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Full Length Research Paper

Alteration in antioxidant and antimicrobial attributes of leaves of *Zizyphus* species in response to maturation

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This study was conducted to assess the antioxidant and antimicrobial potential of leaves extracts of five different species of *Zizyphus*. The methanol extracts of both young and mature leaves were prepared, and their antioxidant phytoconstituents (total phenol, total flavonoid) and activities (inhibition of linoleic acid peroxidation, DPPH scavenging ability, and reducing power) were subsequently determined. The total phenolic (19.0 to 28.2 GAE mg/g) and total flavonoid (38.1 to 61.8 CE mg/g) contents of young leaves were significantly higher ($P < 0.05$) than that of mature one (19.0 to 25.1 GAE mg/g) and (33.6 to 50.3 CE mg/g), respectively. Total phenolic contents, total flavonoid contents and DPPH scavenging activity decreased with maturation. All the extracts also showed remarkable antimicrobial activity against a panel of micro-organisms. Overall results of study showed that *Zizyphus* leaves are a good source of natural antioxidant and antimicrobial compounds, and thus can be utilized in herbal formulations.

Key words: Antimicrobial, antioxidant, extracts, leaves, species, *Zizyphus.*

INTRODUCTION

Free radicals not only cause the deterioration of lipidic food products but also impart some effect on living organisms at cellular and sub-cellular levels (Sarikurkcu et al., 2008). Day by day the interest in medicinal plants being a source of natural products is increasing and various parts of these plants have been extensively studied for their antioxidant and radical scavenging activities (VanderJagt et al., 2002). The antioxidants and antimicrobial activities of plant extract have created the basis of many applications in pharmaceuticals, alternative medicines and natural therapy (Abiy et al., 2005). Especially the phenolics found in vegetables, fruits and other medicinal plants are receiving much attention of medical scientists due to their impending function in the prevention of various diseases. This natural gift, the antioxidants may be taken as dietary, food supplement or as a drug (Cai et al., 2004).

Zizyphus is a genus of family Rhamnaceae comprising of about 40 species. Plants of this genus are widely distributed in Europe and South-Eastern Asia. In Pakistan, it is commonly known as Bayr and used traditionally as tonic and aphrodisiac and sometimes as hypnotic-sedative, It also possess anxiolytic, anticancer, antifungal, antibacterial, antiulcer, anti-inflammatory, antispastic, and wound healing properties (Benammar et al., 2010). Their pounded leaves are applied as a dressing to wounds (Mahajan and Chopda, 2010).

To the best of our knowledge, the antimicrobial and the antioxidant properties of the leave extracts from different species of Bayr, indigenous to Pakistan have not been studied. Therefore, this study was designed with main objective to evaluate the antioxidant and antimicrobial attributes of leaves of different species of *Zizyphus.*

MATERIALS AND METHODS

Samples

Leaves samples of five *Zizyphus* species; *Zizyphus jujuba* mill, *Zizyphus spina-christi*, *Zizyphus mauritiana*, *Zizyphus lotus* and *Zizyphus vulgaris* (Figure 1) were obtained from the square 9 area of Horticulture, University of Agriculture Faisalabad, Pakistan. The

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Figure 1. Different species of *Zizyphus.*

specimens were further identified and authenticated by the taxonomist, Department of Botany, University of Agriculture, Faisalabad. Manually separated young and mature leaves were air-dried and stored in polythene bags for further analysis.

Reagents and chemicals

Linoleic acid, butylated hydroxytoluene (BHT) (99.0 %), DPPH, catechin, gallic acid and Folin-Ciocalteu reagent (2 N) were purchased from Sigma Chemicals Co. (St, Louis, MO, USA). All other chemicals and reagents, all of analytical grade were from Merck (Darmstadt, Germany).

Preparation of antioxidant / antimicrobial extracts

The dried sample of *Zizyphus* leaves was grinded using a commercial grinder (TSK-949, West point, France). Powdered sample (10 g) of each was used for extraction of antioxidant and antimicrobial compounds. Sample was mixed with 100 ml aqueous methanol (methanol: water 80:20 v/v) and kept in an orbital shaker (Gallenkamp, UK) at room temperature for 24 h and then filtered through Whatman No. 1 filter paper. The extracts were

concentrated under reduced pressure at 45°C. The crude extract was weighed to calculate the % yield and stored at -4°C, until used for further analyses.

Evaluation of antioxidant activity of the extracts

Determination of total phenolic contents (TPC)

Total phenolic contents were determined using Folin-Ciocalteu reagent (Chaovanalikit and Wrolstad, 2004). Crude extract (50 mg) was combined with Folin-Ciocalteu reagent (0.5 ml) and deionized water (7.5 ml). After 10 min, 1.5 ml of 20% sodium carbonate (w/v) was added. Then mixture was heated in a water bath at 40°C for 20 min after that cooled in an ice bath. The absorbance of blue colored solution was measured at 755 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). Amount of TPC were expressed as gallic acid equivalents (GAE) mg/g of dry weight.

Determination of total flavonoid contents (TFC)

The TFC were measured following a previously reported method (Dewanto et al., 2002). Briefly, plant extract (0.1 mg/ml) was successively mixed with 4 ml water, 0.3 ml of 5% $NaNO_2$, 0.3 ml of 10% $AlCl_3$, and 2 ml of 1.0 M NaOH. Finally, 2.4 ml of water was added to the reaction flask and mixed well. Then absorbance of the reaction mixture was measured at 510 nm. TFC were determined as catechin equivalents (mg/g of dry weight).

DPPH· scavenging assay

The antioxidant activity of the leaves extract was also evaluated by measuring their free radical scavenging capacity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as free radical (Bozin et al., 2006). The samples (0.5 to 100 µg/L) were mixed with 1 ml of DPPH solution (90 µM) and made up to a final volume of 4 ml with 95% MeOH, butylated hydroxytoluene (BHT) was used as positive control. After 1 h incubation period, the absorbance was recorded at 515 nm. Inhibition of free radical by DPPH in % was calculated using the following formula:

$$I\ (\%) = 100 \times [(A_b - A_s)/A_b]$$

Where A_b is the absorbance of the control reaction (containing all reagents except the test extract), and A_s is the absorbance of the tested extract.

Determination of antioxidant activity in linoleic acid system

The inhibition of peroxidation of linoleic acid capacity of the extracts was measured following the reported method of Iqbal et al. (2005). Leave extracts (5 mg) were added separately to a mixture of linoleic acid (0.13 ml), 99.8% ethanol (10 ml) and 10 ml of 0.2 M sodium phosphate buffer (pH 7). The mixture was made up to 25 ml with distilled water and incubated at 40°C for 360 h. Degree of oxidation was deliberated by peroxide value applying thiocyanate method as described by Yen et al. (2000). Briefly, 10 ml of ethanol (75% v/v), 0.2 ml of aqueous solution of ammonium thiocyanate (30% w/v), 0.2 ml of sample solution and 0.2 ml of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HCl; v/v) added consecutively. After 3 min of stirring, the absorption was measured at 500 nm a control contained all reagents with the exception of extracts. Butylated hydroxytoluene (BHT) was used as positive control. Percent inhibition of linoleic acid oxidation was calculated using the following equation: 100 – [(Abs. increase of sample at 360 h / Abs. increase of control at 360 h) × 100], to express antioxidant activity.

Determination of reducing power

The reducing power of *Zizyphus* leave extracts was determined according to the procedure described by Yen et al. (2000). Extract (2.5 to 10.0 mg) was mixed with sodium phosphate buffer (5.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 ml, 1.0%); the mixture was incubated at 50°C for 20 min. Then 5 ml of 10% trichloroacetic acid was added and the mixture centrifuged at 980 g for 10 min at 5°C in a refrigerated centrifuge (CHM-17; Kokusan Denki, Tokyo, Japan). The upper layer of the solution (5.0 ml) was taken and diluted with 5.0 ml of distilled water and ferric chloride solution (1.0 ml, 0.1%), and absorbance was noted at 700 nm.

Evaluation of antimicrobial activity of the extracts

The *Zizyphus* leaves extracts were individually tested against a panel of microorganisms including three bacteria, *Escherichia coli, Pasturella multocida* and *Staphylococcus aureus* and four pathogenic fungi, *Aspergillus niger*, *Aspergillus flavus*, *Alternaria alternata* and *Ganoderma lucidum* obtained from the Department of Biochemistry, University of Agriculture Faisalabad, Pakistan. Bacterial strains were cultured overnight at 37°C in nutrient agar (Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 30°C using potato dextrose agar (Oxoid).

Disc diffusion method

The antimicrobial activity of the *Zizyphus* leave extracts was determined by disc diffusion method (NCCLS, 2004). The discs (6 mm in diameter) were soaked with 50 µl extract and placed on the inoculated agar. To compare the activity with standard antibiotics, *Rifampicin* (30 µg/disc) (Oxoid) and *Fluconazol* (30 µg/disc) (Oxoid) were used as positive reference for bacteria and fungi, respectively. Disc without samples were used as a negative control. Test discs and standard disc were placed in separate Petri dishes. Petri dishes than incubated at 37°C for 24 h for bacterial and 25°C for 3 days for fungal growth. Antimicrobial activity was evaluated by measuring the inhibition zone (mm).

Microdilution broth method

For calculation of minimum inhibitory concentration (MIC), which represents the concentration that completely inhibits the growth of microorganisms, a microdilution broth susceptibility assay was used (NCCLS, 2004). A series of dilutions of leave extracts was prepared in a 96-well microliter plate, including one growth control and one sterility control 160 µl NB and SDB for bacteria and fungi, respectively, were added onto the micro plates with 20 µl of the tested solution. Then, 20 µl 5 × 105 CFU/ml (confirmed by viable count) of standard microorganism suspension was inoculated onto the micro plates.

These plates were incubated at 37°C for 24 h for bacteria and 28°C for 48 h for fungi. *Rifampicin* was used as a reference compound for antibacterial and *fluconazole* for antifungal activities. The growth was indicated by the presence of a white ‘pellet’ on

Table 1. Percentage yield (g/100 g DW) of methanolic extracts from leaves of different species of *Zizyphus*.

Species	Yield g/ 100 g DW		
	Young leaves	Mature leaves	mean
Z. spina-christi	$21.0 \pm 0.3^{b}{}_{b}$	$23.7 \pm 1.81^{a}{}_{c}$	22.3^{c}
Z. jujuba mill	$26.0 \pm 0.25^{a}{}_{a}$	$21.4 \pm 0.88^{b}{}_{b}$	23.7^{b}
Z. mauritiana	$27.0 \pm 0.57^{a}{}_{a}$	$24.1 \pm 0.20^{b}{}_{a}$	25.5^{a}
Z. lotus	$26.8 \pm 0.82^{a}{}_{a}$	$22.5 \pm 0.23^{b}{}_{b}$	24.1^{ab}
Z. vulgaris	$19.6 \pm 0.56^{b}{}_{a}$	$24.2 \pm 1.01^{a}{}_{a}$	21.9^{d}

Values are mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$. Superscript alphabets within the same rows indicate significant difference ($p < 0.05$) between maturity stages. Subscript alphabets within the same column depicted significant difference among different species of *Zizyphus.*

the well bottom. Then for results calculation were performed in mg/ml.

Statistical analysis

Three samples of each *Zizyphus* species were assayed. Each sample was analyzed individually in triplicate and data was reported as mean (n = 3 × 3) ± SD (n = 3 × 3). Data were analyzed using two way analysis of variance (ANOVA), and Minitab 2000 version 13.2 statistical Software at 5% significance level.

RESULTS AND DISCUSSION

Percentage yield of extracts

The quantity of the desired components that can be extracted from a plant depends on the polarity and amount of the solvent as well as contact time between solvent and plant material (Hsu and Coupar, 2006). Due to excellent solubility potential for antioxidant components, the methanol was used for the extraction of antioxidant compounds from *Zizyphus* leaves. Data for the percentage yield of methanolic (80%) extracts from young and mature leaves of different species of *Zizyphus* shown in Table 1 depicted that the maximum percentage yield was obtained from *Z. mauritiana* followed by *Z. lotus* > *Z. jujuba* mill > *Z. vulgaris* > *Z. spina* christi*.* Results showed significant variation ($P < 0.05$) for percentage yield among species.

Yield of the extracts (g/100 g of DW) from leaves of *Zizyphus* species, ranged 19.65 to 27.04% and 21.48 - 24.2% for young and mature leaves, respectively. From the results, it was cleared that yield of the extracts was greater for young leaves as compared to mature leaves. This variation in the extract yield might be due to the fact that in young leaves, there is greater production of carbon rich secondary compounds like phenols as compared to mature leaves. On the other hand, lesser yields obtained from mature leaves could be justified on the fact that during maturation process large amount of carbon is utilized and production of carbon rich compounds is declined and yield of soluble phytochemicals and antioxidants in methanol also decreased (Riipi et al., 2002).

Total phenolic contents

Determination of total phenolic contents in *Zizyphus* leaves extracts was done by Folin-Ciocalteau method (Chaovanalikit and Wrolstad, 2004) and the results were reported as gallic acid equivalent (GAE). Total phenolic contents were found to be ranged 19.0 to 28.2 and 19.0 to 25.0 mg GAE/g for young and mature leaves, respectively (Table 2). Effect of maturity on the TPC of *Zizyphus* leaves was significant ($p < 0.05$). Results depicted that, TPC of young leaves extracts were found to be generally higher as compared to mature leaves. Results of species means showed that maximum amount of TPC was obtained from leaves of *Z. mauritiana* followed by *Z. vulgaris* > *Z. spina-christi* > *Z. lotus* > *Z. jujuba* mill. No previous reports are available regarding total phenolic contents of *Zizyphus* leaves; however, Kamiloglu et al. (2009) determined total phenolic contents in the fruits of two genotypes of *Z. jujuba* mill. The highest total phenolic content was observed in MHS 6 and MHS7 genotypes (42 and 40 mg gallic acid equivalent (GAE)/g dry weight (DW), while the lowest content was found in MHS 5 and MHS 14 (28 and 25 mg GAE/ g DW). Jain et al. (2011) investigated the pharmacognostic and phytochemical properties of the leaves of *Zizyphus xylopyrus* (retz) wild, phenolic contents were observed in water and ethanol extracts and also in powdered form. Olajuyigbe et al. (2011) determined the phenolic content and antioxidant property of the bark extracts of *Ziziphus mucronata* willd. subsp. The values of total phenolic contents varied from 24.72 to 31.96 mg GAE/100 g dry weight of plant material. These findings are comparable to present results for different species. Choi et al. (2011) measured

Table 2. Total phenolic contents (GAE mg/g of DW) and total flavonoids contents (CE mg/g DW) of the leaves of different species of *Zizyphus*.

Species	TPC			TFC		
	Young leaves	Mature leaves	Mean	Young leaves	Mature leaves	Mean
Z. jujube mill	$23.8 \pm 0.58^{a}{}_{c}$	$19.0 \pm 0.48^{b}{}_{e}$	21.4^{d}	$61.8 \pm 0.69^{a}{}_{a}$	$50.3 \pm 0.63^{b}{}_{a}$	56.1^{a}
Z. spina-christi	$28.2 \pm 0.27^{a}{}_{a}$	$20.1 \pm 0.17^{b}{}_{d}$	24.15^{b}	$38.1 \pm 0.21^{a}{}_{e}$	$33.6 \pm 0.42^{b}{}_{e}$	35.9^{a}
Z. mauritiana	$26.1 \pm 0.56^{a}{}_{b}$	$24.0 \pm 0.45^{b}{}_{b}$	25.0^{a}	$58.3 \pm 0.65^{a}{}_{b}$	$46.7 \pm 0.70^{b}{}_{b}$	52.5^{b}
Z. lotus	$19.0 \pm 0.56^{b}{}_{d}$	$25.1 \pm 0.39^{a}{}_{a}$	22.0^{d}	$53.8 \pm 0.72^{a}{}_{c}$	$45.0 \pm 0.46^{b}{}_{c}$	49.4^{c}
Z. vulgaris	$26.1 \pm 0.21^{a}{}_{b}$	$22.9 \pm 0.66^{b}{}_{c}$	24.5^{b}	$48.5 \pm 0.50^{a}{}_{d}$	$43.3 \pm 0.57^{b}{}_{d}$	45.9^{d}

Values are mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$. Superscripts alphabets within the same rows indicate significant difference ($P < 0.05$) between two maturity stages while subscripts alphabets within the same column depicted significant difference among different species of *Zizyphus.*

free amino acid, individual phenolic, and total phenolic content, and antioxidative activities in three *jujuba* fruit pulp extracts from *Boeun-deachu, Mechu, and Sanzoin* cultivars and two seed extracts (*Mechu and Sanzoin*) from plants grown in Korea. Total phenolic content ranged from 1.1 to 2.4 g/100 g in the pulp and from 3.6 to 4.6 g/100 g in the seed. Chutichudet and Kaewsit (2011) also determined the phenolic contents in the leaves of *lettuce cv. Grand Rapids*. Maximum contents (2439.72 mg/100 g) were found at the early development stages of leaves afterwards level of phenolic decreased with the maturation of leaves (964.49 mg/100 g). Nayeem and Karvekar (2010) also reported that phenolic acid contents of the frontal leaves of *Tectona grandis* extract were higher (26 µg/g) than the mature leaves (17 µg/g). Decreasing trend of phenolic contents with maturity in our present study supported with previous reports revealed that the concentration of phenolic acids is sensitive to maturity stage. This is also supported by the findings of Sponas and Wrolstad (1990) who claimed that changes in the phenolic contents of pear depends mainly on level of maturity.

The fact that young leaves contain much higher contents of TPC compared with mature leaves may indicate that young leaves and branchlets are experiencing more intense selective pressure than mature and in turn more synthesis of plant secondary metabolites having defensive potential, that is, phenolics (Zhang et al., 2009). The decrease in phenolics content in mature leaves may reflect an active turnover of phenolics and an increase in bound or non extractable phenolics during senescence (Riipi et al., 2002).

Total flavonoid contents

Total flavonoids contents (TFC) of different species of *Zizyphus* are shown in Table 2, the results are reported as catechin equivalent (CE). Total flavonoid contents of leaves were found to be ranged 38.1 to 61.8 and 33.6 to 50.3 g/100 g for young and mature leaves, respectively. Effect of maturity on the TFC of *Zizyphus* leaves was significant ($p < 0.05$). Also significant ($p < 0.05$) variation in total flavonoid contents of leaves of different species was observed. The amount of total flavonoids in young leaves from all *Zizyphus* species was found to be higher than the mature one. Results of species means showed that maximum amount of TFC was obtained from the leaves of *Z. spina-christi* followed by *Z. mauritiana* > *Z. lotus* > *Z. vulgaris* > *Z. jujuba mill.* The nature and the quantity of phytochemicals vary and depend on the stages of maturation. This is evidenced by the difference in the amount of flavonoids in the mature (9.2 µg/g) and the frontal leaves (15.07 µg/g) of the methanolic extracts of *Tectona Grandis* (Nayeem and Karvekar, 2010). Veerash and Kambhoja (2011) determined the anthelmintic activity of *Z. jujuba* mill and lamk, and stated that the active principle responsible for anthelmintic activity is due to the presence of flavonoids, steroids and tannins in the extracts. Washid and Ameeta (2011) also confirmed that the ethanolic extracts of *Z. xylopyrus* (Retz.)Willd contains major phytochemicals viz. phenolics, flavonoids, tannins, saponins, and traces of alkaloids. Olajuyigbe et al. (2011) investigated the phenolic content and antioxidant property of the bark extracts of *Z. mucronata* Willd. subsp. The flavonoid contents values ranged from 4.80 to 9.02 mg QE/100 g of dry plant material which are much lower than in the leaves of *Zizyphus* species investigated in the present study. Choi et al. (2011) measured antioxidative activities in three *jujuba* fruit pulp extracts from *Boeun-deachu, Mechu*, and *Sanzoin* cultivars and two seed extracts (*Mechu and Sanzoin)* from plants grown in Korea. Flavonoids were measured and ranged from 0.7 to 1.8 in the pulp and from 3.2 to 4.0 in the seed.

DPPH radical scavenging activity

The antioxidant activities of plant phytochemicals also

Table 3. DPPH radical scavenging activity (%) and inhibition of linoleic acid peroxidation activity (%) of the leaves of different species of *Zizyphus*.

Species	DPPH radical scavenging activity (%)			Inhibition of linoleic acid peroxiodation (%)		
	Young leaves	Mature leaves	Mean	Young leaves	Mature leaves	Mean
Z. jujube mill	$70.2 \pm 0.27^{a}{}_{d}$	$57.1 \pm 0.21^{b}{}_{b}$	63.6^{c}	$37.6 \pm 0.36^{b}{}_{b}$	$40.3 \pm 0.18^{a}{}_{c}$	38.9^{b}
Z. spina christi	$82.6 \pm 0.23^{a}{}_{e}$	$52.9 \pm 0.40^{b}{}_{a}$	62.1^{d}	$21.3 \pm 0.33^{b}{}_{d}$	$45.2 \pm 0.20^{a}{}_{b}$	33.2^{c}
Z. mauritiana	$63.9 \pm 0.22^{a}{}_{b}$	$59.1 \pm 0.22^{b}{}_{e}$	61.5^{e}	$20.2 \pm 0.30^{b}{}_{e}$	$26.2 \pm 0.26^{a}{}_{e}$	23.2^{e}
Z. lotus	$61.4 \pm 0.18^{b}{}_{c}$	$68.9 \pm 0.17^{a}{}_{c}$	71.9^{a}	$54.6 \pm 0.28^{b}{}_{a}$	$63.3 \pm 0.16^{a}{}_{a}$	58.9^{a}
Z. vulgaris	$70.2 \pm 0.70^{a}{}_{a}$	$68.3 \pm 0.35^{b}{}_{d}$	69.2^{b}	$23.3 \pm 0.29^{a}{}_{c}$	$39.6 \pm 0.30^{b}{}_{d}$	31.4^{d}

Values are mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$. Superscript alphabets within the same rows indicate significant difference ($p < 0.05$) between two maturity stages while subscript alphabets with in the same column depicted significant difference among different species of *Zizyphus.*

happens by preventing the production of free radicals or by neutralizing/scavenging free radicals produced in the body or reducing/chelating the transition metal (Amic et al., 2003; Oboh et al., 2007). Prevention of the chain initiation step by scavenging various reactive species is considered an important antioxidant mode of action (Dastmalchi et al., 2007).

DPPH radical scavenging activity of leaves extracts of different species of *Zizyphus* is shown in Table 3. Both young and mature leaves of different species of *Zizyphus* exhibited appreciable scavenging activity ranging from 61.4 – 82.6% and 52.9 – 68.9%, respectively. Results of species means depicted that maximum DPPH radical scavenging activity was obtained from leaves of *Z. lotus* followed by *Z. vulgaris* > *Z. jujuba* mill > *Z. spina-christi* > *Z. mauritiana*. Effect of maturity on the scavenging activity of *zizyphus* leaves was significant ($P < 0.05$). Also significant difference ($P < 0.05$) in radical scavenging activity of different species was observed. The decrease in the radical scavenging ability with maturity might be due to reduction in phenolic contents. Comparable results to our findings have been offered by methanolic extract of *Lantana camara* (L) leaves (Bakhta and Ganjewala, 2009). The DPPH scavenging ability of aqueous and methanolic extract of ripe and unripe pepper fruit (UPF) revealed that UPF had higher free radical scavenging ability than RPF (Adedayo et al., 2010). Olajuyigbe et al. (2011) determined the phenolic content and antioxidant property of the bark extracts of *Ziziphus mucronata* Willd. subsp. The alcoholic and aqueous extracts of *Z. mucronata* stem bark exhibited concentration dependent antiradical activity by inhibiting DPPH radical with inhibitory concentration 50% (IC_{50}) values of 0.0646 mg/ml (aqueous), 0.0482 mg/ml (acetone) and 0.0422 mg/ml (ethanol) while those of the standards were 0.0406 mg/ml (BHT) and 0.0411 mg/ml (vitamin C). In the order of activity, ethanol had the stronger antioxidant activity (0.0422 mg/ml), followed by acetone extract while the least activity was obtained from aqueous extract. However, all the extracts exhibited significant DPPH free radical scavenging activity comparable to our results obtained from leaves extracts (ethanol) of five different species of *Zizyphus.*

Antioxidant activity in linoleic acid system

The antioxidant activity of leave extracts of different species of *Zizyphus* in linoleic acid model system was also assessed as ability to prevent from oxidation. Table 3 depicted the inhibition of peroxidation of linoleic acid after incubation period of 360 h (15 days). BHT was used as positive control to compare the antioxidant activity of leave extracts.

Results of species means showed that maximum activity was offered by the leaves of *Z. lotus* followed by *Z. jujuba mill* > *Z. spina-christi* > *Z. vulgaris* > *Z. mauritiana.* All the extracts of young and mature leaves of different species exhibited appreciable inhibition of peroxidation ranging from 20.2 - 54.6% and 26.2- 45.2% for young and mature leaves, respectively. Effect of maturity on the peroxidation of linoleic acid of *Zizyphus* leaves was significant ($P < 0.05$). In our study, although mature *Zizyphus* leaves have lesser amount of phenolic contents, however greater antioxidant ability, it might be due to fact that mature leaves provides more photosynthetic area and they have to face greater environmental stress and resistance to diseases and loss of pigmentation, so their allocation to defense or antioxidant ability is increased (Riipi et al., 2002). Inhibition of peroxidation in mature leaves might be due to more activity of peroxidase enzyme as compared to young leaves (Sairam et al., 2003).

Reducing power of extracts

Reducing power is a novel antioxidative defense mean and mechanism responsible for this property is by electron transfer or hydrogen atom transfer (Dastmalchi

Table 4. Reducing power of the leaves of different species of *Zizyphus* (absorbance at λ = 700 nm).

Leaves	Conc. mg/ml	*Z. jujuba mill*	*Z. spina-christi*	*Z. mauritiana*	*Z. lotus*	*Z. vulgaris*
Young leaves	0.1	$0.32 \pm 0.03^{d}{}_{d}$	$0.92 \pm 0.04^{b}{}_{b}$	$1.24 \pm 0.02^{a}{}_{a}$	$0.83 \pm 0.04^{c}{}_{c}$	$0.23 \pm 0.02^{e}{}_{e}$
	0.2	$0.44 \pm 0.01^{c}{}_{c}$	$1.22 \pm 0.02^{a}{}_{a}$	$1.25 \pm 0.02^{a}{}_{a}$	$1.10 \pm 0.03^{b}{}_{b}$	$0.26 \pm 0.02^{d}{}_{d}$
	0.3	$0.61 \pm 0.02^{d}{}_{d}$	$1.15 \pm 0.01^{c}{}_{c}$	$1.52 \pm 0.03^{a}{}_{a}$	$1.42 \pm 0.03^{b}{}_{b}$	$0.37 \pm 0.01^{e}{}_{e}$
	0.4	$0.78 \pm 0.02^{d}{}_{d}$	$1.34 \pm 0.01^{b}{}_{b}$	$2.01 \pm 0.03^{a}{}_{a}$	$1.65 \pm 0.02^{c}{}_{c}$	$0.53 \pm 0.01^{e}{}_{e}$
Mature leaves	0.1	$0.51 \pm 0.01^{d}{}_{d}$	$0.97 \pm 0.03^{b}{}_{b}$	$1.04 \pm 0.01^{a}{}_{a}$	$0.60 \pm 0.02^{c}{}_{c}$	$0.1 \pm 0.01^{d}{}_{d}$
	0.2	$0.68 \pm 0.01^{d}{}_{d}$	$1.01 \pm 0.04^{b}{}_{b}$	$1.50 \pm 0.02^{a}{}_{a}$	$0.85 \pm 0.03^{c}{}_{c}$	$0.22 \pm 0.02^{e}{}_{e}$
	0.3	$0.73 \pm 0.02^{d}{}_{d}$	$1.35 \pm 0.02^{b}{}_{b}$	$1.78 \pm 0.30^{a}{}_{a}$	$1.11 \pm 0.04^{c}{}_{c}$	$0.3 \pm 0.04^{e}{}_{e}$
	0.4	$0.92 \pm 0.03^{d}{}_{d}$	$1.59 \pm 0.03^{b}{}_{b}$	$1.88 \pm 0.03^{a}{}_{a}$	$1.21 \pm 0.02^{c}{}_{c}$	$0.47 \pm 0.02^{e}{}_{e}$

Values are mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$. Superscript alphabets within the rows indicate significant difference ($p < 0.05$) between two maturity stages while subscript alphabets within the same column depicted significant difference among different species of *Zizyphus*.

et al., 2007). Reducing power of different species of *Zizyphus* is shown in Table 4. The reducing potential was measured over a concentration range of 0.1 to 0.4 mg/ml. Results showed general increase in activity when concentration increased. Reducing potential of different extracts ranged 0.23 to 2.01 and 0.22 to 1.88 for young and mature leaves, respectively. The effect of maturity stage and species difference on antioxidant potential was significant ($P < 0.05$).

Results depicted that maximum reducing potential was demonstrated by young and mature leaves of *Z. mauritiana*. Overall results showed that reducing power of young leaves was greater as compared to mature. This trend was in accordance the earlier trend of TPC, TFC and DPPH scavenging activity. No previous reports are available about the reducing potential of *Zizyphus* leave however; Adedayo et al. (2010) reported that unripe pepper fruit (UPF) showed a higher reducing power than ripe fruit. The physiological changes that accompanied ripening or maturation brings about such change in phytochemistry that would decrease the antioxidant activities of mature leaves. Moreover, loss of some antioxidant constituents during maturation, results in decreased reducing power of mature leaves (Adedayo et al., 2010). Olajuyigbe et al. (2011) determined the phenolic content and antioxidant property of the bark extracts of *Z. mucronata* Willd. subsp. The reducing ability of the extracts showed a dose-dependent trend increasing with increases in the concentrations of the extracts. While highest reducing ability was observed at the highest concentration of each of the extracts, significant differences existed between the reducing ability of each of the extracts. Of the three extracts, acetone extract exhibited the highest reducing capability (0.454 ± 0.001) at the highest concentration. This was followed by (0.421 ± 0.002) ethanol extract while aqueous extract showed 0.392 ± 0.002. These values are lower than our observed values in other *Zizyphus* species.

Antimicrobial activity

Dubey et al. (2011) performed standardization of leaves of *Ziziphus nummularia* Linn, concluded that the selected species of *Ziziphus* contains various active phytoconstituents which was confirmed by preliminary phytochemical screening.

Antibacterial activity measured by disc diffusion method

The antibacterial activity of leave extracts of different species of *Zizyphus* was determined by disc diffusion method against *E. coli, S. aureus* and *P. multocida* strains. The methanolic extracts were applied on bacterial strains and the zones of inhibition (mm) were measured by zone reader (Table 5). From the data, it was accomplished that methanolic extract of young leaves of *Z. mauritiana* showed strong activity against *S. aureus* (23 mm zone of inhibition) while methanolic extract of young leaves of *Z. lotus and Z. jujuba* mill showed moderate activity against *E. coli* (20 mm zone of inhibition) and *P. multocida* (19 mm zone of inhibition). Overall *Zizyphus* species showed moderate to strong activity against all of the three bacterial strains.

Al-Reza et al. (2010) used methanol extract (300 µg/disc) of *Z. jujuba* which displayed a remarkable antibacterial activity against *S. aureus*, *Listeria monocytogenes*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *E. coli*. Methanol and acetone leaf extracts of *Z. mauritiana* which showed antibacterial effects against *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., *Proteus vulgaris* and *B. subtilis* (Chowdary and Padashetty, 2000). Eiznhamer and Xu (2004) reported antibacterial properties of *Z. jujuba* and inhibition of the growth of both *Staphylococcus aureus and Escherichia coli.*

Table 5. Antibacterial and antifungal activities of Leave extracts of different species of *Zizyphus*.

Microorganisms		*Z. Jujuba* Mill	*Z. spina christi*	*Z. mauritiana*	*Z. lotus*	*Z. vulgaris*
(Bacteria)				**Zone of inhibition (mm)**		
E. coli	Young leaves	20±0.04 $^{b}_{b}$	19±0.09 $^{a}_{a}$	19±0.02 $^{a}_{a}$	20±0.01 $^{a}_{a}$	13±0.01 $^{c}_{c}$
P. multocida		19±0.09 $^{a}_{a}$	15±0.01 $^{c}_{c}$	18±0.09 $^{b}_{b}$	19±0.04 $^{c}_{c}$	15±0.01 $^{c}_{c}$
S. aureus		18±0.01 $^{c}_{c}$	13±0.02 $^{d}_{d}$	23±0.04 $^{a}_{a}$	21±0.02 $^{b}_{b}$	19±0.09 $^{c}_{c}$
E. coli	Mature Leaves	20±0.02 $^{b}_{b}$	15±0.01	18±0.01 $^{c}_{c}$	22±0.02 $^{a}_{a}$	20±0.03 $^{b}_{b}$
P. multocida		16±0.04 $^{b}_{b}$	12±0.01 $^{c}_{c}$	12±0.04 $^{c}_{c}$	18±0.03 $^{a}_{a}$	12±0.03 $^{c}_{c}$
S. aureus		15±0.02 $^{c}_{c}$	17±0.09 $^{b}_{b}$	20±0.03 $^{a}_{a}$	18±0.04 $^{b}_{b}$	12±0.09 $^{d}_{d}$
(**Fungi**)				**Zone of inhibition (mm)**		
G. Lucidum	Young leaves	22±0.02 $^{a}_{a}$	15±0.04 $^{b}_{b}$	17±0.02 $^{b}_{b}$	22±0.04 $^{a}_{a}$	15±0.07 $^{b}_{b}$
A. flavus		15±0.03 $^{a}_{a}$	16±0.02 $^{a}_{a}$	11±0.04 $^{b}_{b}$	15±0.09 $^{b}_{b}$	12±0.07 $^{b}_{b}$
A. niger		13±0.09 $^{b}_{b}$	15±0.04 $^{a}_{a}$	14±0.07 $^{a}_{a}$	15±0.07 $^{a}_{a}$	10±0.02 $^{b}_{b}$
A. alternate		17±0.04 $^{c}_{c}$	20±0.07 $^{b}_{b}$	22±0.07 $^{b}_{b}$	26±0.02 $^{a}_{a}$	18±0.01 $^{c}_{c}$
G. Lucidum	Mature leaves	19±0.03 $^{a}_{a}$	18±0.03 $^{a}_{a}$	20±0.07 $^{a}_{a}$	16±0.04 $^{b}_{b}$	17±0.03 $^{b}_{b}$
A. flavus		16±0.02 $^{a}_{a}$	11±0.09 $^{b}_{b}$	13±0.04 $^{b}_{b}$	12±0.02 $^{b}_{b}$	11±0.01 $^{b}_{b}$
A. niger		15±0.03 $^{b}_{b}$	17±0.03 $^{b}_{b}$	20±0.01 $^{a}_{a}$	16±0.01 $^{b}_{b}$	15±0.07 $^{b}_{b}$
A. alternate		16±0.09 $^{b}_{b}$	18±0.07 $^{a}_{a}$	20±0.06 $^{a}_{a}$	21±0.09 $^{a}_{a}$	19±0.01 $^{a}_{a}$

Values are mean ±SD of three samples analyzed individually in triplicate at $p < 0.05$. Superscript alphabets within the rows indicate significant difference (P <0.05) between two maturity stages while subscript alphabets within the same column depicted significant difference among different species of *Zizyphus.*

Antibacterial activity of *Ziziphus* species might be due to the presence of phytochemicals like flavonoids, glycosides, saponins, betulinic acid, ascorbic acid and phenolic acids. Alkaloids are strong antibacterial agents. Most important alkaloids found in *Zizyphus* leaves were frangufoline, coclaurine, isoboldine, norisoboldine, asimilobine, iusiphine and iusirine (Mahajan and Chopda, 2010). Nisar et al. (2010) performed biological screening of *Zizyphus oxyphylla* edgew leaves and results revealed that ethyl acetate fraction showed some good activity (16 mm zone of inhibition) against *B. subtilus* and (18 mm zone of inhibition) against *S. aureus.*

Antifungal activity of leave extracts of different species of *Zizyphus*

The antifungal activity of leaves extracts of different species of *Zizyphus* was determined by disc diffusion method against *A. flavus, A. niger, A. alternata* and *G. lucidum.* The zones of inhibition are presented in Table 5. From the data, it was concluded that methanolic extract of young leaves of *Z. lotus* showed strong activity against *A. alternata* (26 mm zone of inhibition) nearly equal to control (27 mm) while methanolic extracts of *Z. lotus and Z. Jujuba mill* showed moderate activity against *G. lucidum* (22 mm zone of inhibition) and *A. flavus* (15 mm zone of inhibition). It was observed that methanolic extracts of young leaves are best antifungal source. Mahesh and Satish (2008) reported antifungal activity of leaf extract of *Z. mauritiana* against *A. flavus*, *Dreschlera turcica* (11 mm) and *Fusarium verticillioides* (8 mm) which is comparable to our findings. Nisar et al. (2010) performed biological screening of *Z. oxyphylla* edgew leaves and results revealed that maximum antifungal activity (35%) was shown against *Microsporum canis* by crude, *n*-hexane and aqueous fraction.

Minimum inhibitory concentration (MIC)

MIC represents the minimum concentration that completely inhibits the growth of microorganisms. The accessibility of minimum inhibitory concentration (MIC, mg/ml ± SD) of leave extracts of different species of *Zizyphus,* a microdilution broth susceptibility assay was used, as reported by (NCCLS, 2004). Then for results, calculation was performed in mg/ml.

Minimum inhibitory concentration (mg/ml) against selected bacterial strains

From data given in the Table 6, it was concluded that against *S. aureus* lowest minimum inhibitory concentration (6.18 ± 0.06) was from young leaves extract of *Z. mauritiana,* against *P. multocida* (18.2 ± 0.01) from extract of young leaves of *Z. jujuba* mill and against *E. coli* (19.0 ± 0.01) from young leaves extract of *Z. lotus.*

Table 6. Minimum inhibitory concentration (mg/ml) of leave extracts of different *Zizyphus* species against selected bacterial and fungal strains.

Microorganisms	Leaves	*Z. Jujuba Mill*	*Z. spina christi*	*Z. mauritiana*	*Z. lotus*	*Z. vulgaris*
Bacteria		**Minimum inhibitory concentration (mg/ml)**				
E. coli	Young leaves	$97.9 \pm 0.04^{a}{}_{a}$	$47.0 \pm 0.09^{b}{}_{b}$	$24.0 \pm 0.02^{c}{}_{c}$	19.0 ± 0.01^{c}	$94.0 \pm 0.09^{a}{}_{a}$
P. multocida		$18.2 \pm 0.01^{c}{}_{c}$	$98.0 \pm 0.09^{a}{}_{a}$	$48.0 \pm 0.048^{b}{}_{b}$	49.0 ± 0.05^{b}	$20.0 \pm 0.02^{c}{}_{c}$
S. aureus		$47.9 \pm 0.05^{a}{}_{a}$	$24.4 \pm 0.02^{b}{}_{b}$	6.18 ± 0.06^{d}	48.5 ± 0.048^{a}	$12.7 \pm 0.01^{c}{}_{c}$
E. coli	Mature Leaves	$47.6 \pm 0.05^{b}{}_{b}$	$24.4 \pm 0.02^{c}{}_{c}$	$22.97 \pm 0.03^{c}{}_{c}$	$99.0 \pm 0.09^{a}{}_{a}$	20.2 ± 0.02^{c}
P. multocida		$93.9 \pm 0.09^{a}{}_{a}$	$24.4 \pm 0.02^{c}{}_{c}$	$49.5 \pm 0.049^{b}{}_{b}$	$23.0 \pm 0.02^{c}{}_{c}$	$20.4 \pm 0.02^{c}{}_{c}$
S. aureus		$6.0 \pm 0.06^{c}{}_{c}$	$12.2 \pm 0.01^{b}{}_{b}$	$23.6 \pm 0.02^{a}{}_{a}$	$6.0 \pm 0.06^{c}{}_{c}$	$25.4 \pm 0.03^{a}{}_{a}$
Fungi						
G. lucidum	Young leaves	9.5 ± 0.09^{b}	24.2 ± 0.02^{a}	10.6 ± 0.01^{b}	7.6 ± 0.07^{b}	24.2 ± 0.02^{a}
A. flavus		12.3 ± 0.01^{c}	16.2 ± 0.01^{c}	61.8 ± 0.04^{a}	48.0 ± 0.04^{b}	59.4 ± 0.05^{a}
A. niger		36.2 ± 0.06^{a}	17.2 ± 0.06^{b}	33.2 ± 0.01^{a}	17.2 ± 0.06^{b}	39.2 ± 0.02^{a}
A. alternata		51.2 ± 0.03^{a}	47.9 ± 0.04^{a}	30.5 ± 0.003^{b}	11.2 ± 0.01^{c}	49.5 ± 0.05^{a}
G. lucidum	Mature leaves	50.1 ± 0.05^{b}	52.6 ± 0.05^{b}	19.9 ± 0.01^{c}	71.22 ± 0.03^{a}	66.0 ± 0.02^{a}
A. flavus		47.2 ± 0.01^{b}	95.2 ± 0.09^{a}	55.2 ± 0.02^{b}	91.6 ± 0.09^{a}	93.3 ± 0.09^{a}
A. niger		31.5 ± 0.04^{a}	20.2 ± 0.05^{c}	16.2 ± 0.01^{d}	25.2 ± 0.01^{b}	31.0 ± 0.08^{a}
A. alternata		36.2 ± 0.04^{a}	33.2 ± 0.05^{b}	20.5 ± 0.01^{c}	19.2 ± 0.01^{c}	29.6 ± 0.08^{b}

Values are mean ± SD of three samples analyzed individually in triplicate at $p < 0.05$. Superscript alphabets within the rows indicate significant difference ($p < 0.05$) between two maturity stages while subscript alphabets with in the same column depicted significant difference among different species of *Zizyphus*.

Shahat et al. (2001) reported that the chloroform extract of *Z. spina-christi* leaves showed inhibitory and bactericidal activity against Gram-positive microorganisms such as *Streptococcus pyogenes* and a moderate activity against *Bacillus cereus.* An inhibitory activity against *S. pyogenes* was also showed by the petroleum ether extract of *Z. spina-christi* leaves. A marked inhibitory and bactericidal activity against *B. cereus* as well as an inhibitory effect against *S. aureus* and *S. pyogenes* was shown by the chloroform extract of *Z. spina-christi* seeds. Similarly, Ahmad et al. (2011) reported that n-hexane and ethyl alcohol fractions of *Z. jujuba* were significantly active against *B. pumalis*, *S. epidermidis*, *S. typhi* and *P. aeruginosa* with low values of MIC.

Minimum inhibitory concentration (mg/ml) against selected fungal strains

The minimum inhibitory concentration values of the *Zizyphus* extracts against the test organisms showed that fungi vary widely in the degree of their susceptibility to antifungal agents. From data given in the Table 6 it is clear that against *G. lucidum* and *A. alternata* lowest minimum inhibitory concentration (7.6 ± 0.07) and (11.2 ± 0.01), respectively was from young leaves extract of *Z. lotus;* against *A. flavus* (12.3 ± 0.01) was from young leaves extract of *Z. jujuba* mill; against *A. niger* (17.2 ± 0.06) was from young leaves extracts of *Z. lotus and Z. spina-christi.* From the above data of MIC it was concluded that methanolic extract of *Z. lotus, Z. spina-christi* and *Z. jujuba* mill leaves was potent for detailed studies against fungi. Shahat et al. (2001) reported that chloroform extract *of Z. spina-christi* leaves showed a moderate inhibitory concentration against the fungus *Trichphyton rubrum* (Tr) and low concentration (> 1) against *A. niger* and *Candida albicans*.

Conclusions

In this study, it was demonstrated that extracts from leaves of different *Zizyphus* species exhibited excellent antioxidant activity as measured by different antioxidant assays. An appreciable amount of TPC and TFC were found in analyzed young leaves extracts of different species of *Zizyphus* as compared to mature. Also young leaves exhibited efficient antioxidant and antimicrobial activity. The antimicrobial effects of the major pharmacological components present in the young leave extracts of different species of *Zizyphus* might accelerate the development of new drugs for various diseases caused by microorganisms. However, further research is

needed to identify individual components forming antioxidative and antimicrobial system, and develop their application for food and pharmaceutical industries.

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