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Original Article

Chromatographic Analysis of Phenolic Acids in the Fruit Pulp of Some Citrus Varieties and Their Therapeutic Importance in Human Health

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ABSTRACT

Phenolic compounds play an important role in defense system of the plants and animals against various fungal, bacterial and viral diseases. High performance liquid chromatographic (HPLC) analysis of the pulp of six varieties of Citrus fruits showed that they content good amount of phenolic acid. Lemon pulp (C. lemonum) had five phenolic acids viz., tannic, gallic, ferulic, o-coumaric and cinnamic acids in which gallic acid (32.18 µg/g) was maximum, followed by tannic (12.49 µg/g), ferulic (1.89 µg/g), o-coumeric (1.34 µg/g) and cinnamic (0.26 µg/g fresh wt) acids. Among other varieties having four phenolic acids, viz., tannic, gallic, ferulic and o-coumeric acids were detected. Juice from the pulps of citrus fruits are incorporated in daily dietary food, hence they might be playing a great role in imparting resistance to human body.

INTRODUCTION

Due to increasing demand of plant based healthy food with flavour, aroma, and formulated medicines, considerable interest in the utilization of such materials is gaining importance. The phenolic-based natural substances have shown their possible implication in fulfilling the needs for sound human health. Primary and secondary metabolites are essential for nutrition and proper functioning of physiological systems of the body.

Citrus is a common term genus (*Citrus*) that belongs to the family Rutaceae. It is believed to have originated in the part

of Southeast Asia bordered by Northeastern India, Myanmar (Burma) and the Yunnan province of China[1]. Citrus is the third most important fruit crop in India after banana and mango. Its fruits are the major source of ascorbic acid (Vit. C), as a component of food[2]. Phenolic acids are secondary metabolites, which are abundantly present in citrus fruits after ascorbic acids. They have wide range of therapeutic properties in human beings against various fungal, bacterial and viral diseases[3-6]. Among the secondary metabolites, flavanones, flavones and flavonols are widely distributed in Citrus fruit[7,8,9]. The interest in these classes of compounds is due to their pharmacological activity as radical scavengers[10]. Several reports indicated on the dietary intake of citrus flavonoids improved a reduction in risk of coronary heart disease in humans[11,12]. But in the present scenario citrus is becoming important due to antioxidant, anti-carcinogenic and anti-inflammatory effects[13,14].

Keeping this in view was undertaken to analyze various phenolic acids of the *Citrus* varieties.

MATERIALS AND METHODS

Extraction of phenolic compounds

Six varieties of citrus fruits, viz., Lime (*Citrus aurantifolia*), Orange (*C. reticulata*), Mousammi (*C. sinensis*), Lemon (*C. lemonum*), Hajara nibu (*C. microcarpa*) and Grapefruit (*C. paradisi*) were collected from the Horticultural garden, Banaras Hindu University, Varanasi, India and some were purchased from the local market. Peeling and pulp of each type of fruit were used in the study. Extraction of phenolic acids was done in 80 % ethanol by crushing the sample (1g fresh wt) in a pestle and mortar and stored in screw-capped sample tubes and kept for overnight and the suspension was subjected to ultra-sonication (Branson Sonifier, USA) for 15 min at 4^0C followed by centrifugation at 7,500 rpm for 15 min. The clear greenish supernatant was subjected to charcoal treatment to remove pigments from each sample and was then transferred to glass tubes. They were filtered and evaporated under vacuum and finally re-suspended in HPLC grade methanol and again filtered through 0.4 µm methanol compatible membrane filter. Samples were analyzed in an HPLC system using a C-18 reverse phase column in isocratic mode with mobile phase of methanol: 0.4 % aqueous acetic acid (80:20 v/v) at a rate flow of 1ml/min. Detection was done at 290 nm using a UV-VIS detector. Peaks were identified by comparing their retention time as well as by co-injection with the standard phenolic acids. The residue was re-extracted twice and supernatant was pooled prior to evaporation under vacuum (Buchi Rotavapor Re Type). Dried samples were resuspended in 1.0 ml HPLC grade methanol by vortexing and filtered through membrane filter (pore size 0.45µm, Millipore) before HPLC analysis.

HPLC Analysis

Samples prepared for phenolic acid estimation were analyzed through high performance liquid chromatography (HPLC) according to Maurya *et al*., 2007,[15] with an HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with two Shimadzu LC-10 AT*VP* reciprocating pumps, a variable UV-VIS detector (Shimadzu SPD-10 A*VP*), an integrator and Winchrom software for data recording and processing (Winchrom, India). Reversed phase chromatographic analysis was carried out in isocratic conditions using C-18 reverse phase HPLC column [(250 x 4.6 mm id, particle size 5 µm) Luna 5µ C-18 (2), Phenomenex, USA] at 25°C. Running conditions included mobile phase methanol: 0.4 % acetic acid (80:20, v/v), flow rate 1.0

ml/min, injection volume 5 µl and detection at 290 nm. Samples were injected thrice in the sample loop and the means of the peak areas of individual compounds were taken for quantification. Tannic, gallic, chlorogenic, ferulic, cinnamic and salicylic acids were used as internal and external standards. Phenolic compounds and salicylic acid present in the samples were identified by comparing retention time (Rt) of standards as well as by co-injection. Concentrations were calculated by comparing peak areas of reference compounds with those in the samples run under the same elution conditions.

RESULTS AND DISCUSSION

High performance liquid chromatographic (HPLC) analyses of the pulp of six varieties of Citrus fruits indicated that they contained high amount of phenolic acids. Lemon pulp (*C. lemonum*) had five phenolic acids, viz., tannic, gallic, ferulic, o-coumaric and cinnamic acids. In which gallic acid (32.18 µg/g) was maximum followed by tannic (12.49 µg/g), ferulic (1.89 µg/g), o-coumeric (1.34 µg/g) and cinnamic (0.26 µg/g fresh wt) acids. Among all the remaining fruits only tannic, gallic, ferulic and o-coumeric acids were detected. However, the pulp of Lime (*C. aurantifolia*) had four phenolic acids where gallic (26.85 µg/g) was maximum, followed by tannic (14.27 µg/g) acid, but, ferulic and o-coumeric acids were detected in traces. In the pulp of Orange (*C. reticulata*), gallic acid (10.28 µg/g) and tannic (4.33 µg/g) acids were present in good amount but ferulic and o-coumeric acids were in traces. In Mousammi (*C. sinensis*), tannic (12.33 µg/g) acid was maximum followed by gallic (6.28 µg/g), farulic (0.38 µg/g) and o-coumeric (0.42 µg/g) acids. Hajara nibu (*C. microcarpa*) pulp had four phenolic acids where tannic (10.27 µg/g) fresh wt. was maximum, followed by gallic (8.85 µg/g) but ferulic and o-coumeric acids were in traces. In the pulp of Grapefruit (*C. paradisi*), gallic acid (9.85 µg/g) was maximum followed by tannic (4.27 µg/g), o-coumeric (0.45 µg/g) and ferulic (0.28 µg/g fresh wt.) acids (Table 1).

Plant and its preparations are the major source of phenolic acids in human diet. The intake of plant/plant material containing high amount of phenolic acids as well as favonoids enhance resistance due to activation of immune-stimulation and scavenging the SOx radicals the body. Plant polyphenols have attracted much attention recently due to their role in prevention of illnesses such as heart diseases and diseases of cardiovascular system whose causes are in the oxidation of LDL (low density lipoproteins)[16]. The anti-viral[17] and antimicrobial properties against various fungal, bacterial pathogens[18,15,19] are well documented. Phenols and polyphenolic compounds, such as flavonoids, are widely present in the citrus fruits showing significant antioxidant activities[20].

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Table 1. Quantitative analysis of phenolic acid in the pulp of different types of citrus

Types of citrus	Phenolic acid (µg/g fresh wt) in fruit pulp				
	TA	GA	FA	O-Cou-A	CA
Lemon	12.49	32.18	1.89	1.04	0.26
Lime	14.27	26.85	1.38	1.24	UDL
Orange	4.33	10.284	1.034	0.152	UDL
Mausammi	12.33	6.284	0.87	0.42	UDL
Hazara nibu	10.27	8.85	0.38	0. 24	UDL
Grape fruits	4.27	9.85	0. 28	0.45	UDL
C. D.	1.537	2.917	0.609	0.213	0.045
SE (m)	0.481	0.914	0.191	0.067	0.014
SE (d)	0.681	1.292	0.270	0.094	0.020
C. V.	8.633	10.071	34.007	19.590	56.527

TA: Tannic, GA: Gallic, FA: Ferulic, O-Cou-A: O-Coumeric acid, CA: Cinnamic acid, UDL: Under detection level, CD: Critical difference, SE (m): Standard Error of Mean, SE (d) Standard Error of Standard deviation and CV: Coefficient of variation