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In vitro Assessment of Relief to Oxidative Injury by Different Samples of *Xanthoxylum armatum*

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The methanolic extract of *Zanthoxylum armatum* DC was dissolved in distilled water and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol sucessively. The antioxidant potential of the crude methanolic extract, all the organic fractions and the remaining aqueous fraction was evaluated by four methods: DPPH free radical scavenging activity, total antioxidant activity, FRAP assay and ferric thiocyanate assay and total phonolics were also determined. All the samples showed remarkable antioxidant potential. The results revealed that ethyl acetate fraction showed highest value of percentage inhibition of DPPH radical (92.96 % ± 0.13) with very low concentration (60 µg/mL), IC_{50} of ethyl acetate fraction was found to be 19.76 ± 0.12 µg/mL, relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.1 ± 0.92 µg/mL. The chloroform fraction exhibited highest total antioxidant activity (0.9101 ± 0.3) as compared to other fractions. The ethyl acetate fraction also displayed highest FRAP value (714.45 ± 2.61 µg of trolox equivalents) and total phenolic contents (416.4 ± 7.73 mg of gallic acid equivalents). The *n*-hexane fraction showed highest value of inhibition of lipid peroxidation (57.51 % ± 1.56).

Key Words: *Zanthoxylum armatum*, DPPH radical, Ferric thiocyanate, Gallic acid, Peroxidation.

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide and hydroxyl, nitric oxide and peroxynitrite radicals, play important role in oxidative stress related to the pathogenesis of various important diseases[1,2]. In healthy individuals, the production of free radicals is balanced by the antioxidative defense system. However, oxidative stress is generated when equilibrium favours free radical generation as a result of a depletion of antioxidant levels. The oxidation of lipid, DNA, protein, carbohydrate and other biological molecules by toxic ROS may cause DNA mutation or/and serve to damage target cells or tissues and this often results in cell senescence and death. Cancer chemoprevention by using antioxidant approaches has been suggested to offer a good potential in providing important fundamental benefits to public health and is now considered by many clinicians and researchers as a key strategy for inhibiting, delaying, or even reversal of the process of carcinogenesis. Moreover, knowledge and application of such potential antioxidant activities in reducing oxidative stresses *in vivo* has prompted many investigators to search for potent and cost-effective antioxidants from various plant sources. These research activities have contributed to new or renewed public interests worldwide in herbal medicines, health foods and nutritional supplements[3].

Zanthoxylum armatum DC [syn. *Z. alatum* Roxb] (Rutaceae), an evergreen or sub-deciduous shrub, is extensively used in the Indian system of medicine, as carminative, stomachic and anthelmintic. Various phytopharmaceuticals like berberine, dictamnine, xanthoplanine, armatamid, asarinin and fargesin, α- and β-amyrins and lupeol are present in this plant. The bark is pungent and sick from the plant is used in preventing toothache. The fruits and seeds are employed as an aromatic tonic in fever, dyspepsia and expelling roundworms. The essential oil of its fruit exhibited good antibacterial, antifungal and anthelmintic activities. Its petroleum extract showed significant insecticidal activity against *Culex* spp. with IC_{50} value of 20.45 ppm. The essential oil from seeds also exhibited larvicidal activity against mosquito vectors[4-6]. However, to the best of our knowledge, no detailed study to evaluate the *in vitro* antioxidant potential of *Zanthoxylum armatum* DC has been done so far. Therefore, the present study is undertaken to asses the *in vitro* relief to oxidative injury by different samples of this plant.

EXPERIMENTAL

The plant *Zanthoxylum armatum* DC [syn. *Z. alatum* Roxb] was collected from Kotly, Kashmir in March 2009 and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A voucher specimen (G.C. Herb. Bot. 860) has been deposited in the herbarium of the Botany Department of the same university.

Extraction and fractionation of antioxidants: The shade-dried ground whole plant (8.5 kg) was exhaustively extracted with methanol (12 L × 4) at room temperature. The extract was evaporated to yield the crude methanolic residue (815 g), which was dissolved in distilled water (1.5 L) and partitioned with *n*-hexane (1 L × 4), chloroform (1 L × 4), ethyl acetate (1 L × 4) and *n*-butanol (1 L × 4), respectivley. These organic fractions were concentrated separately on rotary evaporator to get the residues. The remaining aqueous layer was also concentrated in a similar way. For this study, the crude methanolic extract, organic fractions and the aqueous fraction thus obtained were used to evaluate their *in vitro* antioxidant potential.

DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-tripyridyl-*s*-triazine), trolox, gallic acid, Follin Ciocalteu reagent and BHT (butylated hydroxytoluene) were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol), sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride and ferrous chloride from Merck (Pvt.) Ltd. (Germany).

DPPH radical scavenging activity: The DPPH radical scavenging activities of various samples of this plant were examined by comparison with that of known antioxidant, butylated hydroxytoluene (BHT) using the method of Lee *et al.*[7]. In brief, various amounts of the extracts (1000, 500, 250, 125, 60, 30, 15 and 8 µg/mL) were mixed with 3 mL of methanolic solution of DPPH (0.1 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 1 h. Then absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. Lower absorbance of spectrophotometer indicated higher free radical scavenging activity.

The per cent of DPPH decolouration of the samples was calculated according to the formula:

$$\text{Antiradical activity} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Each sample was assayed in triplicate and mean values were calculated.

Total antioxidant activity by phosphomolybdenum method: The total antioxidant activities of various samples were evaluated by phosphomolybdenum complex formation method[8]. Briefly, 500 µg/mL of each extract was mixed with 4 mL of reagent solution (0.6M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in sample vials. The blank solution contained 4 mL of reagent solution. The vials were capped and incubated in water bath at 95 ºC for 1.5 h. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of butylated hydroxytoluene (BHT). All determinations were assayed in triplicate and mean values were calculated.

Ferric reducing antioxidant power (FRAP) assay: The ferric reducing antioxidant power assay was done according to the method of Benzie and Strain[9] with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $CH_3COONa{\cdot}3H_2O$ and 16 mL CH_3COOH), pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl and 20 mM $FeCl_3{\cdot}6H_2O$ solution. The fresh experimental solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3{\cdot}6H_2O$ solution and then warmed at 37 ºC before using. The solutions of plant samples and that of Trolox were formed in methanol (250 µg/mL). 10 mL of each of sample solution and BHT solution were taken in separate test tubes and 2990 µL of FRAP solution was added in each to make total volume up to 3 mL. The plant extracts were allowed to react with FRAP solution in the dark for 0.5 h. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The ferric reducing antioxidant power values were determined as micromoles of Trolox equivalents per mL of volatile oil by computing with standard calibration curve constructed for different concentrations of Trolox. Results were expressed in TE µM/mL.

Total phenolic contents: Total phenolics of various samples were determined by the method of Makkar *et al.*[10]. 0.1 mL (0.5 mg/mL) of sample was combined with 2.8 mL of 10 % Na_2CO_3 and 0.1 mL of 2N Folin-Ciocalteu reagent. After 40 min absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenolics were determined as milligrams of gallic acid equivalents per gram of sample by computing with standard calibration curve constructed for different concentrations of gallic acid. The standard curve was linear between 50-400 µg/mL of gallic acid. Results were expressed in GAE µg/mL.

Ferric thiocyanate (FTC) assay: The antioxidant activities of various samples on inhibition of linoleic acid peroxidation were assayed by thiocyanate method[11]. The 0.1 mL of each of sample solution (0.5 mg/mL) was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2.0 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50.0 mL of phosphate buffer. The reaction mixture was incubated for 5 days at 40 ºC. The mixture without sample was used as control. The 0.1 mL of the mixture was taken and mixed with 5.0 mL of 75 % ethanol, 0.1 mL of 30 % ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5 % HCl and allowed to stand at room temperature. Precisely 3 min after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity was expressed as percentage inhibition of peroxidation (IP %) [IP % = {1-(abs. of sample)/(abs. of control)} × 100]. The antioxidant activity of BHT was assayed for comparison as reference standard.

Statistical analysis: All the measurements were done in triplicate and statistical analysis was performed by Microsoft excel 2003. Results are presented as average ± SEM.

RESULTS AND DISCUSSION

DPPH free radical scavenging method has been widely applied for evaluation of antioxidant activity in a number of

extracts. The method is based on the reaction of DPPH radical that is characterized as a stable free radical with deep violet colour and any substance that can donate hydrogen atom to DPPH thus reduces it to become stable diamagnetic molecule. The proton transfer from antioxidant to DPPH radical depends upon the structure of the antioxidant. The colour changes from purple to yellow and its absorbance at 517 nm decreases[12]. In the present investigation, aforesaid samples from *Zanthoxylum armatum* were evaluated for their free radical scavenging activity using the DPPH radical assay. Reduction of DPPH radicals was observed by the decrease in absorbance at 517 nm. These samples reduced DPPH radicals significantly. Values of percent scavenging of DPPH radicals have been shown in Table-1. It was observad that as we increase the concentration, the value of percentage inhibition also increased. Ethyl acetate fraction showed highest value of percentage inhibition of DPPH radical (92.96 %) with very low concentration (60 µg/mL). The IC_{50} value for each sample, defined as the concentration of sample causing 50 per cent inhibition of absorbance, was determined and presented in Table-2. Since IC_{50} is a measure of inhibitory concentration, its lower value would reflect greater antioxidant activity of the sample. Ethyl acetate fraction displayed the highest antioxidant activity with IC_{50} of 19.76 ± 0.12 µg/mL, followed by chloroform fraction (IC_{50} 60.09 ± 2.91 µg/mL), *n*-butanol fraction (IC_{50} 66.74 ± 1.66 µg/mL), *n*-hexane fraction (IC_{50} 140.21 ± 1.65 µg/mL), crude methanolic extract (IC_{50} 173.53 ± 0.25 µg/mL) and aqueous fraction (IC_{50} 226.19 ± 7.21 µg/mL), respectively, relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.1 ± 0.92 µg/mL.

TABLE-1
FREE RADICAL SCAVENGING ACTIVITY OF METHANOLIC EXTRACT OF *Zanthoxylum armatum*, ITS ORGANIC FRACTIONS AND AQUEOUS FRACTION USING 1,1-DIPHENYL-2-PICRYLHYDRAZYL RADICAL (DPPH)

Sample	Concentration in assay (µg/4 mL)	Percentage scavenging of DPPH radical ± SEM*
Methanolic extract	500	81.13 ± 0.10
	250	63.05 ± 0.34
	125	47.55 ± 0.23
	60	33.18 ± 0.11
n-Hexane soluble fraction	1000	83.68 ± 0.08
	500	68.83 ± 0.41
	250	56.53 ± 0.37
	125	45.43 ± 0.26
Chloroform soluble fraction	250	85.37 ± 0.10
	125	67.88 ± 0.55
	60	54.14 ± 1.15
	30	44.81 ± 0.57
		32.81 ± 0.29
Ethyl acetate soluble fraction	60	92.96 ± 0.13
	30	73.68 ± 0.07
	15	40.77 ± 0.04
	8	32.57 ± 0.31
n-Butanol soluble fraction	250	90.80 ± 0.16
	125	68.29 ± 0.71
	60	52.54 ± 0.37
	30	35.16 ± 0.36
Aqueous fraction	500	74.10 ± 0.43
	250	56.26 ± 0.25
	125	44.46 ± 0.46
	60	29.42 ± 1.07
BHT**	60	91.25 ± 0.13
	30	75.56 ± 0.07
	15	42.67 ± 0.04
	08	23.57 ± 0.31

*Standard mean error of three assays. **Standard antioxidant.

Total antioxidant activity of these samples was determined by phosphomolybdenum method. This method is based on the reduction of molybdenum(VI) to molybdenum(V) by the antioxidats and the subsequent formation of a green phosphate Mo(V) complex at acidic pH. Electron transfer occurs in this assay which depends upon the structure of the antioxidant[8]. The antioxidant activities of various samples of *Zanthoxylum armatum* were compared with the reference standard antioxidant BHT. The results showed that the antioxidant activity of these samples was decreased in the following order: methanol extract > hexane fraction > chloroform fraction > ethyl acetate fraction > *n*-butanol fraction> aqueous fraction (Table-2).

Ferric reducing antioxidant power appears to be related to the degree of hydroxylation and extent of conjugation in polyphenols[13]. Ferric reducing antioxidant power assay measures the change in absorbance at 593 nm forming intense blue Fe^{2+} tripyridyltriazine complex from colourless oxidized Fe^{3+} form by the action of electron donating antioxidants[14]. The ferric reducing antioxidant power value expressed in Trolox equivalents, was used to determine the antioxidant ability of the various samples, relative to BHT, a reference standard. Highest FRAP value was exhibited by ethyl acetate fraction (714.45 ± 2.61 µg of trolox equivalents) as compared to other samples. The lowest FRAP value was observed for that of aqueous extract (Table-2).

Plant phenolics are commonly found in both edible and non-edible plants and have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers[15]. Therefore, the antioxidant activities of plant extracts are often explained by their total phenolics. This assay is an electron transfer based assay and gives reducing capacity, which has normally been used to estimate phenolic contents of biological materials[16]. Total phenolics assay was employed on these samples to measure the total phenolic contents. The ethyl acetate fraction showed the highest total phenolic contents (416.4 ± 7.73 mg of gallic acid equivalents) as compared to other plant samples. Total phenolic contents were found to be in the following order: ethyl acetate fraction, *n*-butanol fraction, aqueous fraction, chloroform fraction, methanolic extract and *n*-hexane fraction respectively (Table-2).

The antioxidant activity of these samples was also tested using the ferric thiocyanate (FTC) method. The ferric thiocyanate method is used to measure the amount of peroxide at the beginning of lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ions. Ferric ions will then unite with ammonium thiocyanate and produce ferric thiocyanate, a reddish pigment[17]. The tested samples of plant, showed low absorbance values, which indicated a high level of antioxidant activity. None of the fractions showed absorbance value greater than the negative controls (without plant

TABLE-2
IC_{50}, TOTAL ANTIOXIDANT ACTIVITY, FRAP VALUES, TOTAL PHENOLICS AND LIPID PEROXIDATION INHIBITION VALUES OF *Zanthoxylum armatum* EXTRACT AND FRACTIONS

Sample	IC_{50} of DPPH assay (µg/mL) ± SEM*	Total antioxidant activity ± SEM*	FRAP value TE (µM/mL) ± SEM*	Total phenolics (GAE mg/g of sample) ± SEM*	Inhibition of lipid peroxidation (%) ± SEM*
Methanolic extract	173.53 ± 0.25	0.6766 ± 0.00	685.93 ± 4.54	336.2 ± 1.6	50.18 ± 0.34
n-Hexane fraction	140.21 ± 1.65	0.8987 ± 0.02	311.91 ± 7.86	315 ± 3.01	57.51 ± 1.56
Chloroform fraction	60.09 ± 2.91	0.9101 ± 0.03	340.53 ± 7.99	349.6 ± 4.12	44.75 ± 1.09
Ethyl acetate fraction	19.76 ± 0.12	0.4034 ± 0.01	714.45 ± 2.61	416.4 ± 7.73	49.20 ± 0.18
n-Butanol fraction	66.74 ± 1.66	0.3326 ± 0.01	606.66 ± 6.57	413.1 ± 8.02	50.38 ± 0.31
Water fraction	226.19 ± 7.21	0.2603 ± 0.02	180.56 ± 7.88	371.04 ± 15.63	43.92 ± 0.84
BHT**	12.1 ± 0.92	1.2186 ± 0.07	–	–	62.91 ± 0.60

*Standard mean error of three assays. **Standard antioxidant.

samples) at the end point of the method, indicating the presence of antioxidant activity. Significantly lower absorbances were observed which indicate that the tested samples of *Zanthoxylum armatum* have good antioxidant activities. High percentage of inhibition of lipid peroxidation was exhibited by *n*-hexane fraction (57.51 % ± 1.56). The inhibition of lipid peroxidation by BHT (standard) was 62.91 % ± 0.48 antioxidant activities of tested samples decreased in the following order: *n*-hexane fraction > *n*-butanol fraction > methanol extract > ethyl acetate fraction > chloroform fraction > aqueous fraction, respectively (Table-2).

Conclusion

When the antioxidant activities of the tested samples were compared by above methods, it is observed that ethyl acetate fraction showed highest DPPH radical scavenging activity, FRAP value and as well as total phenolic contents. The chloroform fraction exhibited highest total antioxidant activity while *n*-hexane fraction displayed highest percentage of lipid peroxidation inhibition. So it was generally concluded that although medium-polar fractions contained strong antioxidants but it was also promising to say that all the fractions of this plant are potentially valuable sources of natural antioxidants and bioactive materials, which would be expected to increase shelf life of foods and protect against peroxidative damage in living systems in relation to aging and carcinogenesis.

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