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ANTIOXIDANT POTENTIAL OF *IMPATIENS BICOLOR* ROYLE AND *ZIZYPHUS OXYPHYLLA* EDGEW.

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Abstract

The present investigation has been carried out to evaluate the antioxidant capacity and phenolic composition of *Impatiens bicolor* Royle and *Zizyphus oxyphylla* Edgew. The content of phenolic compounds ranged from 15.77 to 27.61 mg catechin equivalents/g of different parts of *Zizyphus oxyphylla* Edgew., extract and 17.74 mg catechin equivalents/g for *Impatiens bicolor* Royle extract. The HPLC-ESI-MS/MS analysis of phenolic compounds showed that ferulic acid-hexosides was the only compound detected in *I. bicolor*, while *Z. oxyphylla* fruit, stem and leaves exhibited several compounds. Total antioxidant capacity values measured by TEAC assay were 46.32 ± 0.89, 42.56 ± 1.65, 41.34 ± 0.20, and 48.58 ± 0.21 µmol/g of extract, while those measured by FRAP assay were 102.40 ± 0.18, 207.54 ± 7.91, 254.89 ± 4.20, and 233.00 ± 9.07 µmol Fe^{2+}/g, for *I. bicolor* and *Z. oxyphylla* fruit, leaves and stem, respectively. TRAP values were 43.26 ± 1.27, 112.23 ± 0.00, 102.83 ± 1.66, and 117.37 ± 3.70 µmol/g of extract for *I. bicolor* and *Z. oxyphylla* fruit, leaves and stem respectively. The results indicate that these two plants may be a potential source of antioxidants.

Key words: *Impatiens bicolor* Royle, *Zizyphus oxyphylla* Edew, Antioxidant activity.

Introduction

Epidemiological studies have shown an inverse relationship between the consumption of certain fruits, vegetables, and other plant materials and the risk of many diseases, including cardiovascular and cancers (Gey, 1990). Most of these diseases are initiated by free radicals or reactive oxygen species (ROS), which are produced in human body as a result of aerobic metabolism and other normal processes. Living organisms have built in an antioxidant defense system to scavenge these ROS and free radicals and maintain their concentration within certain range. If, as a result of any stress, the production rate of ROS is so high that defense system is unable to scavenge ROS with the same rate, this state of unbalance between production and scavenging of ROS is called oxidative stress. To counteract this stage, antioxidants are required to be taken from external sources to decrease concentration of ROS in living systems. Previously, synthetic antioxidants like butylated hydroxyanisole and butylated hydroxyl toluene were used as food additives, but serious safety concerns about their usage compelled the researchers to explore some alternative potential sources of antioxidant, which may be safer, easily available from indigenous sources and from natural origin. As a result, a number of sources including seeds, fruits, vegetables and plants was explored and proven to be potential sources of antioxidants (Kaur & Kapoor, 2001; Velavan *et al.,* 2007).

Pakistan exhibits a wide range in vegetation and floristic composition making it a varietal emporium of medicinal plants, many of them are still unexplored. In the present investigation, we have screened *Impatiens bicolor* Royle and *Zizyphus oxyphylla* Edgew., for their potential as antioxidants. Some other species of *Impatients* have been used in management of various diseases, such as *I. balsamina* extract, showing a long lasting skin moisturizing effect and prevent dryness, rough skin chap, dandruff and split hair ends. Such herbs are used to prepare lotions, creams, hair tonics, cosmetics, bath preparations and detergents (Hasan & Tahir, 2005) while *Z. oxyphylla* is used in various condition of pain and fever. Both these plants are analyzed in the present study have been screened for other biological activities, such as antibacterial, antifungal, phytotoxic, cytotoxic and insecticidal activities (Nisar *et al.,* 2010 a, b, c; 2011). However no antioxidant investigation exists on these plant therefore these plants are studied for their antioxidant composition and capacity.

Material and Methods

Plant materials and extraction: The plant materials (fruit, stem and leaves) of *Zizyphus oxyphylla* Edgew., and *Impatiens bicolor Royle* (whole plant) were collected from Swat Valley (KPK, Pakistan). Voucher specimen were placed in the National Herbarium Islamabad with voucher no NH-012 (2004) and No.18-NH-4-008. The material was dried under shade, grinded to powder form, and extracted with 80% methanol. The methanolic extracts were filtered and evaporated under vacuum to obtain crude extracts, which were stored until used for analyses.

Chemicals: The 2, 2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were

procured from Sigma-Aldrich (St. Louis, MO, USA). R-Phycoerythrin (R-PE) was from Prozyme (San Leandro, CA, USA); 2,2-azobis (2-amidinopropane) dihydrochloride (ABAP) from Waco Chemicals (Richmond, VA, USA). All the chemicals and solvents used were HPLC grade and purchased from Carlo Erba (Milan, Italy). High-purity deinionized water was produced in the laboratory by using an Alpha-Q system (Millipore, Marlborough, MA, USA).

HPLC-ESI-MS/MS analysis of phenolic compounds: Extracts were subjected to Water 2695 Alliance separation module equipped with a Micromass Quattro Micro Api mass spectrometer fitted with an electrospray interface (ESI) (Waters, Milford, MA, USA) for the determination of phenolic compounds. Initially, MS Scan analysis was performed to study phenolic profiles of the extracts. System was operated in negative ion mode ranging over 100 to 1000 mass-to-charge ratio (m/z). Afterwards, MS scan data was used for development of Multiple Reaction Monitoring (MRM) methods. Reversed phase analytical column i.e. Waters Atlantis dC18 3 µm (2.1 × 150 mm) (Waters) was employed for separation. Flow rate was adjusted at 0.17 mL/min. The mobile phase used was 30 min linear gradient of acetonitrile (5 to 30% in 1% aqueous formic acid), followed by a 5-min washing of 80% acetonitrile followed by 8 min column re-equilibration at start conditions. The ESI source was kept in negative ionization mode. Source and desolvation temperatures were 120ºC and 350°C respectively, while voltages for capillary and cone were 2.8 kV and 35 V. Nitrogen at 750 L/h desolvation gas (N_2), cone gas (N_2) 50 L/h. The collision energy for MS/MS identifications was set at 30 eV, and the collision gas used was argon.

Determination of total antioxidant capacity (TAC): For determination of TAC of plant extracts, a weighed amount (50-130 mg) of extracts of both plants was dissolved in 10 mL of acidified methanol (1% formic acid). The extract samples were used for antioxidant capacity measurement and total phenol content. The extracts were kept at -20°C at dark prior to the analysis.

Determination of total phenolic content (TPC): Total phenolic content of each extract was determined by a previously described method (Singleton & Rossi, 1965). The extracts after oxidization with Folin-Ciocalteu reagent, were neutralized with Na_2CO_3 and absorbance of resulting blue color was measured at 760 nm. Data are expressed as mg catechin equivalents/g of plant extract.

TAC determination: Plant extracts were analyzed for their antioxidant capacity by three different TAC assays: Trolox equivalent antioxidant capacity (TEAC) assay (Pellegrini *et al.,* 2003), ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1999) and total radical-trapping antioxidant parameter (TRAP) assay (Ghiselli *et al.,* 1995). The TEAC and TRAP values are expressed as µmol Trolox equivalent/g of extract, FRAP values are expressed as µmol of Fe^{2+} equivalents per gram of extract.

Results and Discussion

The content of phenolic compounds ranged from 15.77 to 27.61 mg catechin equivalents/g of different parts of *Zizyphus oxyphylla* Edgew., extract and 17.74 mg catechin equivalents/g for *Impatiens bicolor* Royle extract (Table 1). The mass spectral characteristics of phenolic compounds tentatively identified in both species are reported in Table 2. The major phenolic compounds identified were mainly hydroxycinnamate derivatives, especially hexose esters of coumaric, caffeic, ferulic and sinapic acids, while only caffeic acid was recovered in free form. Instead, vanillic acid in hexose-esterified form was the only hydroxybenzoic acid detected in this study. Among flavonoids, kaempferol-rutinoside was the only compound detected. The Table 3 showed the phenolic compounds identified in the different extracts analysed. Ferulic acid-hexosides was the only compound detected in *I. bicolor*, while *Z. oxyphylla* fruit, stem and leaves exhibited several compounds with respect to *I. bicolor*. MRM chromatograms of caffeic acid-hexosides of *Z. oxyphylla* fruit is shown in Fig. 1. At least three isomers have been identified thanks to their spectrometric behavior. In detail, the hexose esters of caffeic acid were identified by molecular ion at m/z 341, with fragment ions at m/z 179 and 135, typical of caffeic acid. The Fig. 2 shows MRM chromatograms of sinapic acid-hexosides, where at least two isomers have been tentatively identified thanks to same molecular ion at m/z 385, besides their fragment ions at m/z 223 and 149, typical of sinapic acid.

Table 1. Total phenol content of plant extracts. Values are presented as mean value ± SD and expressed as mg catechin equivalents/g of plant extract.

Extract	Total phenol content (mg/g)
Impatiens bicolor Royle	17.74 ± 0.12
Zizyphus oxyphylla Edgew fruit	27.61 ± 0.18
Zizyphus oxyphylla Edgew leaves	15.77 ± 0.07
Zizyphus oxyphylla Edgew stem	17.49 ± 1.00

Table 2. Tentative identification of phenolic compounds based on their mass spectral characteristics.

No.	Compound	$[M-H]^-$ (m/z)	Qualifier ions (m/z)
1.	Caffeic acid	179	135
2.	Coumaric acid-hexosides	325	163, 119
3.	Caffeic acid-hexosides	341	179, 135
4.	Ferulic acid-hexosides	355	193, 134
5.	Vanillic acid-hexosides	329	167
6.	Sinapic acid-hexosides	385	223,149
7.	Kaemferol-rutinoside	593	285, 447

Table 3. Phenolic profile of plant extracts.

Extract	Phenolic compounds						
	1	2	3	4	5	6	7
Z. oxyphylla fruit	+	+	+		+		+
Z .oxyphylla leaves		+*			+*	+	+*
Z. oxyphylla stem		+	+*		+	+*	
I. bicolor Royle				+			

+: present; *: trace (present at limit of detection)

Table 4. Total antioxidant capacity of extracts. Values are presented as mean value ± SD.

Extracts	TEAC (µmol of Trolox/g)	FRAP (µmol of Fe^{2+}/g)	TRAP (µmol of Trolox /g)
Impatiens bicolor Royle	46.32 ± 0.89	102.40 ± 0.18	43.26 ± 1.27
Zizyphus oxyphylla Edgew fruit	42.56 ± 1.65	207.54 ± 7.91	112.23 ± 0.00
Zizyphus oxyphylla Edgew leaves	41.34 ± 0.20	254.89 ± 4.20	102.83 ± 1.66
Zizyphus oxyphylla Edgew stem	48.58 ± 0.21	233.00 ± 9.07	117.37 ± 3.70

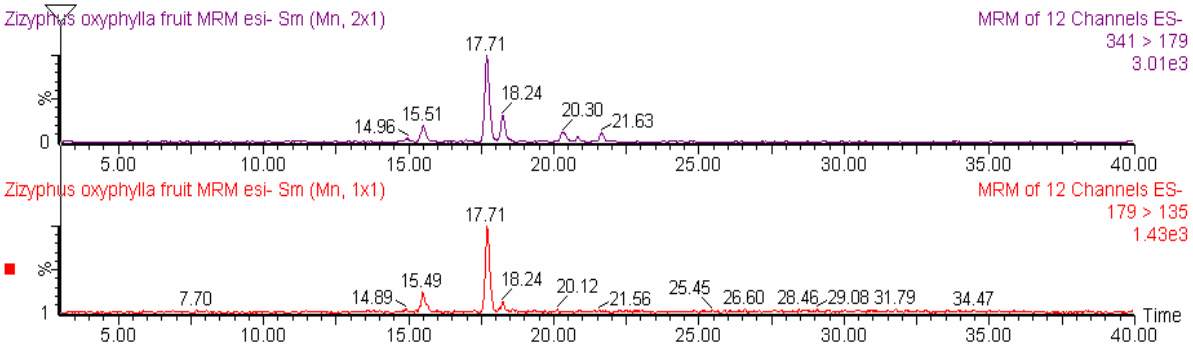

Fig. 1. MRM chromatograms of caffeic acid-hexosides. It is possible to note at least three isomers (at 15.51, 17.71 and 18.24 min.), identified through the loss of hexose moiety (341>179) and further fragmentation of caffeic acid (179>135).

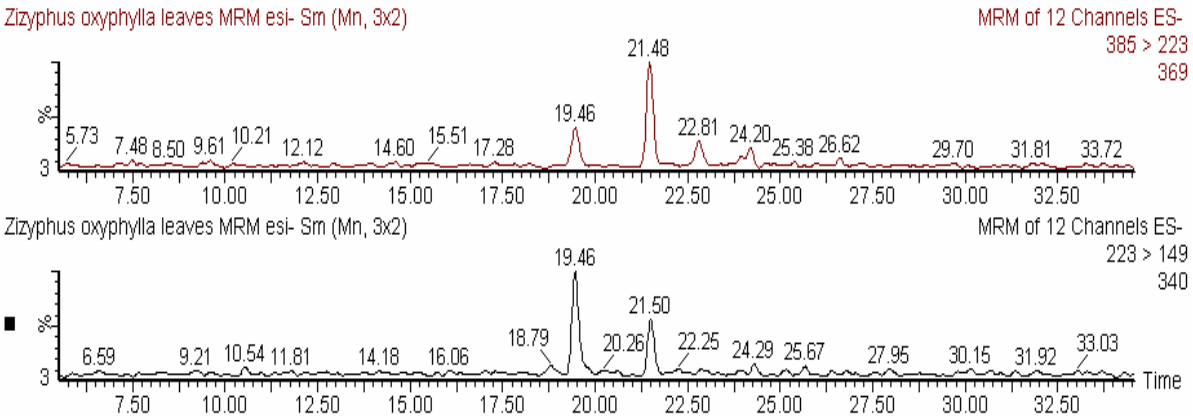

Fig. 2. MRM chromatograms of sinapic acid-hexosides. It is possible to note at least two isomers (at 19.46 and 21.48 min), identified through the loss of hexose moiety (385>223) and further fragmentation of sinapic acid (223>149).

In Table 4, the total antioxidant capacity of extracts is presented. Three parts of *Z. oxyphylla* fruit, stem and leaves have been screened separately for antioxidant activity because they have shown some difference in the extent of various pharmacological activities (Michel *et al.*, 2011). The TAC values measured by TEAC assay were 46.32 ± 0.89, 42.56 ± 1.65, 41.34 ± 0.20, and 48.58 ± 0. 21 µmol TE /g, while those obtained by FRAP assay were 102.40 ± 0.18, 207.54 ± 7.91, 254.89 ± 4.20, and 233.00 ± 9.07 µmol Fe^{2+}/g, for *I. bicolor* and *Z. oxyphylla* fruit, leaves and stem, respectively. Finally, TRAP values were 43.26 ± 1.27, 112.23 ± 0.00, 102.83 ± 1.66, and 117.37 ± 3.70 µmol TE/g for *I. bicolor* and *Z. oxyphylla* fruit, leaves and stem respectively.

Phenolic compounds are important constituents of many plants and have received considerable attention as potentially protective agents against cancer and heart diseases because of their antioxidant activity and their ubiquity in a wide range of commonly consumed foods of plants origin (Rice-Evans, 2001; Muselík *et al.,* 2007). Among different parts of *Z. oxyphylla*, the highest content of total phenols was found in fruit, which was almost double than in leaves and in general it was higher than that of many other fruits, such as podocarpus ones, explored as potent sources of antioxidants (Abdillahi *et al.*, 2011). No previous studies reported the total phenol content of *Z. oxyphylla*, while the TPC of *I. bicolor* found in this work was lowest than that previously reported (Shahwar *et al.,* 2010a,b). However data of total phenolic content of two plants analysed in the present study are higher than ones observed previously for some indigenous species explored (Rizwan *et al.,* 2012; Zia-ul-Haq *et al.,* 2011a,b).

As the antioxidant action in the human body is a very complex process and still its mechanistic details are unclear, therefore, it is generally recommended to determine the antioxidant capacity by different assays to get a more complete picture of the antioxidant activity. Thus, total antioxidant capacity of extracts was determined by three different assays. In particular, TRAP, FRAP, and TEAC were used to measure chain-breaking antioxidant power, the reducing capacity, and the quenching efficacy of extract. FRAP assay is generally preferred for its simplicity and reproducibility of results. All the extracts exhibited appreciable FRAP values (Table 4) comparable to previously explored potential sources (Zia-ul-Haq *et al.,* 2013 a,b,c,d).Scientific literature does not report the antioxidant capacity of *I. bicolor* and *Z. oxyphylla*, making difficult any comparison with other studies. However, based on our results *Z. oxyphylla* stem, although presented few phenolic compounds identified by HPLC-ESI-MS/MS analysis and lower TPC content with respect to other part of this plant, exhibited the higher TAC values for almost all the TAC assays applied. This could be due to the higher antioxidant activity of the individual phenolics present in this plant part.

Conclusion

The present results obtained by all three methods indicate a good potential antioxidant capacity of the investigated species. This report will be a valuable addition in the database of potential sources of antioxidants from medicinal plants.

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