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In vitro Antioxidant Activities of *Ziziphus spina-christi* Fruits (Red Date) Grown in Oman

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Abstract: *Ziziphus spina-christi* Fruits (ZSCF) have been used for human consumption for long time for medicinal purposes in many areas of the world and recommended for diseases in which free radical species are produced as a result of oxidative stress. However, there is lack of systematic study on the antioxidant capacities of ZSCF from Oman. The present study quantifies the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) and investigates the antioxidant ability of the ZSCF grown in the Sultanate of Oman. Antioxidant activity was assessed by using 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Superoxide-radical Scavenging Activity (SRSA), ferric reducing/antioxidant power (FRAP assay), Total Reducing Power Ability (TRPA) and metal chelation assays. Overall the ZSCF showed strong antioxidant ability and high phenolic contents. TPC and TFC contents of ZSCF were 1644 mg Gallic Acid Equivalents (GAE) and 47 mg catechin equivalents (CEQ)/100 g of Dry Weight (DW), respectively. ZSCF showed potent radical scavenging activity in each assay, showing 91% in the ABTS method (at 429 μg mL^{-1}), 51% in the DPPH radical scavenging method (at 140 mg mL^{-1}) and 47% of inhibition in the Superoxide-radical scavenging assay (at 20 μg mL^{-1}). The % chelating effect of ZSCF was 94% (at 100 μg mL^{-1}). The observed antioxidant ability of ZSCF may be due to abundant presence of phenolic contents and high electron donating ability to neutralize free radicals.

Key words: Antioxidant activity, *Ziziphus spina-christi* fruits, flavonoids, reducing power, superoxide anion

INTRODUCTION

The interest of consumers towards natural bioactive compounds as functional ingredients in the food products has arisen due to their various health beneficial effects. Besides their nutritional and sensorial properties, functional foods have been recognised as acting as protective agents. The health promoting benefits of antioxidants from plants is thought to arise from their potential effects on the reactive oxygen/nitrogen species. Epidemiological evidence suggests that antioxidants contained in fruit and vegetables can help to prevent or affect the development of disease. In addition, antioxidants have several industrial uses, such as preservatives in foods to prolong their shelf life. The deterioration of some foods has been linked to the oxidation of lipids which leads to the formation of undesirable secondary lipid peroxidation products causing thus a decrease in the nutritional value of lipid foods, their safety and sensory attributes. Therefore, synthetic commercial antioxidants have been widely used in food industry to retard the oxidation process. However, human and animal studies supported the toxic effect of synthetic antioxidants such as Butylated Hydroxylanisol (BHA), Butylated Hydroxyltoluene (BHT) and tert-butylhydroquinone (TBHQ) (Huang and Wang, 2004; Wang *et al*., 2011). Therefore, restriction on their use are being imposed (Wang *et al*., 2011) and the search for natural antioxidants as safe alternatives is becoming important to the food industry (Pena-Ramos and Xiong, 2001).

Moreover, several studies have indicated that medicinal plants contain a wide variety of natural antioxidants such as phenolic acids, flavonoids and tannins which possess more potent antioxidant activity (Thirumalai *et al*., 2011). Flavonoids which are the most potent nutritional antioxidants (Anderson, 1980), inhibits LDL oxidation, secondary to their abilities to scavenge free radicals and chelate transition metal ions (Mozaffarian *et al*., 2003). It is also most widespread group of natural compounds among the phenolics. These possess wide range of chemical and biological activities

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including antioxidative, anti-mutagenic, anti-allergic and anti-carcinogenic activities (Jimenez and Garcia-Carmona, 1999; Hashimoto *et al*., 1999; Prasad *et al*., 2009).

Ziziphus spina-christi Fruits (ZSCF) belong to the family Rhamnaceae and grow throughout Middle Eastern region including Oman. It is commonly called as jujube and also known as Nabag or Cidr in Oman (Adzu *et al*., 2002) and Dom/Christ thorn in English. It taste like a mixture of apples and dates which can be eaten as fresh as well as in dried form (NAS, 1980). *Ziziphus* species was commonly used in treatment of various diseases such as digestive disorders, diabetes, liver complaints, anodyne, emollient, obesity, urinary troubles, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anemia, diarrhea and insomnia (Kirtikar and Basu, 1984; Han *et al*., 1990; Yossef *et al*., 2011) and also known for medicinal properties such as hypoglycemic, hypotensive, hypolipidemic, anti-inflammatory, antimicrobial, antioxidant, anti-tumour and as an immune system stimulant (Hussein *et al*., 2006; Said *et al*., 2006; Yossef *et al*., 2011). Recently, Yossef *et al*. (2011) reported the hepatoprotective effect of ZSCF extract in animal model. This effect might be due to its antioxidant and free radical scavenger effects. Previous phytochemical studies suggested that ZSCF contains cyclopeptide alkaloids, flavonoids, sterols, tannins and triterpenoid saponin glycosides (Shahat *et al*., 2001). To our knowledge no research has been done on the antioxidant and free radical scavenging activities of ZSCF grown in Oman. Therefore, this study aimed to assess the *in vitro* total phenolic content and antioxidant properties of the ZSCF grown in Oman.

MATERIALS AND METHODS

Chemicals: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS), Nicotinamide Adenine Dinucleotide (NADH), Nitro Blue Tetrazolium (NBT), Phenazine Methosulfate (PMS), were from Sigma (St. Louis, Missouri, USA). All other chemicals used were of analytical grade.

Plant material: ZSCF were purchased from local market of Muscat, Oman at the time of harvesting in the year 2011. Fruits were stored at -40°C until used for the experiments. The fruits were cleaned and washed under tap water. Edible parts of the ZSCF were separated from seeds and then freeze dried and crushed to a fine powder.

Methanolic extract: The freeze dried sample (60 g) was extracted with 150 mL of methanol: water (4:1, v/v), at room temperature (20°C for 24 h using a magnetic stirrer). The extracts were then filtered and centrifuged at 6000 RCF, for 30 min at 3°C using Sanyo, Harrier MSE centrifuge, Manasquan, New Jersey, USA and the supernatant were concentrated under reduced pressure at 40°C for 3-4 h using a rotary evaporator to obtain the ZSCF methanolic crude extract. The crude extract was kept in dark glass bottles at -40°C for further analysis. Extract was analyzed in three replicates and the results were reported as mean values±standard deviation.

Proximate chemical analysis: The proximate chemical analysis was carried out by using the methods described by Association of official analytical chemist (AOAC, 1995). The moisture content was carried out by heating 1 g of the samples in a thermostatically controlled oven to a constant weight at 105°C while the ash content was obtained by heating 1 g of the sample in a muffle furnace at 550°C for 24 h. The crude fat content was determined by solvent extraction using petroleum ether at 60°C for 8 h using Soxhlet apparatus. The crude protein content was analyzed by Kjeldahl method using Foss, 2300 Kjeltec analyzer unit, Burladingen Germany. The carbohydrate content was determined by subtracting total protein, fat, moisture and ash content from 100.

Total antioxidant activity (TAA): Total Antioxidant Activity (TAA) was measured using a modified method as described by Cai *et al*. (2004) and Re *et al*. (1999). The ABTS radical cation ($ABTS^{-+}$) solution was pre-generated by mixing 7 mM ABTS and 2.45 mM potassium persulphate and incubating for 16-20 h in the dark at room temperature. The $ABTS^{-+}$ solution was then diluted with 100% methanol to obtain an absorbance of 0.700±0.005 at 734 nm. Methanolic stock solution of ZSCF extract was prepared to have concentration of 5 mg mL^{-1}. Then serial dilutions of ZSCF extracts were prepared from stock solution and the volume was adjusted to 1 mL using methanol. $ABTS^{-+}$ solution (2 mL; absorbance of 0.700±0.005) was added to different concentration of the test sample and mixed vigorously. The reaction mixture was allowed to stand at 23°C for 6 min and the absorbance at 734 nm was immediately recorded. A standard curve was obtained by using Trolox standard solution at various concentrations (ranging from 0 to 15 mM) in 80% methanol. The percentage inhibition by ZSCF was calculated using equation:

$$\text{ABTS inhibition (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

DPPH radical scavenging activity: The ability of the extract to scavenge stable DPPH radicals was assessed as described by Gyamfi *et al*. (1999):

$$\text{DPPH inhibition (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Superoxide-radical scavenging assay (SRSA): The superoxide scavenging ability of the extract was assessed by the method of Nishikimi *et al.* (1972):

$$\text{SRSA (\%)} = \frac{1 - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Ferric reducing/antioxidant power assay (FRAP): The antioxidant activity of ZSCF extract was determined using a method of Ferric Reducing Antioxidant Power (FRAP) assay of Benzie and Strain (1999) with slight modifications. The FRAP reagent was prepared by mixing 100 mL of 0.1 M acetate buffer, pH 3.6, 10 mL of 10 mM Tripyridyltriazine (TPTZ) solution in 40 mM HCl and 10 mL of 20 mM Ferric chloride solution ($FeCl_3$) and was warmed to 37°C in a water bath. A stock solution of ZSCF extract was prepared (100 mg mL^{-1}). Serial dilutions of ZSCF extract was mixed with 2 mL of freshly prepared FRAP reagent. Reaction mixtures were incubated at 37°C for 4 min. Absorbance was recorded at 593 nm with a reference to a reagent blank (containing FRAP reagent and diluents except sample).

Total reducing power ability (TRPA): The reducing power of ZSCF was quantified by the method of Yen and Chen (1995) with minor modifications. Stock solution of ZSCF extract was prepared to a concentration of 200 mg mL^{-1}. Serial dilutions from the stock solution were made and the volume was adjusted to 1 mL using same solvent. Then 2.5 mL of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide were added to each dilution and reaction mixture was incubated at 50°C for 20 min. After incubation, reaction was terminated by adding 2.5 mL of 10% trichloroacetic acid (TCA) solution and the mixture was centrifuged at 2000 g for 10 min. The supernatant in each tube (3 mL) was mixed with 3 mL deionized water and 0.5 mL of 0.1% ferric chloride solution. The absorbance was measured at 700 nm against a blank. Total reducing power of ZSCF at different concentrations was compared to ascorbic acid as a positive control. All the measurements were taken in triplicate and the mean values were calculated.

Metal chelating assay: The metal chelating ability of the ZSCF extract was determined according to the method of Decker and Welch (1990) with slight modification. Stock solution (200 mg mL^{-1}) of ZSCF extract was prepared. Then serial dilutions from the stock solution were taken and total volume was made up to 3 mL with methanol. 120 μL of 2 mM $FeCl_2$ was then added and the solution was activated by the addition of 240 μL of 5 mM Ferrozine solution. After vortex, reaction mixture was incubated for 15 min at room temperature under shaking conditions. The chelating activity was then measured at 562 nm using CB5 8HY, Thermo spectronic, spectrophotometer (Cambridge, UK). All the measurements were taken in triplicate and the mean values were calculated. The ability of the extract to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating effect (\%)} = \frac{1 - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Total phenolic content (Folin-Ciocalteu assay): Total phenolics of ZSCF were measured by the Folin-Ciocalteu assay of Singleton and Rossi (1965) with slight modifications. 250 μL of Folin-Ciocalteu reagent was mixed with 10 μL of ZSCF extract. After a short incubation of 5 min, 750 μL of sodium carbonate (1.9 M) was added and incubated for 2 h at 25 °C. The absorbance at 765 nm was measured and compared with that from gallic acid standards. The concentration of phenolics in ZSCF extracts was expressed as Gallic Acid Equivalents (GAE). All the measurements were taken in triplicate and the mean values were calculated.

Total flavonoids: Flavonoids were determined according to the aluminium chloride colorimetric assay of Kim *et al.* (2003).

Determination of ascorbic acid (AA) content: Ascorbic acid content (mg/100 g of sample) was determined by direct titration with 2,6-dichloro-indophenol (AOAC, 1995).

Statistical analysis: Data are presented as Mean±standard deviation. Graph Pad Prism 5.00 for windows, GraphPad Software, San Diego California USA was used.

RESULTS AND DISCUSSION

The results of the proximate chemical composition of dry ZSCF (Table 1) indicated that carbohydrate was the main component of dry ZSCF (76.5%), while protein (5.4%), moisture (13.3%) and fat (0.66%) contents were low. These results are in agreement with those obtained by other researchers (Berry-Koch *et al.*, 1990; Abdelmuti, 1991). Also, Saied *et al.* (2008) observed that the flesh of ZSCF is rich in carbohydrates. Nour *et al.* (1987) and Abdelmuti (1991) reported that 100 g dried fruit pulp contains 314 calories, 4.8 g protein and 0.9 g fat.

Phytochemicals have various protective and therapeutic effects which are essential to prevent diseases and maintain a state of well-being. The phytochemical constituents of ZSCF extract show that it is rich in total phenols, total flavonoids (Table 1). Polyphenolic compounds are present in significant amounts in most fruits and vegetables, emphasizing its significance as dietary antioxidants (Pulido *et al*., 2000). Phenolic compounds are important contributors to functional quality and have important role to play in counteracting Reactive Oxygen Species (ROS), this minimizing molecular damage. Phenolic compounds are known as powerful chain breaking antioxidants. The phenolic compounds may contribute directly to the anti-oxidative action (Lu *et al*., 2011). This shows a strong association between anti-oxidative activities and phenolic compounds which suggested that phenolic compounds are probably responsible for the anti-oxidative activities of ZSCF. They are also effective hydrogen donors which makes them good antioxidants (Dudonne *et al*., 2009).

It is well known that plant phenolics in general are highly effective free radical scavengers and hence are antioxidants. Thus, the *in vitro* antioxidant and therapeutic properties of ZSCF may be possibly attributed to the phytochemicals (polyphenol and flavonoids) present. The present results indicated that ZSCF contained high level of TPC (1644 mg GAE/100 g DW) (Table 1), which was higher than the TPC of ZSCF from Yemen (1190 mg GAE/100 g DW) (Alzoreky and Nakahara, 2001) and Egypt (755 mg GAE/100 g DW) (Yossef *et al*., 2011). This is a rather high value compared to dried dates (220 mg GAE/100 g) (Singh *et al*., 2012), mango (240 mg GAE/100 g DM), Tamarind (390 mg GAE/100 g DM), Longan (160 mg GAE/100 g DM), avocado (130 mg GAE/100 g DM), Jackfruit (90 mg GAE/100 g DM) (Soong and Barlow, 2004). It has been reported that compounds such as the flavonoids which contain hydroxyls, are responsible for the radical scavenging effects of most plants (Das and Pereira, 1990). The mechanisms of action of the flavonoids are through scavenging or chelating processes (Cook and Samman, 1996). In the present study, the TFC of ZSCF from Oman was found to be 47 mg CEQ/100 g DW (Table 1) which might contribute to its high antioxidant activity. Polyphenols particularly flavonoids could be oxidized by polyphenol oxidases and may lose its antioxidant activity as it form corresponding quinines (Jimenez and Garcia-Carmona, 1999).

Table 1: Proximate chemical composition of dry ZSCF

Composition	Values
Protein (%)	5.40±0.13
Moisture (%)	13.30±0.16
Fat (%)	0.66±0.05
Carbohydrate (%)	76.50±0.27
Ash (%)	4.11±0.11
TPC (mg GAE/100 g)	1644.00±3.20
TFC (mg CEQ/100 g)	47.00±1.87
Ascorbic acid (mg/100 g)	14.20±0.46

Values in the table were expressed as Mean±SD (n = 3) and dry weight basis, TPC: Total phenolic content, TFC: Total flavonoid content, GAE: Gallic acid equivalents, CEQ: Catechin equivalents

According to other researchers, ZSCF contains different active compounds such as peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, betulinic acid and triterpenoid saponin glycosides (Cheng *et al*., 2000; Shahat *et al*., 2001; Tripathi *et al*., 2001). Total Ascorbic acid content of ZSCF was found to be 14.2±0.46 mg/100 g DW which was much lower than the 30 mg/100 g of DW in ZSCF from Saudi Arabia (Duke, 1985).

Total antioxidant activity of ZSCF extract was calculated by ABTS method. $ABTS^{-+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate. This pre-formed radical cation reduced it to ABTS in a concentration-dependent manner when combined with plant extract. The results of this assay were compared with that of trolox (standard). The total antioxidant activity of the ZSCF showed 91% inhibition in the ABTS method (at 429 μg mL^{-1}) (Fig. 1). The TEAC value revealed that extract considerably neutralized the radical ion and hence it is a potent antioxidant. The most effective antioxidants in this respect are reported to be phenolics and flavonoids which eventually facilitate the scavenging of free radicals by donating electron. DPPH (1,1-diphenyl-2-picrylhydrazyl) assay is a rapid and sensitive way to survey the antioxidant activity of natural compounds. It is a stable free radical (with red color) and its color changes to yellow when it scavenged. The degree of its discoloration is attributed to hydrogen donating ability of test compound. Hydrogen and electron transfer from

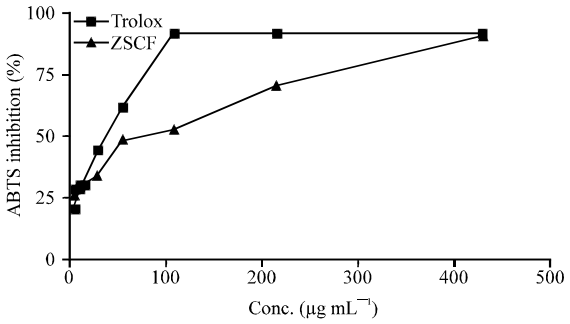

Fig. 1: Trolox antioxidant capacity (ABTS assay) of *Ziziphus spina-christi* fruits extract (ZSCF). The values are Mean±SD

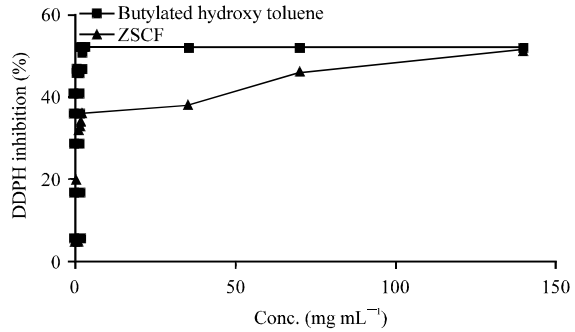

Fig. 2: DPPH radical scavenging activity of *Ziziphus spina-christi* fruits extract (ZSCF). The values are Mean±SD

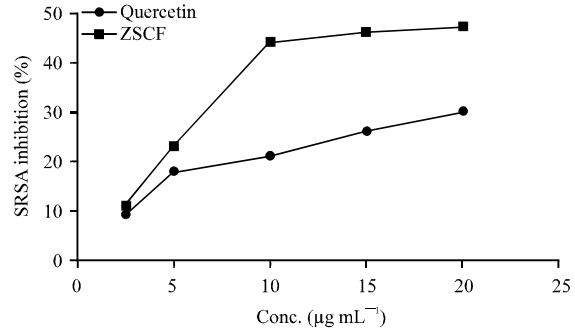

Fig. 3: Superoxide anion scavenging activity of *Ziziphus spina-christi* fruits extract (ZSCF). The values are Mean±SD

antioxidant analyses to DPPH occur in the DPPH assay methods. Neutralizing effect of different concentrations of ZSCF extract on DPPH free radical has been investigated and compared with BHT (Fig. 2). The scavenging effect of extract and standard on the DPPH$^{\bullet}$ radical was expressed as percentage inhibition. ZSCF exhibited effective scavenging of DPPH$^{\bullet}$ radical and hence antioxidant activity, showing 51% inhibition (at 140 mg mL^{-1}). The scavenging property of ZSCF extract can be attributed to the presence of hydroxyl groups which can donate the electron and neutralize the existing free radical in the reaction mixture. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented (Williams and Jeffrey, 2000). A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases. The transfers occur at different redox potentials in this assay and also depend on the structure of the antioxidant. Ascorbic acid, glutathione, cysteine, tocopherols, polyphenols and aromatic amines have the ability to donate hydrogen and electrons and can thus be detected by DPPH assay model (Marwah *et al*., 2007).

The superoxide radical scavenging ability of ZSCF has been presented in Fig. 3. In the PMS-NADH-NBT system, superoxide anion is derived from dissolved oxygen by PMS-NADH coupling reaction and reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The inhibition of O_2^{2-} was found to be concentration dependent. ZSCF showed 47% of inhibition in the SRSA assay (at 20 µg mL^{-1}). Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as singlet oxygen and hydroxyl radicals. Furthermore, superoxide radical is considered to play an important role in the peroxidation of lipids. Therefore, studying the scavenging effects of ZSCF on superoxide radicals is one of the most important ways of clarifying the mechanism of antioxidant activity. Previous reports suggested that flavonoids are effective antioxidants mainly because they scavenge superoxide anions (Robak and Gryglewski, 1988). This scavenging of superoxide anion is increased markedly with increase in the concentration due to presence of hydroxyl group of the phenolics which may contributes by their electron donation (Bravo, 1998). Similarly, in this study ZSCF extract might scavenge the superoxide anion in a similar way since they are rich in phenolics containing more hydroxyl groups. The results suggest that the ZSCF extract is a more potent scavenger of superoxide radical might be due to the presence of phytochemicals such as flavonoids, alkaloids, phenolics.

The FRAP assay is a simple and reproducible method which can be applied to the study of the antioxidant activity of food extracts and beverages. Therefore, we used this *in vitro* assay system to assess the ability of ZSCF extract. Fe^{2+} ion is measured spectrophotometrically through the determination of its coloured complex with 2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ) at 593 nm. Figure 4 shows reducing ability of ZSCF extract at different concentrations. Since the antioxidant activity of a substance is usually correlated directly to its reducing capacity, these results directly evidenced the iron reducing capability of ZSCF extract.

Figure 5 shows the reductive capabilities of ZSCF extract. The reducing power increased with the increasing amount of extract. The reducing capacity of ZSCF may serve as a significant indicator of its potential antioxidant activity. Recently, Khennouf *et al*. (2010) reported that the reducing power of tannins prevents liver injury by inhibiting the formation of lipid peroxides.

Metal chelating effect of ZSCF and EDTA (standard) is shown in Fig. 6. The ZSCF extract harboring chelating Ferrozine and form complexes with ferrous ions. In the

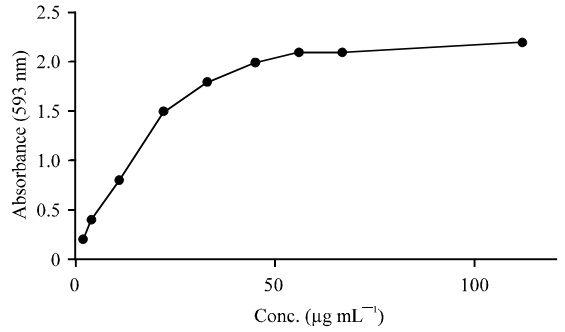

Fig. 4: Ferric reducing power assay of *Ziziphus spina-christi* fruits extract. The values are presented as Mean±SD

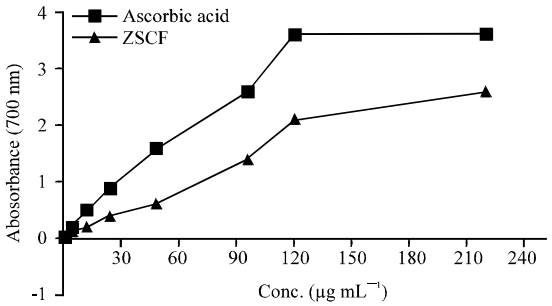

Fig. 5: Reducing power ability of *Ziziphus spina-christi* fruits extract (ZSCF). The values are Mean±SD

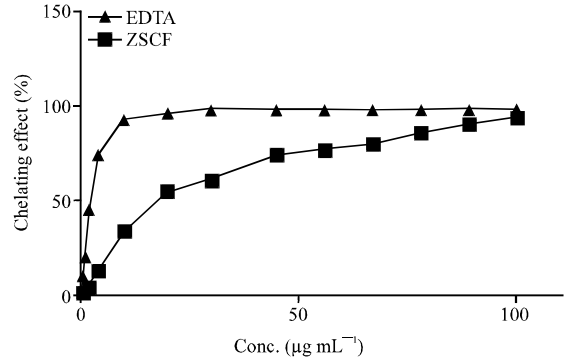

Fig. 6: Metal chelating activity of *Ziziphus spina-christi* fruits extract (ZSCF). The values are Mean±SD

presence of chelating agents, purple colored complex formation is interrupted and as a result, there is decrease in purple color of the complex. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine-Fe^{2+} complex is interrupted with the increase in concentration of sample.

CONCLUSION

To our knowledge, this is the first study dealing with the free radical scavenging capacity of ZSCF grown in Oman. The antioxidant activities of ZSCF were determined by using available different *in vitro* antioxidant assay systems along with total phenolic and flavonoid contents were also estimated. Over all, ZSCF showed high antioxidant capability in all tested assays and concurrently showed higher phenolic contents. This study proved the potential antioxidant activity of ZSCF grown in Oman which can be used as easy accessible source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits. The exact mechanism is still unclear and further studies are needed to validate the therapeutic potential of this fruit by assessing the activity and protective action of ZSCF antioxidants against cell damage, first *in vitro* and then *in vivo*.

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