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PHENOLIC ACIDS PROFILING AND ANTIOXIDANT POTENTIAL OF MULBERRY (*MORUS LAEVIGATA* W., *MORUS NIGRA* L., *MORUS ALBA* L.) LEAVES AND FRUITS GROWN IN PAKISTAN

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Mulberry trees are distributed throughout Pakistan. Besides its use in forage and food for animals, it is also used as herbal medicine. This study is carried out to determine phenolic acids profile, sugar content, and the antioxidant activity of the leaves and fruits of three mulberry species (*Morus alba* L., *Morus nigra* L. and *Morus laevigata* W.) grown in Pakistan. Dried fruits and leaves of three mulberry plant species were extracted by three different methods. The extracts were analysed by HPLC with diode array detection for phenolic acids. Antioxidant activity and sugar content were determined using spectrophotometric assays. The structure of chlorogenic acid was confirmed by LC-MS/MS analysis. The major phenolic acids were identified as gallic, protocatechuic, p-hydroxybenzoic, vanillic, chlorogenic, syringic, *p*-coumaric, ferulic and *m*-coumaric acids. Chlorogenic acid was the prominent phenolic acid in all leaves samples collected from three mulberry species and optimum extraction of chlorogenic acid was achieved using 80% aqueous methanol with ultrasonic-assisted extraction. Total phenolic compounds as determined by Folin-Ciocalteu assay were in the range of 3.89 to 11.79 mmol/ 100 g gallic acid equivalent, whereas antioxidant activities by DPPH assay ranged from 22.85–76.88 μmol/100 g quercetin equivalent. The concentration of sugars in different samples ranged between 36.56-82.15 mmol/ 100 g sucrose equivalent. This is the first report on identification of phenolic acids and antioxidant activity in the extracts of three cultivars of mulberry plants grown in Pakistan. This information will be of considerable value to the commercial producers of mulberry trees in the country.

INTRODUCTION

Recent interest in bioactive phenolic compounds have resulted in increased attention for the optimization of procedures for extraction of bioactive phytochemicals from different natural sources [Szajdek & Borowska, 2008; Naczk & Shahidi, 2005]. Several studies on some plants such as rosemary, sage, berries, ginkgo, and oregano have resulted in development of natural antioxidant formulations for food, cosmetic, dietary supplements, and other applications [Dillard & Bruce German, 2000]. As over 8,000 different phenolic phytochemicals have been documented in the literature, the task for accurate quantification of phenolic compounds is challenging and complex [Luthria, 2006b]. Phenolic compounds can be grouped into three broad categories: polyphenols, phenolic acids, and a miscellaneous group. Polyphenols can be arranged into two broad classes: tannins (condensed and hydrolysable) or flavonoids (flavones, flavonols, flavanones, flavanols, anthocyanidins). Similarly, phenolic acids can be further subdivided into two main subgroups, hydroxycinnamic and hydroxybenzoic acids and the miscellaneous group comprises lignans, coumarins, stilbenes, and other phenolic compounds not included in the other two subgroups [Luthria, 2006a,b]. Phenolic acids are known to exhibit significant biological activity [Robbins & Bean, 2004].

Morus plant species possess enormous importance in medicinal, economical, industrial, clinical, and domestic fields [Andreoni, 2005; Sánchez, 2000]. Work carried out on some selected species of this plant demonstrates bioactivities for which plant has been used as folk medicine, *e.g.* mulberry fruit juice has been used as folk remedies for tumors of fauces, aptha, asthma, cold, cough, diarrhea, dyspepsia, edema, fever, headache, hypertension, and wounds. Roots and bark are purgative, anthelminthic, astringent, while leaves of mulberry play vital role in rearing silkworm [Datta, 2000; Sánchez, 2000; Stalikas, 2007]. Its leaves and young bark are recognized as excellent animal feed and are healthy, pleasant and non-toxic, it enhances milk yield of dairy animals as well [Dillard & Bruce German, 2000]. Mulberry leaves are also used as a purpose-grown fodder for larger live stock [Sánchez, 2000]. It is reported that mulberry leaf contains moisture, carbohydrates, and proteins that are utilized by silkworm larvae [Absar *et al.*, 2005; Ghoush & Alam, 2003].

Mulberry tree is distributed worldwide with greater concentration in India and China [Datta, 2000; Sánchez, 2000]. In colder regions of Pakistan, such as Chitral, Quetta, Azad Jammu and Kashmir, growing conditions are suitable for cultivating mulberry, mainly *Morus alba* L., *Morus nigra* L., *Morus laevigata* W., and *Morus rubra* L., which are used for rearing

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silk worm, in promoting silk quality and quantity, improvement in productivity of cocoons and making solidified multinutrient feed blocks for live stock [Ginai, 1968; Habib, 2004; Mahmood & Jan, 2001; Younas *et al.*, 2004]. These mulberry species are also cultivated in a warmer region of the country. In Hyderabad Division-Pakistan (semi-arid zone), *M. laevigata* species is grown on large area as compared to others species. Moreover, many species of the family *Moraceae* have been studied enlightening the presence of phenolic compounds; however, the potential health benefits of *Morus* species grown in Pakistan have not been studied yet in detail. In *Morus* family, *M. alba* has been reported for the antioxidant potential measurements [Bae & Suh, 2007; Chu *et al*., 2006; Ercisli & Orhan, 2008; Katsube *et al.*, 2006; Yen *et al.*, 1996] while antioxidant properties of various solvent extracts of *M. indica* leaves grown in India [Arabshahi-Delouee & Urooj, 2007], chemical composition in terms of phenolic compounds, fatty acids, total suspended solids (TSS), ascorbic acid and mineral content of *M. alba*, *M. rubra,.* and *M. nigra* grown in the eastern region of Turkey are also reported. Du *et al*. [2003] have identified antiviral flavonoids from root bark of Morus alba L. while *M. atropupurea Roxb* is the only species investigated in detail by Isabelle *et al.* [2008].

Quantitative extraction of active constituents is an important step before analysis. Quantity of analyte extracted from different matrices depends on the type of matrix, extraction solvent composition, extraction techniques and its conditions [Stalikas, 2007]. Very little emphasis is usually given to the optimization of extraction conditions prior to analysis. However, with recent development of new automated high throughput extractors such as microwave-assisted extraction (MAE) and pressurized-liquid extraction (PLE), interest in bioactive phytochemicals, and wide variation in reported bioactivities and quantity of phytochemicals has resulted in increased attention to sample preparation techniques [Camel, 2001; Huie, 2002; Letellier & Budzinski, 1999; Luthria *et al.*, 2007; Ong, 2004; Tura & Robards, 2002].

In the present work, *Morus alba* L., *Morus nigra* L., and *Morus laevigata* W. leaves and fruits grown in Pakistan were separately assayed for antioxidant potential using DPPH scavenging ability, total phenolics by Folin-Ciocalteu (FC) method, total sugars by anthrone method and phenolic acids by high performance liquid chromatography. In addition, comparison of the influence of three commonly deployed extraction techniques (ultrasonic assisted extraction (UAE), magnetic stirring, and homogenization) was studied as well.

MATERIAL AND METHODS

Chemicals

All standards were purchased from Tokyo Chemical Industry Ltd. (Japan). Methanol (HPLC grade), ethanol, acetonitrile, ethylacetate and formic acid were purchased from Fischer Scientific (UK). Sodium dodecylsulfate (Fluka, Germany), cetyltrimethylammonium bromide (Acros Organics, USA), DPPH (MP Biomedicals Inc., France) were purchased from (Fluka, Switzerland). Folin-Ciocalteu reagent and sodium carbonate were purchased from (Merck, Germany). All chemicals were used as received.

Plant material

The leaves and fruits of three species of mulberry were included in this study. *M. laevigata* W. was collected from the National Center of Excellence in Analytical Chemistry, University of Sindh, Jamshoro, Pakistan and the other two species (*M. alba* L. and *M. nigra* L.) were gathered from Sindh Agriculture University, Tandojam, Hyderabad in Sindh, Pakistan.

Sample preparation

Fruit and leaves samples were stored at -4°C immediately after collection. Fresh fruit and leaves samples (25 g) of each mulberry species were lyophilized (CHRIST Alpha 1-4, Martin Christ, Germany) at -30°C. After drying the samples were ground and sieved through 0.3 μm mesh. The powdered dried samples were stored at -4°C until analysed.

Ultrasonic-assisted extraction

Freeze-dried mulberry samples (200 mg) were extracted with 10 mL of MeOH:H_2O (80:20 v/v) in a sonicator bath (Model No. SC 121 TH, Sonicor Instrument Corp., Copiague, NY, USA). Sonication was carried out at ambient temperature for 10 min. The mixture was centrifuged, solvent was decanted, and the residue was re-extracted with a fresh 10 mL solvent. The two extracts were combined, and the volume was adjusted to 20 mL with the extraction. Appropriate aliquots of extracts were filtered through a 0.45 μm Swinney syringe filter for the assay of phenolic compounds by the FC method and phenolic acids by the HPLC analysis. Same extracts were used for the measurement of scavenging capacity by DPPH.

Extraction using magnetic stirrer

Freeze-dried mulberry samples (200 mg) were weighed in a screw-cap glass vial fitted with a small magnetic bar (10 × 2 mm). The mixture was stirred with 10 mL of MeOH/H_2O (80:20 v/v) for ten minutes at ambient temperature. The mixture was centrifuged, supernant was decanted, and the residue was re-extracted with a fresh 10 mL of solvent for ten minutes. The mixture was worked up as described above and analysed by HPLC and FC methods.

Extraction using homogenization

Freeze-dried mulberry samples (200 mg) were weighed in a 100 mL beaker. The mixture was homogenized (Unipan, Type 302, Mechanika Precyzyjna, Poland) with 10 mL of MeOH/H_2O (80:20 v/v) at ambient temperature for 10 min. The mixture was centrifuged, solvent was decanted, and the residue was re-extracted with a fresh 10 mL of solvent. The mixture was worked up as described above and analysed by HPLC.

HPLC analysis of phenolic acids

The phenolic acids analysis was carried out in a Spectra system SCM 1000 (ThermoFinnigan, California, USA) liquid chromatograph equipped with a vacuum degasser and a DAD system. A Hypersil Gold C-18 (250 mm×4.6 mm, 5 μm) column (Thermo Corporation, USA) was used for separation. The mobile phase consisted of 0.1% formic acid in water (B) and methanol (A), a linear gradient of 5% (A) to 28%

(A) for 50 min followed by 28% (A) for 10 min was used. After 60 min, the mobile phase concentration was brought back to 5% (A) and held for 10 min for column equilibration. The flow rate was 0.7 mL/min while the injection volume was 20 µL. UV detection was performed at 270 nm. Software used for data acquisition and evaluation was Chromquest, Version 4.2. Identification of phenolic acids was based on retention time and UV spectrum with those of standards. The solutions of standards at various concentrations from 1 to 40 µg/mL were injected into the HPLC-DAD system and the calibration curves were established for each standard compound. The concentrations of the compounds were calculated from peak area according to calibration curves.

The presence of chlorogenic acid was verified by LC-MS (LCQ Advantage Max, Surveyor with quadruple and ion-trap system by ThermoFinnigan, California, USA) comprising a Surveyor MS Pump and an autosampler with 20 µL sample volume. LC was operated on similar conditions as mentioned for LC-UV but with the flow rate of 0.5 mL/min. MS was operated in ESI (negative ion) mode; needle voltage of 4.5 kV, probe temperature of 200°C, cone voltage of -10V, collision energy 25%, sheath gas flow rate of 40 arbitrary units, and auxiliary gas flow rate of 20 arbitrary units. Samples were run in SIM mode (for chlorogenic acid m/z 353-354) while MS-MS was operated in full scan mode. All the data was processed using Xcalibur software.

The DPPH radical-scavenging ability of plant extracts

The assay of DPPH radical-scavenging potential was carried out according to the reported method [Rubens & Wagner, 2004] with some modifications. Briefly, 2 mL of 0.1 mmol/L DPPH in methanol solution was added to 2 mL of plant extract (extracted by stirring) and then the mixture solution was placed in dark for 30 min. The quercetin standards (1-10 µmol) were treated in similar way and used as standard reference to measure the scavenging potential of sample. The absorbance was measured on Perkin Elmer lambda 35, UV/VIS Spectrophotometer at 517 nm for 0-30 mins with different time intervals. Constant absorbance at 30 min was used to construct a calibration graph. The amount equivalent to quercetin was calculated from the calibration graph using an equation of straight line $y = -0.0206$ µmol/L $+0.3563$ ($R^2 = 0.966$) and expression for concentration was converted to µmol/ 100 g of the sample.

Total phenolics by FC method

The total phenolic contents of mulberry species were determined using the FC reagent method [Iqbal *et al.*, 2005]. The reaction mixture contained 200 µL of diluted sample extracts, 800 µL of freshly prepared diluted FC reagent and 2 mL of 7.5% sodium carbonate. The final mixture was make-up to 7 mL with deionized water. Mixtures were kept in dark at ambient temperature for 2 h to complete the reaction. The absorbance at 765 nm was measured on a Perkin Elmer lambda 35, UV/VIS Spectrophotometer, with 1 cm cell. Gallic acid was used as a standard, the amount of phenolic compounds was calculated from a calibration graph $y = 0.0035$ ppm $+ 0.0248$ ($R^2 = 0.996$) and results were calculated as gallic acid equivalents (mmol/100 g) of mulberry species. The reaction was conducted in triplicate and results were averaged.

Total sugar contents

Anthrone method was used for the analysis of total sugars from mulberry leaves and fruits, according to the reported method [Sawhney & Singh, 1996] with some modifications. Briefly, 1 mL of each standard (as well as for sample extracts), was added to 1.5 mL of anthrone reagent (0.2% in H_2SO_4) and mixed thoroughly. The sample was placed in boiling water bath and brought to boiling. The solution was cooled to room temperature and absorbance was measured for each sample and standards at 620 nm. The formation of blue-green complex indicates the presence of total sugars. Sucrose was used as a standard for a calibration graph $y = 0.0145$ ppm $+ 0.0375$ ($R^2 = 0.9909$) and results were calculated as sucrose equivalents (mmol/100 g) of dried plant material.

RESULTS AND DISCUSSION

Extraction and analysis of phenolic acids

Chromatographic separation of sixteen phenolic acids was carried out with minor modifications to the procedure reported by Robbins & Bean [2004]. The minor variation was of change in mobile phase from 30% (B) 60% (A) to 28% (B) and 62% (A). Figure 1 shows the chromatogram of mixed standards with good resolution and sharp symmetrical peaks for all sixteen phenolic acids included in this study. Using this procedure, fruits and leaves of three different mulberry species were assayed for individual phenolic acids profiles (Figure 2). Eleven phenolic acids and their aldehyde derivatives namely, gallic, protocatechuic, protocatechuic aldehyde, *p*-hydroxybenzoic, vanillic, chlorogenic, syringic, syringealdehyde, *p*-coumaric, ferulic, *m*-coumaric were identified and quantified. Identification was done by comparing retention times and UV spectra of individual phenolic acids. The identification of chlorogenic acid, the major phenolic acid in leaf samples was carried out by LC-MS-MS analysis. Using the same optimized HPLC procedure, the peak at retention time 35.2 min produced a molecular ion peak of 353.5 (for caffeoylquinic acid) and for MS/MS fragmented peaks at m/z 191 (quinic

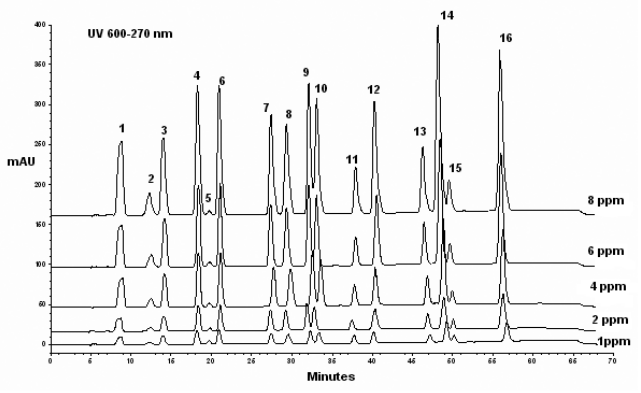

FIGURE 1. HPLC chromatograms of standard mixture composed of (1) Gallic acid, (2) 2, 4, 6-trihydroxy benzoic acid, (3) Protocatechuic acid, (4) Protocatechuic aldehyde, (5) Gentisic acid, (6) *p*-Hydroxybenzoic acid, (7) Vanillic acid, (8) Chlorogenic acid, (9) Vanillin, (10) Syringic acid, (11) Syringealdehyde, (12) *p*-Coumaric acid, (13) Ferulic acid, (14) *m*-Coumaric acid, (15) Sinapic acid, and (16) *o*-Hydroxycinamic acid. Conditions: as in the text.

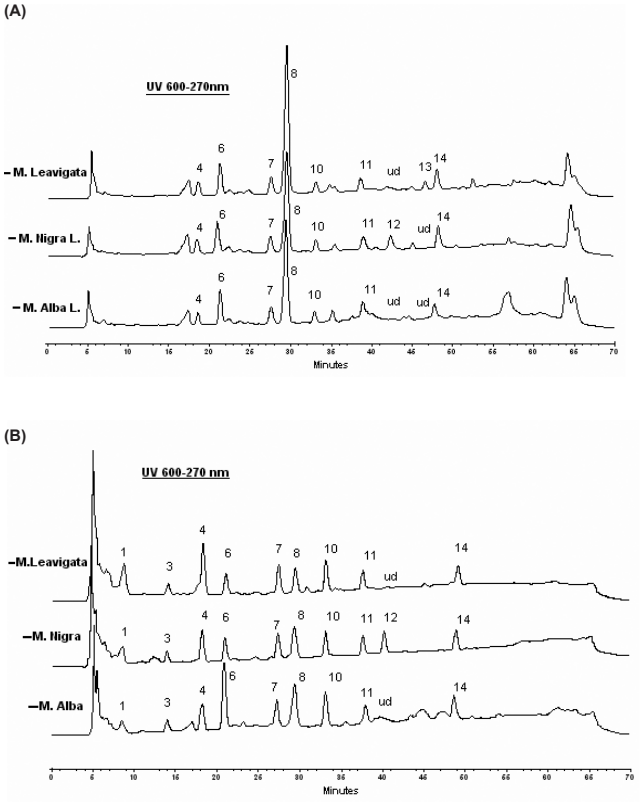

FIGURE 2. HPLC chromatograms of leaves (A): identified peaks are: Protocatechuic aldehyde (4), *p*- Hydroxybenzoic acid (6), Vanillic acid (7), Chlorogenic acid (8), Syringic acid (10), Syringealdehyde (11), *p*-Coumaric acid (12), *m*-Coumaric acid (14); and of fruits (B): identified peaks: Gallic acid (1), Protocatechuic acid (3), Protocatechuic aldehyde (4), *p*- Hydroxybenzoic acid (6), Vanillic acid (7), Chlorogenic acid (8), Syringic acid (10), Syringealdehyde (11), *p*-Coumaric acid (12), and *m*-Coumaric acid (14).

acid) and 179 (caffeoyl moiety). This mass spectrum data for chlorogenic acid matches with the data reported in literature [Troung *et al*., 2007] and confirms its presence in mulberry samples.

The total phenolic acids in fruits and leaves samples of three mulberry species extracted by UAE varied from 71.7 mg/100 g to 184.3 mg/100 g of dried plant material (Table 1). The phenolic acid profiles varied with the type of the species and the matrix (leaves or fruits) analysed. Optimum total phenolic acids (184.3 mg/100 g) were obtained in *M. leavigata* leaves and minimum level (71.7 mg/100 g) was extracted from *M. leavigata* fruit. Chlorogenic acid was the predominant phenolic acid in all three mulberry species leaves samples and its abundance ranged between 60.5% to 67.2% of total detected and quantified phenolic acids. Chlorgenic acid (24%) was the abundant phenolic acid in the fruits of *M. alba* whereas protocatecheuic acid (41%) was observed in highest concentration in fruits of *M. nigra*. However, different phenolic acids were evenly distributed in the fruits of *M. leavigata.*

The extraction techniques show differences in the quantity of phenolic acid extracted from both flowers and leaves extracted by three different extraction procedures (ultrasonic assisted extraction, stirring, and homogenization). The maximum yield of phenolic acids was obtained from samples extracted by UAE. The total phenolic acids content extracted from leaves and flowers by stirring method was reduced by 17.8% and 24.3% respectively. Intermediate yields of total phenolic acids were obtained when extraction was achieved by homogenization. Only 7.2% and 11.0% reduction in yields of total phenolic acids was obtained as compared to UAE procedures (Figure 3B). The total phenolic yields as determined by FC method for the extracts prepared by three extraction procedures were different as compared to total phenolic acids as determined by HPLC (Figure 3A). The above variation can be attributed to differences in the assay procedures. With HPLC only phenolic acids were analysed while with colorimetric FC assay all phenolics as well as other reducing com-

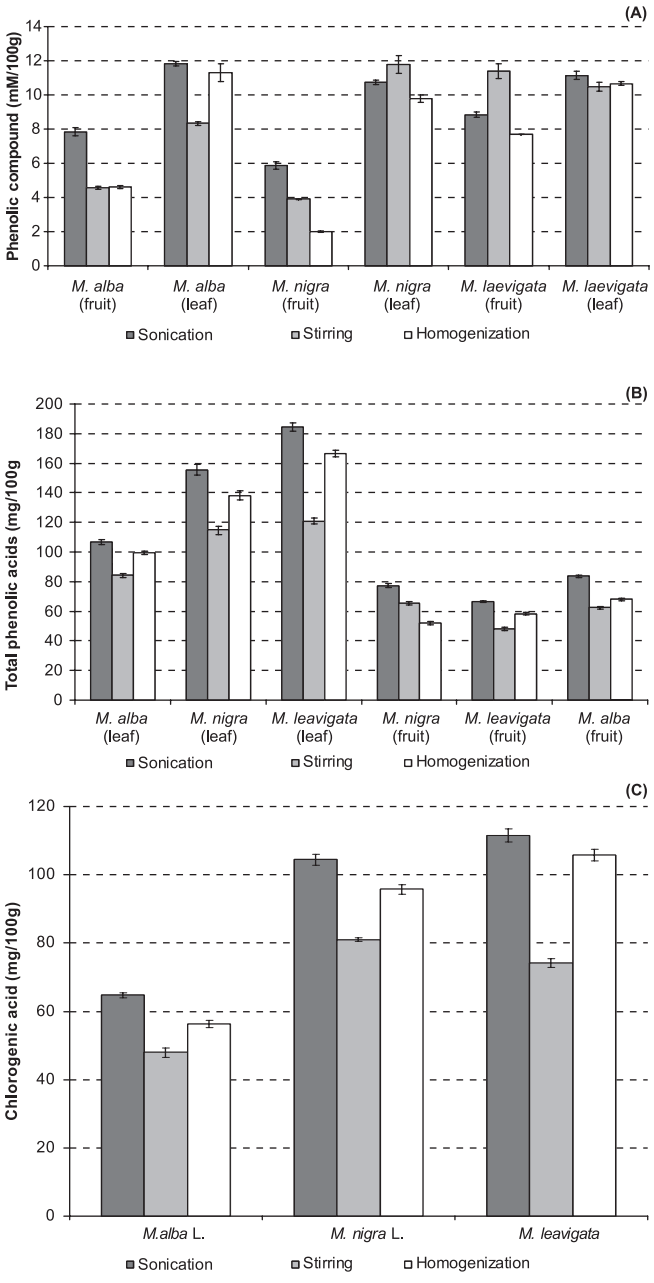

FIGURE 3. (A) Effect of various techniques on extraction of total phenolic compounds using 80% methanol as extracting solvent, (B) Effect of various techniques on extraction of phenolic acids using 80% methanol as extraction solvent, (C) Extraction of chlorogenic acid from leaves of mulberry species using various techniques with 80% methanol as extraction solvent

TABLE 1. Comparison of extraction techniques for phenolic acids analysis by three different extraction procedures in three species of mulberry found in Pakistan (each data point is average of three replicates, n.d indicates not detected).

Sample	Extract	Ingredient	Sonication		Extraction Techniques			
					Magnetic Stirring		Homogenization	
			Found (mg/100 g)	RSD (%)	Found (mg/100 g)	RSD (%)	Found (mg/100 g)	RSD (%)
Morus alba	Leaf	Gallic acid	n.d.	–	n.d.	–	n.d.	–
		Protocatechuic acid	n.d.	–	n.d.	–	n.d.	–
		Protocatechuic aldehyde	5.06	1.5	3.81	2.7	3.70	0.9
		p-Hydroxybenzoic acid	7.52	1.6	9.82	1.3	13.95	0.8
		Vanillic acid	7.34	2.1	5.70	1.4	5.55	1.8
		Chlorogenic acid	64.73	1.2	47.90	2.7	56.29	2.0
		Syringic acid	5.04	1.2	3.53	1.8	3.78	0.8
		Syringealdehyde	11.10	2.7	9.92	0.8	11.66	0.3
		p-Coumaric acid	n.d	–	n.d	–	n.d	–
		Ferulic acid	n.d	–	n.d	–	n.d	–
		m-Coumaric acid	5..96	1. 3	3.43	1.4	4.47	1.7
	Fruit	Gallic acid	5.81	1.1	4.32	2.6	3.57	0.9
		Protocatechuic acid	3.49	0.4	2.30	0.9	2.69	2.6
		Protocatechuic aldehyde	8.10	0.2	6.31	1.5	5.72	1.8
		p-Hydroxybenzoic acid	15.27	0.8	13.40	0.8	5.21	1.8
		Vanillic acid	4.57	1.0	3.95	2.5	3.70	0.8
		Chlorogenic acid	20.47	1.1	17.03	1.4	24.45	1.2
		Syringic acid	9.19	1.7	6.31	1.7	8.48	1.2
		Syringealdehyde	14.06	1.6	6.35	0.6	11.33	0.6
		p-Coumaric acid	n.d	–	n.d	–	n.d	–
		Ferulic acid	n.d	–	n.d	–	n.d	–
		m-Coumaric acid	2.63	1.3	2.33	2.2	2.78	0.5
Morus nigra	Leaf	Gallic acid	n.d.	–	n.d.	–	n.d.	–
		Protocatechuic acid	n.d.	–	n.d.	–	n.d.	–
		Protocatechuic aldehyde	6.04	0.2	4.38	3.6	5.14	1.3
		p-Hydroxybenzoic acid	3.09	0.6	1.72	0.5	2.18	3.6
		Vanillic acid	10.08	5.9	8.21	2.3	8.84	0.2
		Chlorogenic acid	104.39	1.5	81.01	0.7	95.70	1.5
		Syringic acid	6.53	2.9	3.80	1.7	4.55	2.3
		Syringealdehyde	14.83	4.2	10.24	2.2	12.99	2.2
		p-Coumaric acid	4.24	1.9	3.61	1.3	6.67	0.6
		Ferulic acid	n.d	–	n.d	–	n.d	–
		m-Coumaric acid	6.21	1.3	1.83	3.0	2.10	5.4
	Fruit	Gallic acid	5.81	1.7	1.72	2.5	4.21	0.8
		Protocatechuic acid	31.66	0.1	23.34	0.9	26.34	0.7
		Protocatechuic aldehyde	7.43	2.0	3.82	1.7	6.89	2.0
		p-Hydroxybenzoic acid	4.70	0.5	3.35	3.4	4.73	2.9
		Vanillic acid	2.31	1.6	2.25	1.9	1.63	0.5
		Chlorogenic acid	4.41	3.1	5.43	2.2	7.44	2.4
		Syringic acid	1.81	1.2	1.63	0.9	1.59	1.3
		Syringealdehyde	7.94	2.8	6.75	2.1	6.78	0.4
		p-Coumaric acid	8.66	2.8	2.27	1.2	4.89	1.1
		Ferulic acid	n.d	–	n.d	–	n.d	–
		m-Coumaric acid	2.43	2.4	1.49	3.1	1.33	2.7
Morus leavigata	Leaf	Gallic acid	n.d.	–	n.d.	–	n.d.	–
		Protocatechuic acid	n.d.	–	n.d.	–	n.d.	–
		Protocatechuic aldehyde	6.40	2.2	6.52	1.8	5.62	1.3
		p-Hydroxybenzoic acid	15.67	0.2	7.59	2.5	13.52	0.9
		Vanillic acid	10.87	0.3	6.83	1.4	8.47	0.2
		Chlorogenic acid	111.59	1.7	74.12	1.8	105.85	1.6
		Syringic acid	6.26	2.7	5.15	3.0	5.54	0.4
		Syringealdehyde	14.30	2.6	9.09	1.1	13.68	2.1
		p-Coumaric acid	n.d.	–	n.d.	–	n.d.	–
		Ferulic acid	13.48	0.5	5.88	2.0	7.47	2.7
		m-Coumaric acid	5.69	1.7	5.76	0.7	6.56	1.4
	Fruit	Gallic acid	10.88	1.6	10.02	1.7	9.69	1.5
		Protocatechuic acid	5.61	1.7	3.38	2.7	1.67	1.5
		Protocatechuic aldehyde	8.79	1.2	7.42	1.9	5.07	1.4
		p-Hydroxybenzoic acid	3.01	0.6	1.31	3.2	2.79	0.1
		Vanillic acid	8.20	0.5	4.63	2.3	7.95	2.1
		Chlorogenic acid	6.94	1.6	3.79	3.4	7.05	2.6
		Syringic acid	8.11	1.9	3.94	2.9	7.78	1.5
		Syringealdehyde	9.71	0.3	7.25	2.6	9.21	1.6
		p-Coumaric acid	n.d.	–	n.d.	–	n.d.	–
		Ferulic acid	8.42	0.6	4.93	0.3	6.71	1.1
		m-Coumaric acid	2.01	1.5	1.14	2.9	0.45	3.2

pounds are assayed due to lack of specificity of the FC assay. In addition, other researchers have isolated other classes of phenolic phytochemicals, namely anthocyanins, flavonols form different mulberry species [Arabshahi-Delouee & Urooj, 2007; Tsai *et al.*, 2005]. Chlorogenic acid, the most abundant phenolic acid showed similar extraction trend between the three extraction procedures as compared to total phenolic acids (Figure 3C).

Higher yields with UAE can be attributed to ultrasonic waves that affect the cell wall and cause destruction of thin skin and assisting in release of phenolic compounds trapped inside the cell-wall or by facilitating swelling and hydration of plant material to cause enlargement of pores which will improve mass transfer hence extraction if proper solvent employed [Huie, 2002]. The results obtained in the present study are comparable to those reported by Luthria *et al*. [2007], where UAE was identified as a better technique as compared to other classical extraction procedures except PLE. Waksmundzka-Hajnos *et al.* [2007] have also reported that improved extraction yields of phenolic acids from foliage, *i.e.* UAE at elevated temperature is better technique next to MAE. To evaluate the influence of extraction solvents, various solvents like: methanol, ethanol, ethylacetate, acetonitrile, were tried for extraction of chlorogenic acid. Besides organic solvents, 0.1 mol/L sodium dodecylsulfate (SDS) and 0.1 cetyltrimethylammonium bromide (CTAB) were also used, as the last two solvents are considered as green alternative to harmful organic solvents. Ethanol, ethylacetate, acetonitrile and CTAB did not show good extractability of chlorogenic acid, while 0.1 mol/L SDS was able to extract 83.84 mg/100 g as compared to 101.17 mg/100 g (100% methanol) and 104.39 mg/100 g (80% methanol). The percent recovery obtained with 80% methanol was the highest while with SDS (0.1 mol/L) was the lowest but the advantage of using SDS is the environment-friendly solvent system. Also the chromatogram (not shown) obtained with SDS injection produces sharper peaks and better resolution than methanol and other solvent systems.

Antioxidant activity assays, phenolic content and total sugars analysis

Table 2 shows the total phenolic (TP) content, DPPH scavenging potential, and the total sugar content in the leaves and fruits extract of three mulberry species grown in Pakistan. Extracts derived from stirring method were used for determination of the antioxidant activity by TP and DPPH assays. The TP, total sugars and DPPH scavenging activity were in the range of 3.89 to 11.79 and 36.56 to 82.15 mmol/100 g and 22.85–76.88 μmol/100 g dry weight of fruit and leaves, respectively. Results showed that *M. laevigata* leaves and fruits extracts showed the highest antioxidant activity as determined by both assay procedures. This was in good agreement with the total phenolic acid content as determined by HPLC in leaves and fruit extracts of *M. laevigata*. Intermediate antioxidant activity was shown in the extracts of leaves and flower of *M. nigra* and the lowest antioxidant activity was exhibited by the *M. alba* leaves and fruit extracts. Leaves had comparatively high values of phenolic compounds, while fruit contained higher total sugars.

A positive correlation (R=0.803) was obtained for total phenolics and DPPH scavenging activity extracted by stirring.

TABLE 2. Antioxidant activities by FC and DPPH methods and total sugar content in leaves and fruit extracts of three mulberry species grown in Pakistan. All samples were extracted with 80% methanol using magnetic stirring technique. All extracts were analyzed in triplicates.

Samples	Total phenolics by FC method (mmol/100 g GA Eq.)	Total Sugars (mmol/100 g Sucrose eq.)	DPPH° Scavenging as Quercetin Eq. μmol/100 g
Leaves			
Morus nigra L.	11.79 ±0.51	50.33 ± 0.08	65.99 ± 3.27
Morus alba L.	8.33 ±0.11	36.56 ± 0.05	48.13 ± 1.20
Morus leavigata W.	10.48 ±0.26	50.69 ± 0.11	76.88 ± 4.20
Fruits			
Morus nigra L.	3.89 ±0.04	54.68 ± 0.03	42.57 ± 3.77
Morus alba L.	4.56 ±0.08	60.15 ± 0.09	22.85 ± 2.78
Morus leavigata W.	11.38 ±0.43	82.15 ± 0.41	54.99 ± 2.66

TABLE 3. Comparison of phenolic content and antioxidant assay data from mulberry plants grown in other regions of the world.

Plant material	Part of plant (origin)	Phenolic content *(mg/100 g)	DPPH*	Reference
Morus nigra L.	Fruit (Turkey)	1422	–	[Ercisli & Orhan, 2007]
Morus alba L.	Fruit (Turkey)	181	–	
Morus rubra L.	Fruit (Turkey)	1035	–	
Morus nigra L.	Fruit (Turkey)	1943-2237	< BHA	[Ercisli & Orhan, 2008]
Morus alba L.	Fruit (Korea)	257	< Std.	[Bae & Suh, 2007]
Morus indica L.	Leaves (India)	9320	< Std	[Arabshahi-Delouee & Urooj, 2007]
Morus atropurpurea Roxb	Fruit (southern China)	441-1710	–	[Isabelle *et al.,* 2008]
M. nigra L.	Leaves (Pakistan)	1380	≅Querectin	Current work
M. alba L.	Leaves (Pakistan)	2230	≅Querectin	
M. laevigata W.	Leaves (Pakistan)	2130	>Querectin	
M. nigra	Fruit (Pakistan)	1020	<Querectin	Current work
M. alba	Fruit (Pakistan)	1570	<Querectin	
M. laevigata	Fruit (Pakistan)	1260	>Querectin	

*For comparison DPPH activity was calculated as%DPPH Scavenging activity with reference to Querectin as standard and phenolic compound as mg/100g

The good correlation shows that phenolic compounds contribute to scavenging activity in mulberry species. The correlation of total sugars with DPPH was negative (-0.122) though the strength of correlation was poor. In Table 3, the available data for antioxidant activities and phenolic compounds from various regions is compiled. The phenolic content in the assayed samples here is comparable with the reported values for Turkey, China and Korea but reported values from India are high. This may be attributed to variation in the species assayed (genetically modified) and the growing conditions [Arabshahi-Delouee & Urooj, 2007].

This is the first report on identification of phenolic acids and antioxidant activity in the extracts of three cultivars of mulberry plants grown in Pakistan. This information will be of considerable value to the commercial producers of mulberry trees in Pakistan.

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