



Phenolic Acids Composition of Fruit Extracts of Ber (*Ziziphus mauritiana* L., var. Golo Lemai)

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Abstract

Fruits of *Ziziphus mauritiana* L. (ber) are consumed in fresh and dried/processed form in many countries across Asia including Pakistan. In the present study, we analyzed the composition of total phenolic acids (free, soluble-bound and insoluble-bound) from ber fruit extracts by applying a pressurized liquid base hydrolysis extraction (PLBHE) using Dionium cells. Nine phenolic acids (protocatechuic, *p*-hydroxybenzoic, ferulic, chlorogenic, vanillic, caffeic, vanillin, *o*- and *p*-coumaric acids) were extracted, separated, and quantified by HPLC-DAD. Identification of phenolic acids was achieved by comparison of retention times, ultraviolet, and mass spectral data with authentic commercial standards. Results showed that *p*-coumaric acid (3719 ± 22 µg/g) was the predominant phenolic acid extracted from ber samples. In addition, four phenolic acids, namely *p*-hydroxybenzoic (2187 ± 71 µg/g), vanillin (2128 ± 20 µg/g), ferulic (2629 ± 96 µg/g), and *o*-coumaric acids (2569 ± 41 µg/g) were obtained in intermediate amounts from dried *Ziziphus mauritiana* L. fruit. The total phenolic acids content was determined as 18231 ± 306 µg/g dry matter basis (DMB). This study indicates that ber fruit is a good natural source of phenolic acids and that PLBHE can be used for the assay of phenolic acids.

Keywords: *Ziziphus mauritiana* L; Phytochemicals; Phenolic acids; Pressurized Liquid Extraction.

Introduction

Among the various varieties of Rhamnaceous *Ziziphus* (formerly known as *Zizyphus*) species, *Ziziphus mauritiana* L. is a most common fruit tree found in rural areas of Sindh, Pakistan [1]. *Z. mauritiana* L. (locally known as ber) fruit is known to contain several bioactive phytochemicals such as phenolic acids, amino acids, phosphorus, calcium, iron, carbohydrates, ascorbic acid, and vitamins A and C [1-5]. Phenolic acids are secondary metabolites that belong to the group of phenolic compounds that are ubiquitously distributed throughout the plant kingdom [6, 7]. Phenolic phytochemicals play an important role in the normal growth, development and protection in

plants [8]. There has been significant interest in plant phenolics during the last couple of decades due to their health beneficial effects arising from antioxidant, anti-inflammatory, anti-hepatotoxic, antitumor, and antimicrobial activity [7, 9-13].

Phenolic acids are known to occur in free and conjugated forms within cells. In a bound form phenolic acids commonly occur as ester linked to other biomolecules. Free phenolic acids are determined by extraction of plant material with aqueous methanol, while soluble-bound phenolic acids are released by hydrolysis of the plant extract, and the total phenolic acids are determined

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by direct hydrolysis of the plant material [14-18]. Total phenolic acids (sum of free and bound) are frequently measured after base, and/or acid, or enzyme hydrolysis of plant material. [14-17]. Base hydrolysis with NaOH and protecting agent (EDTA and ascorbic acid) is commonly used for the determination of free, bound, and conjugated phenolic acids from plant materials [15, 16, 19, 20]. The quantity and the identity of phenolics extracted from plant material are dependent upon the extraction technique and solvent composition. Classical extraction methods for phenolic compounds use large quantity of organic solvents with and without acid and/or base [21, 22].

During the past decade, conventional liquid extraction (water bath, ultrasonic assisted extraction (UAE), magnetic stirring, etc.) methods have been replaced with automated and efficient extraction techniques such as pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE). The primary advantages of these newer techniques over classical method are; automation, increased throughput, extraction in an inert atmosphere at high temperature and pressure, and a significant reduction in solvent usage and waste generation [3, 23]. There are several recent research publications that show that extraction yield of phenolics is significantly improved with PLE as extraction can be carried out at higher temperatures and pressures in an inert nitrogen atmosphere [23, 24].

In the present study, we have evaluated the extraction of free and bound phenolic acids from ber fruit using pressurized liquid base hydrolysis extraction (PLBHE) procedure with Dionium cells.

Material and Methods

Plant material

The fresh fruit of *Z. mauritiana* L. was collected from the backyards of the Tando kesar district Hyderabad, Sindh, Pakistan during the month of February 2010. The species name was confirmed by the Department of Plant Protection, Sindh Agriculture University, Tandojam, Sindh, Pakistan and the plants were identified as a Gola Lemai variety of *Z. mauritiana* L. Fruit samples were stored at 4 °C immediately after collection.

After not more than two days, the pericarp was then separated from the seed and the samples were freeze-dried and stored in a freezer (-70 °C).

Chemicals

All reagents were analytical or HPLC grade. Methanol, ethanol, and acetone were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Formic acid and ascorbic acid were procured from Aldrich Chemical Company (Milwaukee, WI, USA). EDTA (Ethylenediamine tetraacetic acid) was purchased from EMD Chemicals (Gibbstown, NJ, USA). Diatomaceous Earth (ASE Prep DE) for PLE was purchased from Dionex Corporation (Sunnyvale, CA, USA). Deionized water (18Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA, USA). Polyvinylidene difluoride (PVDF) syringe filters with a pore size 0.45 μm were obtained from National Scientific Company (Duluth, GA, USA).

Pressurized liquid extraction and base hydrolysis of phenolic acids

Pressurized liquid extraction and simultaneous base hydrolysis of dried fruit samples were carried out with Dionium cells (Dionex Corp, Sunnyvale, CA, USA) using a pressurized liquid extractor. Dried ber samples (500 ± 1 mg) and 4 gm Diatomaceous Earth (DE) were mixed thoroughly and loaded in the Dionium cells in the following order: Two fiber glass filters were placed at the bottom of the extraction cell (66 ml), followed by 4 gm of Ottawa sand. The ber sample was thoroughly mixed with 4 gm of DE. The well mixed sample was loaded into the Dionium cell and 10 ml of base hydrolysis solution (0.372 gm of EDTA and 1 gm ascorbic acid in 2N NaOH) was added. The void volume of the Dionium cell was filled with 2 gm DE and Ottawa sand. Two fiber glass filters were placed at the top and the cap was screwed on firmly. The cells and the cleaned empty collection vials were loaded into the extractor racks. The conditions used for the hydrolysis were as follows: temperature-100 °C, pressure: 1500 psi, preheating equilibration time: 5 min, static extraction time: 5 min, number of cycles: 3, purge time with N₂: 200 sec. Initially

extraction was carried out with acidified water. The same cells were re-extracted with EtOAc. Both acidified water and EtOAc extracts were collected in the same collection vial.

The pH of the combined extract was adjusted to 2.5 with 6N HCl. The aqueous organic extract was mixed well by shaking the bottle and transferred into two 50 ml disposable tubes. The mixture was centrifuged in a low speed bench top centrifuge (Damon IEC HN-SII, Ramsey, Minnesota, USA) at 5000 rpm for 10 min. The top organic layer was transferred into a round bottom flask, and the aqueous portion was re-extracted twice with 10 ml of ethyl acetate. The 20 ml of the combined organic layer was evaporated in a rotary evaporator. The dried material was re-dissolved in 2 ml of 80% methanol (MeOH: H₂O) filtered through a 0.45 μ m PVDF filter and the extract was analyzed by HPLC. Four replicate extractions and analysis were carried out with each sample.

Determination of phenolic acids by HPLC-DAD, LC-ESI-MS

Separation of phenolic acids by HPLC-DAD.

Analysis of phenolic acids from all extracts was carried out using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) system consisting of a quaternary pump with a vacuum degasser, a thermostatic column compartment, an auto-sampler, and a diode array detector (DAD). Separation of phenolic acids was achieved using a reversed phase C18 Luna column (Phenomenex, Lorange, CA, USA, 150 x 4.6 mm; particle size 5 μ m), preceded by a guard column (Phenomenex, 4 x 3.0 mm) of the same stationary phase as described earlier [20]. Solvents A and B consisted of 0.1% (v/v) formic acid in water and methanol respectively. The flow rate was set to 1 ml/min. A linear gradient went from 5% (B) to 30% (B) in 25 min, was held at 30% (B) for 35 min, then gradient elution was changed from 30% (B) to 100% (B) for 10 min and a linear mode was used as 100% (B) for 5 min. After 75 min, the mobile phase concentration was brought back to 5% (B) and held for 10 min for column equilibration. For quantification of phenolic acids,

calibration curves were prepared with authentic phenolic acid standards obtained commercially.

Identification of phenolic acids by LC-DAD-ESI-MS.

A mass spectrometer detector (MSD) (Agilent, Palo Alto, CA, USA) with electron spray ionization (ESI) coupled to the Agilent 1100 was used for identification of phenolic acids from ber fruit varieties. For LC-MS analysis, the same column, flow rates, and gradients were used as described for HPLC. Mass spectra were acquired in the positive and negative ion modes at both low and high fragmentor voltages (70V and 250 V) as described by Lin and Harnly [15]. The instrument was set to scan from 100 to 2000 mass units. The temperature of the drying gas was 350 °C at a flow rate of 13 L min⁻¹ and a nebulizer pressure of 50 psi. The LC system was directly connected to the mass spectrometer with no stream splitting. Phenolic acid identification was achieved by comparison of the LC-MS data with authentic commercial standards and data reported in the literature.

Results and Discussion

Separation and identification of phenolic acids in ber fruit by HPLC-DAD and HPLC-ESI-MS

Fig. 1 shows the HPLC separation with diode array detection of phenolic acids extracted from saponified Gola lemai ber sample. In the HPLC chromatogram, peaks 1, 6, 8, and 9 are the four major phenolic acids identified as protocatechuic (1), vanillin (6), *p*-coumaric (8), and ferulic (9) acid. In addition, *p*-hydroxybenzoic (2), chlorogenic (3), vanillic (4), caffeic (5), and *o*-coumaric acids (10) were also present in comparatively lower quantities (Fig. 1). Identification of the phenolic acids was achieved by comparison of retention times and ultraviolet and mass spectral data with authentic commercial standards (Table 1). Peak (7) was tentatively identified as an isomer of caffeic acid as it showed an ion at m/z 181 ($M+H$)⁺ in the positive ion mode and an ion at m/z 179 ($M-H$)⁺ in the negative ion mode, and its UV spectra was also similar to that of caffeic acid (Fig. 2).

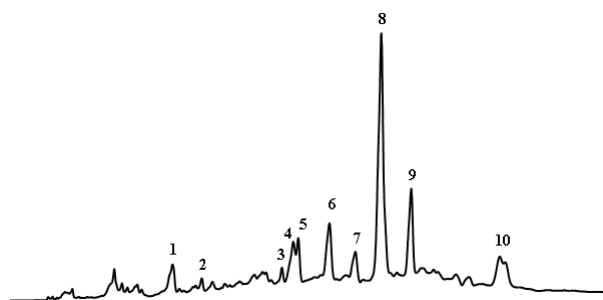


Figure 1. Chromatographic separation of 10 phenolic acids [Protocatechuic acid (1), *p*-hydroxybenzoic acid (2) Chlorogenic acid (3), Vanillic acid (4), Caffeic acid (5) Vanillin (6), Unknown (7), *p*-Coumaric acid (8), Ferulic acid (9), *o*-Coumaric acid (10)] with diode array detector extracted from saponified Gola lemai ber sample.

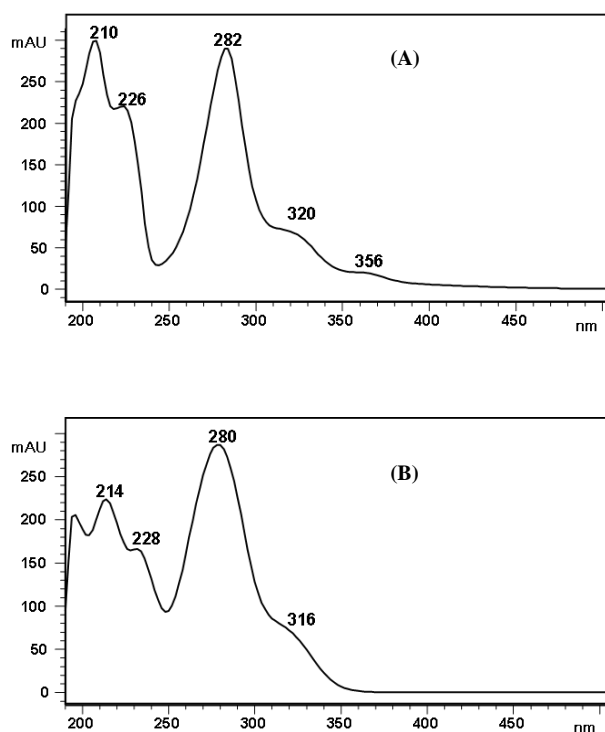


Figure 2. Comparison of UV spectra of unknown-1 (Unk.) with standard of caffeic acid.

Quantification of phenolic acids in ber samples

The amount of individual and total phenolic acids extracted by pressurized liquid extraction from Gola lemai ber variety of *Z. mauritiana* L. fruit is shown in Table 1. The nine major identified phenolic acids (protocatechuic, *p*-hydroxybenzoic, ferulic, chlorogenic, vanillic, caffeic, vanillin, *ortho*- and *para*-coumaric acids)

was quantified using external calibration with commercially available standards and diode array detection. Results show that, *p*-coumaric acid (3719 ± 22 $\mu\text{g/g}$) is the predominant phenolic acid. In addition, four phenolic acids namely, *p*-hydroxybenzoic (2187 ± 71 $\mu\text{g/g}$), vanillin (2128 ± 20 $\mu\text{g/g}$), ferulic (2629 ± 96 $\mu\text{g/g}$), and *o*-coumaric acids (2569 ± 41 $\mu\text{g/g}$) were obtained in intermediate amounts from dried *Ziziphus mauritiana* L. fruit. The total phenolic acids content was determined as 18231 ± 306 $\mu\text{g/g}$ (Table 1). In previously published reports the two predominant phenolic acids in Gola lemai ber (GLB) were vanillin (773 $\mu\text{g/g}$ DMB) and *p*-coumaric acid (699 $\mu\text{g/g}$ DMB). In a separate report on Zimbabwean wild ber fruits, the authors detected the presence of *p*-hydroxybenzoic acid (366 $\mu\text{g/g}$) in addition to the two identified phenolic acids listed above [33]. However, the quantity of phenolic acids extracted from ber fruit determined in the present study is more than five times than the previous literature values [1]. This increase in extraction yield of phenolic acids in the present study may be attributed to multiple factor: In earlier publications, the authors used UAE technique for extraction and analysis of free soluble phenolic acids [1, 34, 36]. The PLE extractions were carried out at a higher temperature in an inert nitrogen atmosphere. The increased extraction efficiency at higher temperature is due to greater equilibrium (solubility) and mass transfer rate (diffusion coefficient) [32], while pressure assists greater solvent penetration into sample matrix. The accelerated solvent extractor process allows use of temperatures well above the normal boiling point of the solvent, which is not possible with other extraction techniques like, UAE, Stirring, Soxhlet, and other classical extraction procedures. In addition, improved in extraction yields of phenolic acids from eggplants and black cohosh, have been observed when extractions were performed at elevated temperature using pressurized solvent extractor as compared to extraction performed at ambient room temperature [25, 31, 35]. In the present study, both free and bound phenolic acids were analyzed; however, previous literature reports were only on free phenolic acids. 4) The natural variability of phenolic acids present in food can also be due to differences in growing and environmental conditions [16, 20, 27].

Table 1. Quantification (A) and Identification (B) of phenolic acids from Gola ber varieties of *Z. mauritiana* L. fruit by Pressurized liquid base hydrolyzed extraction (PLBHE) procedure using Dionium cells

samples		A. Quantification		B. Identification		
Scientific name	No	Phenolic acids	Extraction method	HPLC-DAD		HPLC/ESI-MS
			Pressurized Liquid Extraction ($\mu\text{g/g} \pm \text{stdev}$)	t_R (min)	λ_{max} (nm)	$[\text{M}+\text{H}]^+ / [\text{M}+\text{H}]^-$
<i>Ziziphus mauritiana</i> L.	1	Protocatechuic acid	$374. \pm 11$	10.9	218, 260, 294	155/153
	2	<i>p</i> -hydroxybenzoic acid	$2,187 \pm 71$	13.4	216, 254	139/137
	3	Chlorogenic acid	$1,127 \pm 58$	18.2	244, 294, 324	355/353
	4	Vanillic acid	$1,574 \pm 24$	19.0	218, 260, 294, 324	169/167
	5	Caffeic acid	$1,925 \pm 41$	19.3	222, 240, 296, 324	181/179
	6	Vanillin	$2,128 \pm 20$	21.4	230, 274, 308	153/151
	7	Unknown	$*487 \pm 17$	23.1	226, 282, 320, 356	181/179
	8	<i>p</i> -Coumaric acid	$3,719 \pm 22$	24.9	210, 228, 296, 310	165/163
	9	Ferulic acid	$2,629 \pm 96$	26.9	218, 238, 296, 326	195/193
	10	<i>o</i> -Coumaric acid	$2,569 \pm 41$	32.8	228, 276, 330	165/163
	Total		$18,231 \pm 306$	--	--	--

Conclusions

The purpose of the research was to determine the composition of phenolic acids from ber fruit (*Ziziphus mauritiana* L.). The results indicate that there is a significant increase in the extraction yield of phenolic acids by PLE technique from ber fruit. Nine phenolic acids were separated and identified as protocatechuic, vanillin, *p*-coumaric, ferulic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, and *o*-coumaric acids. The *p*-coumaric acid was the most predominant phenolic acid of the nine phenolic acids while the remaining phenolic acids were present in higher concentrations than previously reported.

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