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Antioxidant Activity of Various Extracts and Organic Fractions of *Ziziphus jujuba*

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Abstract

Antioxidant effectiveness of indigenous medicinal plant *Ziziphus jujuba* shoots extracts and fractions with different polarity solvents (*n*–hexane, ethylacetate, methanol, chloroform) was assessed for total phenolics content (TPC), total flavonoid contents (TFC), DPPH radical scavenging activity and percentage inhibition of peroxidation in linoleic acid system. The shoots extracts and fractions contained appreciable levels of total phenolic contents 310-823 GAE (mg/100g Dry plant matter) and total flavonoid contents 210-650 CE (mg/100g of Dry plant matter). The *Ziziphus jujuba* extracts and various organic fractions also exhibited good DPPH 50% inhibition (IC_{50}) ranges from 23.1µg/mL to 52.5 µg/ml and Inhibition of Peroxidation in Linoleic Acid 20.1 to 70.1%., respectively. Of the *Ziziphus jujuba* shoots extracts and fractions tested, 100% methanolic extract and 100% chloroform fraction exhibited the maximum antioxidant activity, the results of the present investigation demonstrated significant ($p< 0.05$) variations in the antioxidant activity. The results of the present comprehensive analysis demonstrated that *Ziziphus jujuba* shoots extracts and organic fractions are a viable source of natural antioxidants and might be exploited for functional foods and nutraceutical applications

Keywords: Total phenolic contents; flavonoids; antioxidant activity; DPPH free radical scavenging; *Ziziphus jujuba.*

Introduction

Ziziphus jujuba L. commonly known as Ber in Pakistan, belonged to the family Rhamnaceae that consists of 45 genera and 550 species is widely distributed in tropical and subtropical climates in the world (1). *Ziziphus jujuba* L is a hardy tree of arid region which can be grown successfully in saline soil under hot, arid environment (2). Its fruits are palatable and delicious with a good amount of vitamin A, C and B complexes and minerals. Some species, like *Z. mauritiana* and *Z. jujuba,* occur on nearly every continent, whereas other species, like *Z. nummularia, Z. spina-Christi* and *Z. mucronata,* are restricted in their distribution to distinct areas.

The chemical composition of the oil of *Ziziphus* Leaves obtained by hydrodistillation has the major components: geranyl acetone (14.0%), methyl hexadecanoate (10.0%), methyl octadecanoate (9.9%), farnesyl acetone C (9.9%), hexadecanol (9.7%) and ethyl octadecanoate (8.0%) (3). Alkaloids, flavonoids, sterols, tannins, saponin, and fatty acids have been isolated and chemically identified from the different species of the genus *Ziziphus* (4). Besides, it has significant levels of antioxidant activity, reducing power, scavenging effect on free radicals. Because of these properties, *Ziziphus* species are used in folk medicine for the treatment of some diseases in the world (5).

Flavonoids from *Ziziphus jujuba* help a lot to treat various diseases and disorders like cancer and diarrhea. Important flavonoids present in *Ziziphus jujuba* are quercetin and epicatechin. Flavones, isoflavones and anthocyanidins are also present in *Ziziphus* species.

Materials and methods

Plant material

The selected medicinal plant *Ziziphus jujuba* shoots were collected from the Botanical Garden, University of Agriculture, Faisalabad, and identified from the Department of botany University of Agricultural Faisalabad, Pakistan where a voucher specimen number has been deposited.

Preparation of extracts and fractions

2Kg fresh shoots of *Z. jujuba* washed with distilled water. Then they were shade dried. The dried shoots were powdered and stored in container till further experiment. In the weighed amount of powder the measured amount of 100% methanol (2×15 L) was added and kept it for 4-5 days at room temperature. Rotary evaporator was used to remove solvent. Extract (140 g) became viscous which were dried on water bath and then stored at – 4 ^{0}C. The process was repeated three times with intervals of four days. The methanolic extract was dissolved in distilled water and fractionation was done by using different polarity based solvents and got *n*-hexane (50g), 100% chloroform (42g), 100% ethyl acetate (40g) successively. The 100% ethylacetate fraction (E) was further subjected to column chromatography over a flash silica gel eluting with *n*-hexane and gradient of ethyl acetate upto 100%. Four fractions (E1-E4) were collected. The same procedure was repeated with remaining residue but solvents used for elution were methanol and chloroform with varied polarity and four fractions (M1-M4) were collected as shown in Fig 1. All fractions and extracts obtained were investigated for antioxidant activity using different assays.

Determination of Total Phenolic Contents

Amount of TP contents were determined by using Folin Ciocalteu reagent (6). Briefly 0.05g of extract/fraction was mixed with 0.5mL of 50 times diluted Folin Ciocalteu reagent and 7.5mL deionized water, the mixture was kept at room temperature for 10 min. and then 1.5 mL of 10% sodium carbonate(w/v) was added, mixed it thoroughly. The mixture was heated in a water bath at 40^{o}C for 20 min. and then cooled in an ice bath; absorbance at 755nm was measured using a spectrophotometer (U-2001, Hitachi Instrument Inc. Tokyo, Japan). Amounts of total phenolic contents were calculated using Gallic acid calibration curve within range of 10-100ppm (R^2 = 0.9952). The results were articulated as Gallic acid equivalents (GAE) g/100gm of dry plant matter. All samples were analyzed thrice and results averaged.

Determination of Total Flavonoid Contents (TFC)

TFC were measured by a spectrophotometric method following a previously reported method (7). Each extract and fraction material (1mL containing 0.1 mg/mL) was placed in a 10 mL volumetric flask, then added 5mL of distill. Water. 0.3mL of 5% $NaNO_2$ was added to each of volumetric flasks; after 5 min. 0.6mL of 10% $AlCl_3$ was added. After another 5 min. 2mL of 1 M NaOH was addedn and then volume made up

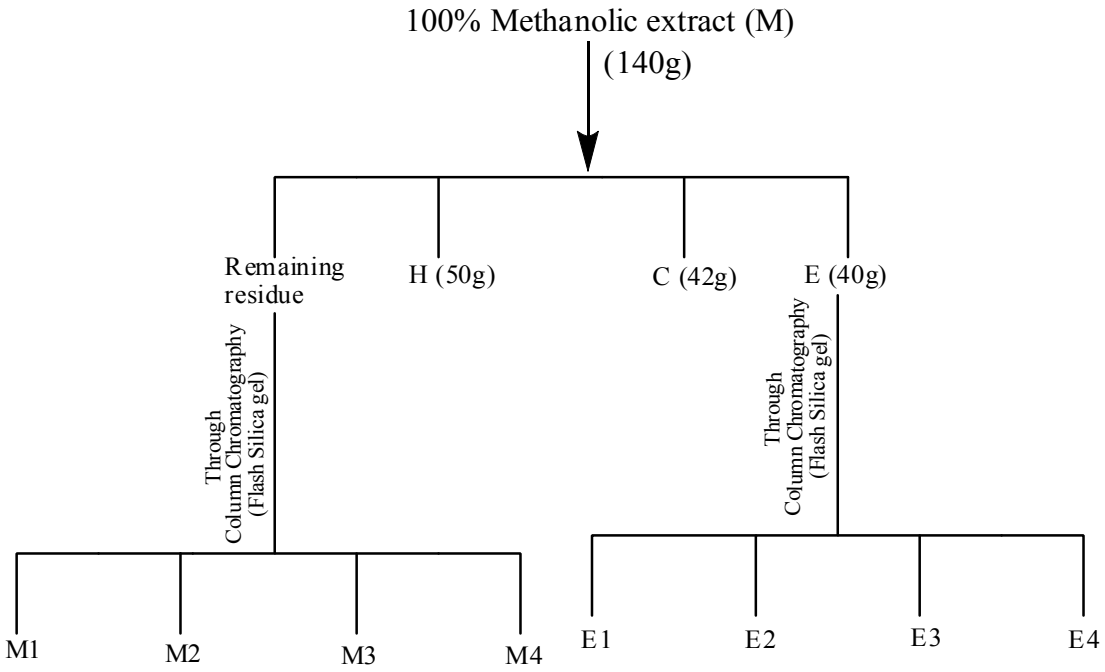

Fig.1 Schematic diagram showing preparation of extracts and fractions of *Z. jujuba* shoots

M; 100% methanol

M1; 20% methanol and 80% chloroform

M2; 40% methanol and 60% chloroform

M3; 60% methanol and 40% chloroform

M4; 80% methanol and 20% chloroform

E; 100% ethylacetate

E1; 20% ethylacetate and 80% *n*-hexane

E2; 40% ethylacetate and 60% n-hexane

E3; 60% ethylacetate and 40% *n*-hexane

E4; 80% ethylacetate and 20% *n*-hexane

C; 100% chloroform

H; 100% *n*-hexane

with distilled water. Absorbance of the reaction mixture was measured at 510 nm using a spectrophotometer (U-2001, Hitachi Instrument Inc. Tokyo, Japan). TFC were determined as Catechin equivalents (g/100g of dry plant matter). Three readings were taken for each sample and results were averaged.

DPPH Free Radical Scavenging Assay

2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was carried out spectrophotometrically as described by (8). Stock solution was prepared by dissolving 0.001g of extract in 10mL of methanol. Now dilution formula was applied and ppm solutions of different concentrations like 20ppm, 40ppm, 60ppm, 80ppm and 100ppm were made in methanol. Then 0.025ml of each ppm solution was taken in a dry test tube with the help of micropipette and 2.5 mL of DPPH was added in them. The absorbance of resulting solution and blank were measured at 517nm after 30 min at room temperature. For each sample, three replicates were recorded. Then inhibitory percentage of DPPH was calculated according to the following equation:

$$\% I = [(A_{blank} - A_{sample}) / A_{blank}] X 100$$

Where: A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. IC_{50} value (mg/mL) is the concentration at which the scavenging activity was 50% and it caused 50% neutralization of DPPH radicals, were calculated from the plot of inhibition percentage against concentration.

Antioxidant Activity Determination in Linoleic Acid System

The antioxidant activities of *ziziphus jujuba* shoots extracts and fractions were also determined in terms of measurement of percentage inhibition of peroxidation in the linoleic acid system, following the method described by (9) with some modifications. Extracts (5mg) of each treatment were added to a solution mixture of linoleic acid (0.13mL), 99.8% ethanol (10mL) and 10mL of 0.2M sodium phosphate buffer (pH=7). Mixture was diluted to 25mL with distilled water. Then the solution was incubated at 40^{o}C for 175 hours, and the degree of oxidation was measured following thiocyanate method (10). With 10mL of ethanol (75%), 0.2mL of an aqueous solution of ammonium thiocyanate (30%), 0.2ml of sample solution and 0.2mL of ferrous chloride ($FeCl_2$) solution (20mM in 3.5% HCl) being added sequentially. After 3 min of stirring the absorption values of mixtures measured at 500 nm were taken as achievable contents. A control was performed with linoleic acid but without extracts. Synthetic antioxidant butylated hydroxytoluene (BHT) was used as a positive control. In the sample the maximum peroxidation level was observed as 175 hrs. (7 days and 7hrs.), the sample that contained no antioxidant component was used as a test point. Percent inhibition of linoleic acid per oxidation was calculated to express antioxidant activity as:-

100 - [(Abs. increase of sample at 175 hrs. / Abs. increase of control at 175 hrs.) X 100

Statistical Analysis

All the experiments were conducted in triplicate unless stated otherwise and statistical analysis of the data was performed by analysis of variance (ANOVA), using STATISTICA 5.5 (Stat Soft Inc, Tulsa, Oklahoma, USA) software. A probability value of difference $p \leq 0.05$ was considered to denote a statistically significance. All data were presented as mean values ± standard deviation (SD).

Results and discussion

Total Phenolic and total flavonoid Contents

The total phenolic contents (TPC) and total flavonoid contents (TFC) of *Ziziphus jujuba* shoots different extracts and fractions are presented in Table 1. The amounts of TPC and

TFC extracted from *Z. jujuba* shoots in different solvent systems were in the ranges 310–823 GAE (mg/100 g) and 210– 650 CE (mg /100 g), respectively. Methanol extract (100%) of the *Z. Jujuba* shoots showed the significantly ($p< 0.05$) highest TPC and TFC, 823 and 650 mg/100g, respectively. These differences in the amount of TPC and TFC may be due to varied efficiency of the extracting solvents to dissolve endogenous compounds. The ability of different solvents to extract TPC and TFC was of the order: M> C> M4> E> M3> M2> M1> E2> E1> E4> E3>H. *n*-hexane extracted least amount of TPC and TFC 310 and 210 (mg/100g) respectively. Methanol is efficient and most widely used to extract antioxidative components including phenolic acids and other phenolic components (11). Although chloroform, ethylacetate also extracted reasonable amounts of TPC, however *n*-hexane being non- polar in nature was least effective for extraction of phenolic contents. Chloroform and methanol is preffered for the extraction of antioxidant compounds mainly because of their lowers toxicity (12). The differences in the amounts of TFC in different extracts and fractions could be explained by the fact that presence of phenolics is affected by plant species, maturity at harvest, growing conditions, soil conditions and post harvest treatments.

DPPH Free Radical Scavenging Assay

We investigated the free radical scavenging activity and lipid oxidation inhibition of *Z. jujuba* shoots extracts and fractions. Free radical scavenging activities of the samples were measured by DPPH assay. Free radical scavenging capacity increased with increasing extracts and fractions concentration (Table 1). *Z. jujuba* 100% methanolic extract and 100% Chloroform fraction showed excellent radical scavenging activity, with IC_{50} (the extract concentration providing 50% of inhibition) values of 23.1 and 25.2µg/ml, respectively. The free radical scavenging activity of other fractions was lower to that of 100% methanolic extract. When compared with the synthetic antioxidant BHT (IC_{50}=19.1), all extracts and fractions offered significantly ($p< 0.05$) lower antioxidant activity. The radical scavenging activity of different extracts and fractions was of the order:

BHT> M> C> M4> E> M3> M2> M1> E2> E1> E4> E3>H.

Antioxidant Activity in Linoleic Acid System

The antioxidant activity has also been assessed as ability to prevent from oxidation. Therefore, inhibition of linoleic acid oxidation was also used to assess the antioxidant activity (AA) of *Ziziphus jujuba* shoots. Antioxidant activity of different jujuba extracts and fractions were determined by inhibition of linoleic acid system using thiocyanate method described by Yen et al (10). When the inhibition of linoleic acid oxidation of *Ziziphus jujuba* extracts and fractions were compared with synthetic BHT all the extracts and fractions exhibited significantly ($p< 0.05$) lower antioxidant activity than shown by BHT (89.2%). Maximum antioxidant activity in linoleic acid system was found in 100% Methanolic extract (70.1%) and 100% Chloroform fraction (62.1%). Tocopherols, carotenoids also contribute to antioxidant activity in terms of measurement of % inhibition of peroxidation. Chloroform fraction showed good inhibition of peroxidation so there is possibility of presence of these compounds in it. The order of inhibition of linoleic acoid oxidation offered by jujuba shoots extracts and fractions was as under:-

BHT> M> C> M4> E> M3> M2> M1> E2> E1> E4> E3>H.

Due to lack of data on the inhibition of linoleic acid oxidation of jujuba extracts, the results determined cannot be compared.

Table.1 Antioxidant activities of *Z. jujuba* shoots extracts and various fractions.

Tested extracts and fractions	Total phenolic contents GAE (mg/100g) of Dry plant matter	Total flavonoid contents CE (mg/100g) of Dry plant matter	DPPH, IC_{50} (µg/ml)	Inhibition in linoleic acid system (%)
M	823±15.2	650±15.0	23.1±12.1	70.1±4.11
M1	510±12.0	432±14.1	41.2±1.10	42.1±3.10
M2	512±11.2	450±15.2	34.1±0.86	48.1±2.10
M3	610±10.8	490±14.3	32.1±1.10	50.3±1.22
M4	714±11.5	580±14.6	28.4±0.99	61.2±1.51
E	632±9.32	510±12.3	29.5±1.10	56.1±2.60
E1	420±8.91	312±12.5	47.1±1.40	28.3±2.71
E2	440±8.45	406±11.3	45.4±2.11	32.2±2.53
E3	398±12.1	240±11.5	50.1±1.81	22.4±1.10
E4	410±8.11	298±10.9	49.2±2.12	26.2±1.13
C	750±8.60	620±13.0	25.2±1.81	62.1±2.50
H	310±9.31	210±10.8	52.2±2.83	20.1±1.52
BHT	------	------	19.1±2.10	89.2±3.41

M= 100% methanol: M1= 20% methanol and 80% chloroform: M2= 40% methanol and 60% chloroform: M3= 60% methanol and 40% chloroform: M4=; 80% methanol and 20% chloroform: E= 100% ethylacetate: E1=20% ethylacetate and 80% *n*-hexane: E2= 40% ethylacetate and 60% n-hexane: E3= 60% ethylacetate and 40% *n*-hexane: E4= 80% ethylacetate and 20% *n*-hexane: C=100% chloroform: H= 100% *n*-hexane. *V*alues are mean ± SD of samples analyzed in triplicate. All calculations made on dry basis.

Conclusion

In this study, it was demonstrated that Extracts and fractions from *Z. jujuba* shoots exhibited good antioxidant activity as measured by various antioxidant assays. Sizeable amounts of TPC and TFC were found in analyzed extracts and fractions of plant. The results of the present study would certainly help to ascertain the potency of the crude extracts and fractions of *ziziphus jujuba* shoots as potential source of natural antioxidants. Discussed study offer some preliminary information about antioxidants potential in this source, but to develop useful antioxidants more work is required to further establish composition of components involved and how to isolate them in appropriate form for particular application

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