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Phenolic Acids in Selected Tropical Citrus

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ABSTRACT

In spite of wide research on plant phenolics, limited data are available on the phenolic acid content in selected tropical citrus. Phenolic acids are known to contribute health benefits to humans. In this study, free and ester conjugated phenolic acid in selected tropical citrus was successfully identified and quantified using Gas Chromatography Mass Spectrometry (GCMS). *Citrus microcarpa, Citrus medica, Citrus hystrix* and *Citrus suhuiensis* were among the tropical citrus analysed for their free and ester conjugated phenolic acids. *C. microcarpa* contains high amount of free and ester conjugated phenolic acid, which may suitable to be applied in health food products.

INTRODUCTION

Phenolic acids have been reported for their health benefits such as antioxidant, block the biosynthesis of leukotrienes (components involved in immunoregulation diseases, asthma and allergic reaction) and anti-HIV [1]. These compounds contain two distinguishing constitutive carbon frameworks such as hydroxycinnamic and hydroxybenzoic structures. Among different types of plant polyphenols, phenolic acids contributes one-third of the dietary phenols in plant foods. Phenolic acids are present in plants as free and bound form. Bound phenolics may link with ester, ether or acetal bonds [2]. For instance, ferulic acid was found to be ester linked such as C-2 hydroxyl group of arabinofuranose or C-6 hydroxyl group of galactopyranose residue of the pectic side chain [3]. Although different types of enzyme such as pectinase and cellulase were used to release phenolic acids from the acetal and hemiacetal bonds, however, there are no studies has been conducted on the use of enzymes for the ester cleavage reactions. Sun et al [4] reported that some phenolic acids such hydroxycinnamic acid could be obtained by participating in either ester or ether linkages using 1M NaOH at 170°C for 2 hours.

Despite this, there is no definitive method for hydrolysis has yet been developed since 2003. Zadernowski et al [2] had successfully developed techniques to release phenolic acids from ester and glicosidic bonds. For instance, alkaline conditions are employed to isolate phenolic acids from citrus by using sodium hydroxide for 4h at room temperature under nitrogen and the total phenolic acid are recovered using ethyl acetate.

In spite of wide research on plant phenolics, limited data are available on the analysis of phenolic acids in tropical citruses. In this study, the determination of free and ester conjugated phenolic acid in selected tropical citrus have been successfully carried out.

MATERIALS AND METHODOLOGY

Plant materials

Tropical citrus fruits were bought from Kuala Krau, Pahang, Malaysia. Peel and flesh were separately dried using an oven for 6 h at 40°C and stored at -20°C prior to analysis. Phenolic acid standards were obtained from Sigma-Aldrich (Poole, Dorset, UK). *o*-Hydrocinnamic acid was purchased from Fisher (Leicestershire, UK). Ferulic acid was obtained from AASC Chemicals (Southampton, UK). Derivatization reagent [(*N, O*-bis(trimethylsilyl)acetamide (BSTFA) + 1% trimethylchlorosilane (TMCS)] was purchased from Sigma-Aldrich (Poole, Dorset, UK). Ethylacetate (HPLC grade) and methanol (HPLC grade) were bought from Rathburn Chemicals Ltd. (Walkerburn, Scotland). All other chemicals including phenolic acid standards were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated.

Extraction of free phenolic acids

Extraction, fractionation, purification and formation of trimethylsilyl derivatives (TMS) of phenolic acids was carried out using method developed by Borges et al [5], Roowi et al. [6-8]

and Zadernowski et al [2]. Soluble phenolics from freeze-dried citrus (3.0 g) were extracted three times with methanol: water (80:20) at room temperature for 1 h using an orbital shaker (IKA KS 130 Basic U.K.). The crude extract was acidified to pH 2 with 6M HCl and extracted three times with ethyl acetate at room temperature. The ethyl acetate layer which contained free phenolic acids was evaporated to dryness under vacuum at < 40°C.

Extraction of conjugated phenolic acids

Water fraction obtained from free phenolic extraction was adjusted to pH 7.0 with 2M NaOH and evaporated under vacuum at < 40°C. The residue was treated with 20 mL 4M NaOH and placed under N_2 atmosphere for 4 h at room temperature. The solution was acidified with 6M HCl to and extracted with ethyl acetate at (1:1) ratio.

Purification of phenolic acids fractions

Each residue of the phenolic acid fractions, obtained as described above, was dissolved in 50 mL of 5% (wt/vol) $NaCO_3$ (pH 8) and extracted three times with ethyl acetate to remove fatty acid. The aqueous water phase was then acidified to pH 2.0 with 6M HCl and extracted three times with ethyl acetate. Excessive water in the ethyl acetate fraction was then dried using molecular sieves. Dried extracts which contained free and conjugated phenolic acids were placed into glass vials, silylated using 300 µL of *N, O*-bis(trimethylsilyl)acetamide (BSTFA) and heated at 80°C for 80 min on a heating block. The silylated samples were directly analyzed by GCMS (Thermo Finnigan and Trace DSQ).

GC-MS conditions

Phenolic acids were separated using a ZB-5MS 30 m x 0.25 i.d. x 0.25 µm capillary column (Phenomenex, Cheshire, UK) with helium as a carrier gas (1.0 ml/L). The GC-MS conditions were as follows: injection volume (1 µL), initial temperature at 80°C for 5 min to 160°C at 10°C/min for 10 min and 235°C at 5°C/ min for 10 min, injector temperature (280°C), MS transfer line (290°C), ion source (200°C) and split ratio (1:100). Mass spectra were scanned at *m/z* 50-650 at 0.82 scans/sec. Electron impact energy was 70eV. Phenolic compounds in citrus were identified according to their retention time, mass spectra of authentic standards and NIST 98 library screening. Quantifications were based on a standard curve of 2,4,5-trimethoxycinnamic acid (internal standard). All standards and samples were analyzed in triplicate.

RESULTS AND DISCUSSION

Free phenolic acids

Ferulic acid and benzoic acid are free phenolic acids and were found in all selected citrus. Manach et al [9] also reported that ferulic acid is the most abundant phenolic acid found in cereal grains, which constitute its main dietary source. Ferulic acid was a major phenolic acid in *C. microcarpa* peel and *C. suhuiensis* and *C. microcarpa* fruit flesh. *p*-Coumaric and vanilic were found in high quantity in *C. medica* and *C. hystrix* peels. 4-Hydroxybenzoic was only found in *C. microcarpa* and *C. medica* peel, whereas chlorogenic acid was only detected in *C. microcarpa* flesh. Benzoic acid, which is known as a preservative, was found in high quantity in *C. microcarpa* peels (Table 1 and 2). Free phenolic acids identified in tropical citrus may possess various antioxidant activities. According to Kim and Lee [10], the ranking of antioxidant capacity was as follows: *p*-coumaric, sinapic, ferulic acid, vanilic acid, caffeic acid and chlorogenic acid. The higher the number of OH groups attached to aromatic ring, the higher value of antioxidant capacity.

The total free phenolic content in the *C. microcarpa* peel was higher than flesh, or even higher than any citrus. Goreinstein et al [11] also reported that free phenolic acids such as ferulic, sinapic, *p*-coumaric and caffeic were significantly higher in the peel than flesh.

Bound phenolic acids

Goreinstein et al [11] studied on *C. microcarpa* and *C. suhuiensis* reported that bound and free phenolic acids liberated from ester bonds were found higher in peel than flesh. Ferulic acid was found highest at 48.16 ± 8.47 mg/g dwt. followed by vanilic and benzoic acid. Ester bonded benzoic- and *p*-coumaric acids were found in *C. suhuiensis* peel. *C. hystrix* flesh and *C. medica* flesh also contains bound phenolic acid, but not as high as *C. microcarpa* peel (Table 3). In total, ester bound phenolic acids in *C. microcarpa* peel was higher than other citrus or even higher than free phenolic acids (Figure 1). In our previous study, we reported that *C. microcarpa* peel also contained higher amount of flavonoids than flesh [12]

Table 1: Retention times and identified ions in the mass spectra of silylated derivatives of phenolic standards

Peak	t_R	Compounds	Identified ions (m/z)
1	7.22	Benzoic acid	194, 178, 147, 135, 105, 77
2	11.86	trans-Cinnamic acid	220, 205, 131, 103, 161, 75
3	13.87	4-Hydroxybenzoic acid	267, 233, 193, 129, 73
4	17.74	Vanilic acid	297, 267, 223, 126, 73
5	18.89	o-Hydrocinnamic acid	308, 293, 147, 73
6	19.17	2,3-Dihydrobenzoic acid	369, 355, 281, 193, 137, 73
7	22.07	p-Coumaric acid	308, 293, 249, 219, 147, 73
8	22.47	Gallic acid	281, 179, 73
9	25.26	Ferulic acid	338, 249, 323, 146, 73
10	26.19	Caffeic acid	396, 381, 219, 191, 147, 73
11	28.14	Sinapic acid	338, 368, 353, 73
12	40.31	Chlorogenic acid	345, 307, 255, 219, 191, 73

Table 2: Free phenolic acids tropical citrus (mg/g dwt.)

Peak	Sample	*C. microcarpa* peel	*C. medica* peel	*C. hystrix* peel	*C. suhuiensis* fruit flesh	*C. microcara* fruit flesh
1	Benzoic acid	1.95 ± 0.6	0.14 ± 0.04	0.70 ± 0.07	0.91 ± 0.19	0.04 ± 0.00
3	4-Hydroxybenzoic acid	0.66 ± 0.19	0.90 ± 0.3	n.d	n.d	n.d
4	Vanilic acid	n.d	3.67 ± 1.5	1.48 ± 0.19	0.05 ± 0.03	n.d
7	*p*-Coumaric acid	4.13 ± 0.99	2.30 ± 0.07	n.d	0.28 ± 0.06	n.d
9	Ferulic acid	6.85 ± 0.16	1.66 ± 0.14	0.60 ± 0.16	1.53 ± 0.55	0.13 ± 0.02
10	Caffeic acid	3.98 ± 1.06	n.d	n.d	0.16 ± 0.05	0.02 ± 0.01
11	Sinapic acid	2.79 ± 0.96	n.d	n.d	0.45 ± 0.1	0.01 ± 0.00
12	Chlorogenic acid	n.d	n.d	n.d	-nd-	0.03 ± 0.01
	Total	**20.36**	**8.67**	**2.78**	**3.38**	**0.23**

Mean values expressed as mg ± standard error (n=3)

Table 3: Ester-conjugated phenolic acid in tropical citrus (mg/g dwt.)

Peak	Sample	*C. microcarpa* peel	*C. suhuiensis* peel	*C. hystrix* flesh	*C. medica* flesh
1	Benzoic acid	13.58 ± 3.16	0.09 ± 0.01	0.91 ± 0.19	0.00 ± 0.00
3	4-Hydroxybenzoic acid	1.30 ± 0.24	n.d	n.d	n.d
4	Vanilic acid	39.20 ± 7.78	n.d	0.05 ± 0.00	n.d
5	*o*-Hydrocinnamic acid	n.d	n.d	n.d	n.d
7	*p*-Coumaric acid	19.17 ± 3.00	0.07 ± 0.02	0.28 ± 0.06	0.00 ± 0.00
9	Ferulic acid	48.16 ± 8.474.2	n.d	1.53 ± 0.55	0.01 ± 0.00
10	Caffeic acid	1.76 ± 0.40	n.d	0.16 ± 0.05	0.00 ± 0.00
11	Sinapic acid	1.92 ± 0.24	n.d	0.45 ± 0.10	0.00 ± 0.00
12	Chlorogenic acid	n.d	n.d	n.d	n.d
	Total	**113.09**	**0.16**	**3.38**	**0.02**

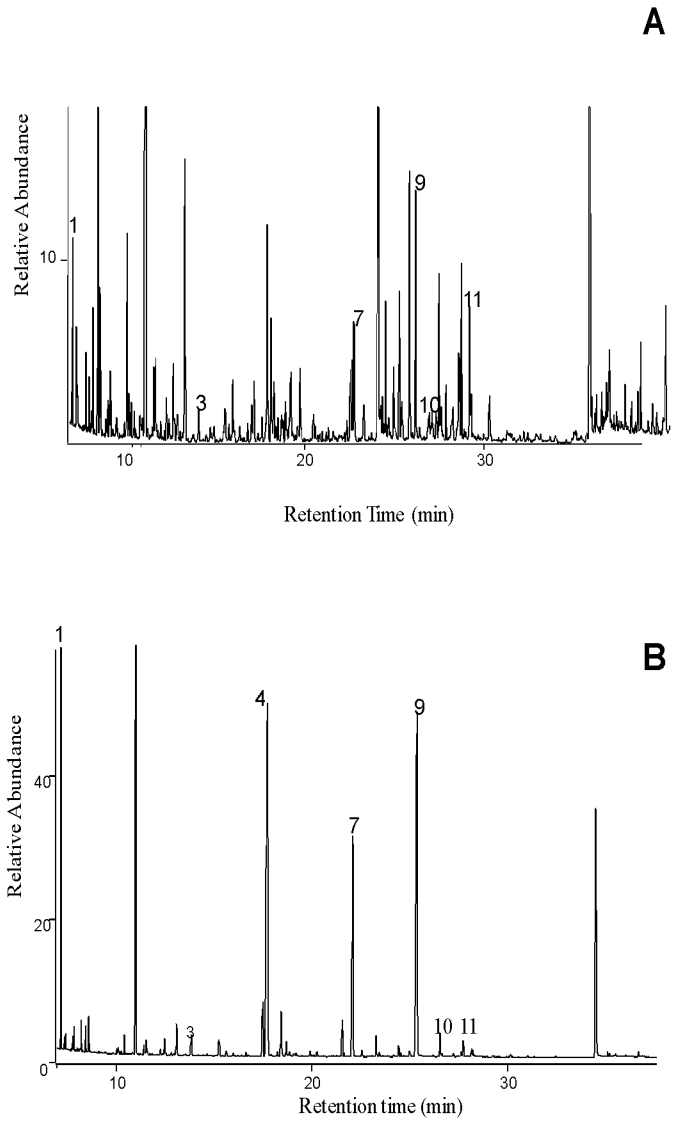

Figure 1: Free (**A**) and ester-conjugated (bound) (**B**) phenolic acids in *C. microcarpa* fruit peel

CONCLUSION

C. microcarpa may be considered to be a good source for free and ester conjugated phenolic acid, which may suitable to be applied in food and drinks.

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