79% increase in the MDA content of the brain homogenates when compared to the basal brain homogenates. However, *Z. alatum* caused a significant inhibition ($p<0.05$) in Fe (II) induced lipid peroxidation in the brain in a dose-dependent manner (0.05-1 mg/mL) (Table 2).

Table 3 shows the inhibition of the extracts with Fe (II) and SNP induced lipid peroxidation in kidney of rat. The results revealed that incubation of tissue in the presence of 10 μM Fe (II) and 5 μM SNP caused 59% and 65% increase in the MDA content of the kidney homogenates when compared to the normal kidney homogenates. Plant extract decreased the level of TBARS in kidney but the inhibition was less compared to the liver and brain tissues as shown in Table 3. Here, the extract afforded greater protection against SNP (30.4%) induced lipid peroxidation compared to iron (17.6%) at a concentration of 1mg/mL. It is suggested that reactive oxygen species such as superoxide anion (O2-), hydroxylradical (OH.) andnitricoxide inactivates enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation.

Table 1. Antioxidant activity of ethanolic fruit extract of *Zanthoxylum alatum* (ZA) on iron sulphate induced and sodium nitroprusside (SNP) induced lipid peroxidation in a rat liver homogenate *In vitro*.

Treatments	ZA concentration (mg/mL)	MDA (µmoL/g.tissue)[a]	MDA inhibition (%)
Normal	-	0.228 ± 0.04[a]	-
Control	-	0.767 ± 0.10[b]	-
Iron + ZA	0.04	0.702 ± 0.11[c]	8.47
Iron + ZA	0.06	0.684 ± 0.39[c]	10.8
Iron + ZA	0.1	0.643 ± 0.03[d]	16.1
Iron + ZA	0.5	0.61 ± 0.07[d]	20.4
Iron + ZA	1	0.42 ± 0.16	45.2
Normal	-	0.152 ± 0.11[a]	-
Control	-	0.43 ± 0.125[b]	-
SNP + ZA	0.05	0.37 ± 0.11	13.9
SNP+ZA	0.1	0.30 ± 0.07	30.2
SNP + ZA	0.2	0.33 ± 0.06	23.2
SNP + ZA	0.5	0.21 ± 0.02	51.1
SNP + ZA	1	0.186 ± 0.05	56.7

[a]Results are expressed as means of three experiments in duplicate ± SD, [a,b,c]means without a common superscript differ significantly ($p \leq 0.05$) by Duncan's multiple range test (DMRT).

Table 2. Antioxidant activity of ethanolic fruit extract of *Zanthoxylum alatum* (ZA) on iron sulphate induced and Sodium nitroprusside (SNP) induced lipid peroxidation in rat brain homogenate *In vitro*.

Treatments	ZA concentration (mg/mL)	MDA (µmoL/g.tissue)[a]	MDA inhibition (%)
Normal	-	0.057 ± 0.057[a]	-
Control	-	0.40 ± 0.133[b]	-
Iron + ZA	0.05	0.38 ± 0.059	5
Iron + ZA	0.1	0.36 ± 0.05	10
Iron + ZA	0.2	0.30 ± 0.13[c]	25
Iron + ZA	0.5	0.26 ± 0.9[cd]	35
Iron + ZA	1	0.243 ± 0.54[d]	39.2
Normal	-	0.07 ± 0.045[a]	-
Control	-	0.33 ± 0.042[b]	-
SNP + ZA	0.05	0.31 ± 0.032[b]	6
SNP+ZA	0.1	0.29 ± 0.034	12.1
SNP + ZA	0.2	0.25 ± 0.01	24.2
SNP + ZA	0.5	0.21 ± 0.07	36.4
SNP + ZA	1	0.155 ± 0.06	53

[a]Results are expressed as means of three experiments in duplicate ± SD, [a,b,c]means without a common superscript differ significantly ($p \leq 0.05$) by Duncan's multiple range test (DMRT).

Table 3. Antioxidant activity of ethanolic fruit extract of *Zanthoxylum alatum* (ZA) on iron sulphate induced and Sodium nitroprusside (SNP) induced lipid peroxidation in kidney homogenate *In vitro*.

Treatments	ZA concentration (mg/mL)	MDA (µmoL/g.tissue)[a]	MDA inhibition (%)
Normal	-	0.20 ± 0.038[a]	-
Control	-	0.51 ± 0.03[b]	-
Iron + ZA	0.05	0.462 ± 0.096[c]	9.41
Iron + ZA	0.1	0.46 ± 0.025[c]	9.8
Iron + ZA	0.2	0.44 ± 0.03[d]	13.7
Iron + ZA	0.5	0.43 ± 0.023[d]	15.6
Iron + ZA	1	0.42 ± 0.14[d]	17.6
Normal	-	0.15 ± 0.038[a]	-
Control	-	0.345 ± 0.08[b]	-
SNP + ZA	0.05	0.34 ± 0.045[b]	2.31
SNP+ZA	0.1	0.33 ± 0.05[b]	4.34
SNP + ZA	0.2	0.263 ± 0.03	23.7
SNP + ZA	0.5	0.28 ± 0.06	18.8
SNP + ZA	1	0.24 ± 0.034	30.43

[a]Results are expressed as means of three experiments in duplicate ± SD, [a,b,c]means without a common superscript differ significantly ($p \leq 0.05$) by Duncan's multiple range test (DMRT).

Table 4 shows the total content of phenolics and flavomoids and the results indicate that *Z. alatum* ethanolic extract contains significant amounts of flavonoids and phenolic compounds. Both these classes of compounds have good antioxidant potential and their effects on human nutrition and health are considerable.

Table 4. Total Content of phenolics and flavonoids in hot water extract of *Zanthoxylum alatum* (ZA) whole fruit.

Material	Total phenolics (GA^E mg/g)	Total flavonoids ($Quer^B$ mg/g)
Whole fruit	33.3 ± 0.3	6.66 ± 0.06

Values represent the means ± SD (n=3)
GA^E is gallic acid equivalents., $Quer^B$ is quercetin equivalents

The radical scavenging activity of the ethanolic extracts was tested against two important radicals, DPPH and hydroxyl (Fig. 1 and 2). The extract exhibited a stronger antioxidant activity against OH radical as it caused 47 % scavenging of the radical at 2 mg/mL and also showed its ability to scavenge the radical even at a low concentration (0.25 mg/mL). The ethanolic extract also showed its ability to quench the stable DPPH radical (IC_{50} = 4.56 ± 1.3 mg/mL) which is less compared to standard ascorbic acid (IC_{50} = 21.4 ± 1.6 µg/mL) (Fig. 2). Oxidative damage to lipids and DNA is of particular significance in carcinogenesis. *Z. alatum* with its antioxidant activity was expected to protect against OH mediated damage of deoxyribose. The extract afforded significant protection ($p<0.05$) against both iron and hydrogen peroxide induced damage of deoxyribose (Fig. 2) which is in close agreement to the results obtained on benzoic acid hydroxylation assay (Fig. 2A).

The metal chelating activity of the extract was determined by reaction with 1,10-phenanthroline which can quantitatively form complex with Fe^{2+}. In the presence of the extract the complex formation was disrupted which results that color of the complex is decreased. The results obtained on iron chelation assay demonstrated that the extract possesses strong Fe (II) chelating activity and even at the minimal concentration of 0.1 mg/mL, its chelation rate was higher than 50% (Fig. 4). The total antioxidant activity of extract expressed as ascorbic acid equivalent in phosphomolybdenum assay was 132.3 ± 1.13 µg/mL and increased linearly with the increasing concentration of extract (Fig. 4). This assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green complex at acid pH.

Discussion

Oxidative stress, the consequence of an imbalance of pro-oxidants and antioxidants in the organism, is rapidly gaining recognition as a key phenomenon in chronic diseases. It is directly involved in the pathogenic mechanism of risk factors and in the protection exerted by various environmental factors (Ines and Federico, 2000). And the quantification of oxidative stress in populations appears to be a possible indicator for the magnitude of environmental risk factors.Oxidative stress is now recognized to be associated with more than 100 diseases, as well as with the normal aging process (Ghasanfari *et al*., 2006). Antioxidants are intimately involved in the prevention of cellular damage -- the common pathway for cancer, aging, and a variety of diseases. In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases and extensive lysis. Here Fe^{2+} and SNP were used as a tool to induce lipid peroxidation and they showed promising results. The ethanolic extract of *Z. alatum* exhibited good antioxidant activity against two pro-oxidants in tissues. However, in brain, liver and kidney it was more effective against SNP induced inhibition compared to Fe^{2+} induced TBARS. Increases in the formation of TBARS in Fe^{2+} (10 µM) induced oxidative stress as compared to the basal suggest possible damage of tissues with an overload of iron. Rats overloaded with iron showed toxic effects such as hepatocellular hypertrophy, cardiomyopathy, pancreatic atrophy, splenic white pulp atrophy and hemosiderosis in the liver, heart, pancreas and endocrine glands, respectively (Whittaker, 1997). The protections offered by the *Z. alatum* suggest that the aqueous extract may protect the liver and brain against toxicities resulting from potential overload of iron.

Sodium nitroprusside is an anti-hypertensive drug that acts by relaxation of vascular smooth muscle and consequently dilates peripheral arteries and veins. However, SNP has been reported to cause cytotoxicity through the release of cyanide and or nitric oxide (Bates *et al*., 1991). The protection offered by *Z. alatum* extract on tissues (brain, liver and kidney) confirms the antioxidant activity of extract and indicate its use in accidental intoxications resulting from the overload with SNP.

Phytochemical analysis of the ethanolic extract showed high content of total phenolics (33.3 mg/g) and flavonoids (6.66 mg/g) which may be responsible for the antioxidant activity of the extract beside other phytochemicals (Table 4). The results of TLC analysis showed the presence of quercetin (R_f = 0.91) in the ethanolic extracts of fruit and two unidentified yellow spots at R_f values of 0.85 and 0.21, respectively (Fig. 5).

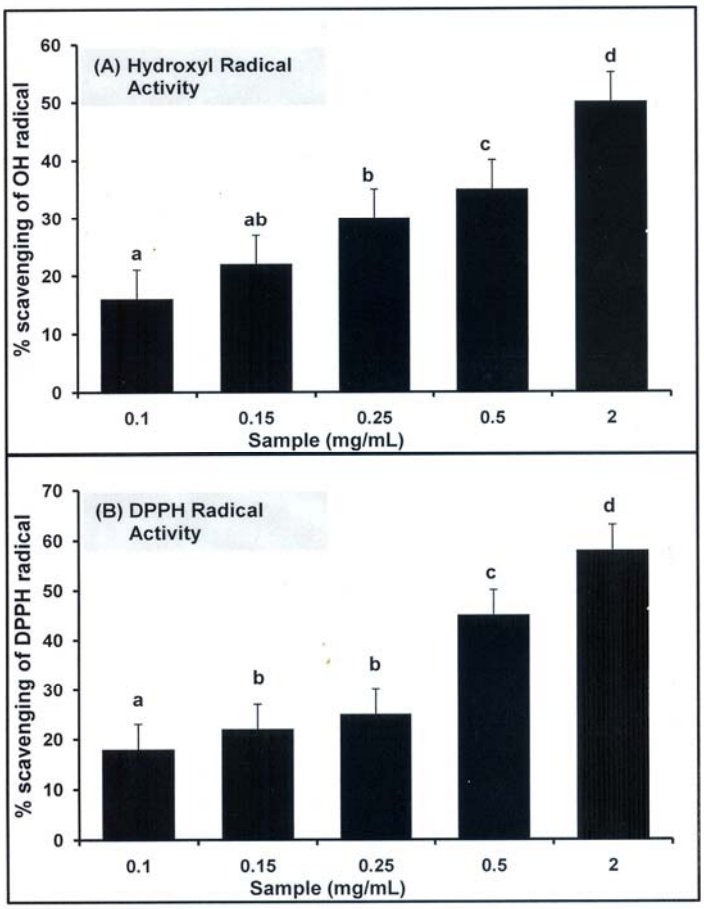


Fig. 2. *In vitro* antioxidant activity of *Zanthoxylum alatum* (ZA) fruit: (A). Hydroxyl radical and (B). DPPH radical scavenging activities. Values are means ± SD (n = 12). Bars with different letters (a, b, c & d) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT).

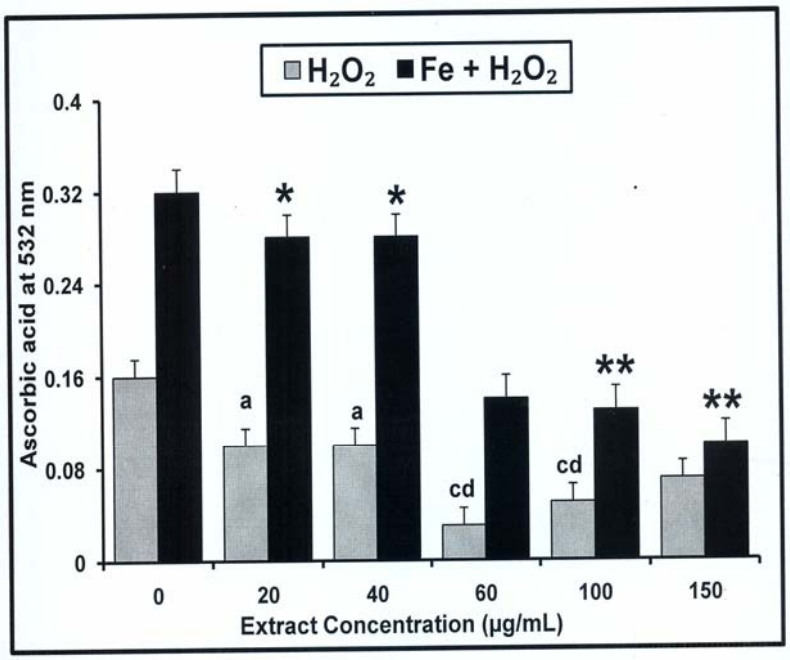


Fig. 3. Hydroxyl radical scavenging potential of ethanolic extract of *Zanthoxylum alatum* (ZA) fruit in deoxyribose degradation assay induced by iron and hydrogen peroxide. Values are means ± SD (n = 12). Bars with different letters (a, b, c & d) and **p<0.05 are significantly different from each other by Duncan's multiple range test (DMRT).

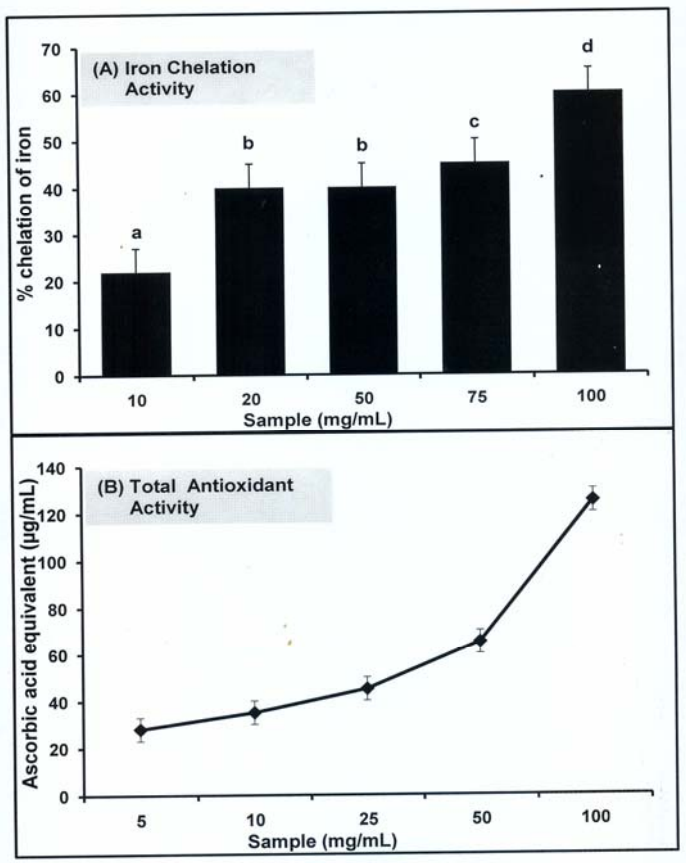


Fig. 4. *In vitro* antioxidant activity of *Zanthoxylum alatum* (ZA) fruit. (A). iron chelation activity of fruit extract. (B). total antioxidant activity of ethanolic extract in phosphomolybdenum assay. Values are means ± SD (n = 12). Bars with different letters (a, b, c & d) are significantly (p<0.05) different from each other by Duncan's multiple range test (DMRT).

Scavenging of hydroxyl radical is an important antioxidant activity because of very high reactivity of the OH radical which enables it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids and nucleotides. The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (Yasuda *et al*., 2000). Hydroxyl radicals are generated by direct addition of Fe (II) salts to a reaction mixture containing phosphate buffer. Benzoate is hydroxylated to hydroxybenzoates. Benzoate is weakly fluorescent but, after monohydroxylation, forms highly fluorescent products (Gutteridge, 1987). Measurement of spectrofluorometric changes has been used to detect damage by hydroxyl radical. *Z. alatum* fruit extract was found to be a powerful scavenger of hydroxyl radicals on both deoxyribose assay (Fenton's reaction) and benzoic acid hydroxylation assays keeping in view the fact that the quenching properties were only obtained by crude ethanolic extracts. The DPPH assay also validated the free radical scavenging activity of the ethanolic extract. However, at lower tested concentrations the scavenging ability of the extract was found to be weak. The antiradical and scavenging activity of the extract in this study is expected due to flavonoids which usually contain the high metal chelating activity (Dorman *et al*., 2003; Oboh *et al*., 2007). In the phosphomolybdenum assay, which is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity), the extract demonstrated high electron-donating capacity showing its ability to act as chain terminators, transforming

relative free radical species into more stable non-reactive products (Zheng *et al*., 1992; Dorman *et al*., 2003). The antioxidant activity of ethanolic extracts of *Z. alatum* may be attributed to the presence of high content of phenolics and flavonoids especially quercetin which are well recognized as potential antioxidants and free radical scavengers and inhibit lipid peroxidation via the scavenging of radicals and metal chelation (Dorman *et al*., 2003; Majid *et al*., 2004). The mechanism of action of flavonoids is through scavenging or chelation. Phenolic compounds are also very important plant constituents because their hydroxyl group confers scavenging ability (Wattenberg *et al*., 1989; Zheng *et al*., 1992; Dorman *et al*., 2003).

Fig. 5. TLC analysis of ethanolic extract of *Zanthoxylum alatum* extract showing the presence of quercetin on top (y) which is at the same distance with the standard quercetin (2) and below are two unidentified spots.

Conclusions

On the basis of the considerations obtained in the present study, it is concluded that ethanolic extracts of *Z. alatum* which contain large amounts of flavonoids and phenolic compouns, exhibit high antioxidant and free radical scavenging activities. The extracts also chelate iron and have reducing power. These *In vitro* assays indicate this plant extract has a significant source of antioxidant, which might be useful in preventing the progress or various oxidative stresses. The scavenging activities observed against DPPH and hydroxyl radicals, as well as the protective activities against lipid peroxidation, lead us to propose *Z. alatum* fruit extract as a promising natural source of antioxidants suitable for application in nutritional/pharmaceutical fields, and in the prevention of free radical-mediated diseases. Hence, more queries will be addressed in future studies to explore the

potential of *Z. alatum* fruit bioactive compounds as chemo preventive and therapeutic agents. Moreover, these findings could result in the development of more effective and selective new medications from functional foods that are capable of blocking the action of reactive oxygen species involved in oxidative stress.

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