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Research Article

Citrus Sinensis Tissue-Specific Specialized Metabolism Elucidated *via* Non-Targeted Metabolomics Strategy

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Abstract

Background: Citrus species are known to contain active phytochemicals that can protect health. Processing of citrus by-products potentially represents a rich source of phenolic compounds and dietary fiber.

Methods: To study the natural variation and spatial accumulation of metabolites, Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed on *C. sinensis* flavedo and leaves.

Results: Results demonstrated that leaf tissues has the highest phenolic, flavonoid and condensed tannin contents, besides to a higher antioxidant activity compared to flavedo. GC-MS was further utilized to localize primary metabolites and revealing differential accumulation patterns of mainly identified metabolites in various tissues. Further investigation indicated that leaves accumulate more amino acids, fatty acids and organic acids while flavedo contain more carbohydrates. Among carbohydrates, monosaccharide's were abundant in flavedo at 72.54% *versus* enrichment of disaccharides and trisaccharides in *C. sinensis* leaves.

Conclusions: This study provides the most comprehensive map for metabolites distribution *C. sinensis* tissues. It could be also concluded that extracts of *C. sinensis* tissues residues, could be explored as an economically viable source of natural antioxidants and nutraceuticals.

Keywords: Metabolome; *C. sinensis* tissues; Gas chromatography-mass spectrometry; Non-targeted metabolomics

Introduction

Being one of the most widespread fruit crops ,Citrus species are known to contain active phytochemicals that can protect health. Processing of citrus by-products potentially represents a rich source of phenolic compounds and dietary fiber. Thus, the exploitation of these bioactive rich residues can provide an inexpensive, efficient, and environment friendly platform for the production of novel nutraceuticals or for the improvement of older ones.

Antioxidants such as Butylated-Hydroxyanisole (BHA) and Butylated-Hydroxytoluene (BHT) could be either toxic and could sometimes stimulate the development of cancerous cells [1,2]. These outcomes have moved the researchers as well as consumers to look for natural foods and constituents that are believed to be health-giving and unadulterated than their synthetic analogues [3]. Therefore, the need for the identification and the isolation of bioactive compounds from by-products of the food processing industries can result in value addition [1].

The contribution of citrus plants in prevention of menacing diseases has been widely assessed [4]. Moreover, it has been reported that citrus fruits and their extracted bioactive compounds exhibit a wide spectrum of promising biological properties due to their rich phenolic and metabolite profiles and their antioxidant properties [5,6]. It was also mentioned that citrus tissues, as leaves and other by-products, have nutritional value, unique flavor and medicinal properties even more exceeding the edible fruit parts [7,8].

In addition, the accumulation of specified metabolites fluctuates in a tissue and species-specific manner, increasing the fitness of the plant in producing these compounds. These latter are known to procure to the plant the chemical defense and communication [9-11].

Taking together the importance of Citrus plants and its national economic importance, this work was designed in order to assess and to compare the phenolic composition and the antioxidant potentialities of the methanolic extracts of *C. sinensis* tissues; namely leaves and flavedo. The results of this work emphasized the potentialities of *C. sinensis* tissues to be used as a prospective source of natural antioxidants and as an antimicrobial agent in the food industry.

Material and Methods

Sampling and metabolite extraction

Healthy and mature leaves and fruits were collected from three *C. sinensis* species. Collected biological replicates were instantly frozen in liquid nitrogen and kept at -80°C.

Next, crushed plant material(≈1g) was dissolved in 10 ml of methanol, and then sonicated for 30 min at ambient temperature. After a 10 min centrifugation step at 3500 rpm the supernatant was recovered and finally vacuum dried for further derivation.

Total phenol contents

Total phenol contents were assessed using the Folin-Ciocalteu reagent as described by Dewanto et al. [12]. Thus, an aliquot of 0.125 mL of the diluted methanol sample was added to the Folin-Ciocalteu

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reagent (0.125 mL) and deionized water (0.5 mL). After adding 1.25 mL of sodium carbonate (Na_2CO_3, 7%) solution to the mixture, the solution was adjusted with deionized water to a final volume of 3 mL and mixed thoroughly. After a 90 min incubation step at 23°C, the absorbance *vs*. prepared blank was read at 760 nm. Total phenol contents were measured as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW) through the calibration curve [0 µg/ml to 400 µg/ml (R^2=0.99)] with gallic acid.

Total flavonoid contents

Total flavonoid contents were measured according to the method of Dewanto et al. [12]. Accordingly, 250 µL of the diluted sample was added to 75 µL $NaNO_2$ (sodium nitrite, 5%). After 6 min incubation step, 10% aluminium chloride ($AlCl_3$) and 1 M of NaOH were added to the mixture which was adjusted with distilled water to 2.5 mL. The absorbance was read at 510 nm and the total flavonoid contents were expressed as mg catechin equivalents per gram of dry weight (µg CE/g DW) through the calibration curve [0 µg/mL to 500 µg/mL (R^2=0.987)] with catechin.

Total tannin contents

Total tannin contents were measured using the modified vanillin method [13]. Therefore, 4% methanol vanillin solution and a concentrated H_2SO_4 solution were added to 50 µl of the appropriately diluted sample. After an incubation step of 15 min, the absorbance was measured at 500 nm against methanol as a blank. Expressed as mg (+)-catechin equivalent per gram of dry weight (µg CE/gDW), the amounts of total condensed tannins were determined through the calibration curve [0 µg/mL to 400 µg/mL (R^2=0.999)] with catechin. It should be mentioned that all measurements were taken as triplicates for all samples.

Antioxidant activity: DPPH radical-scavenging assay

The electron donation capacity of the obtained extracts was measured by bleaching of the purple-coloured solution of 1,1-Diphenyl-2-Picrylhydrazyl radical (DPPH) [14]. Hence, 1 mL of various extracts concentrations was added to 0.5 mL of a 0.2 mM DPPH methanolic solution. After 30 min incubation at room temperature, the absorbance was measured at 517 nm. The antiradical activity was expressed as IC50 (µg/mL), the concentration required to cause a 50% DPPH inhibition. The ability to scavenge the DPPH radical was calculated according to the formula:

$$\text{DPPH scavenging effect (\%)} = 100 \times (A_{blank}-A_{sample})/A_{blank}$$

Metabolome analysis

About 5 mg of the collected residue was mixed with 100 µL of pyridine followed by 30 min incubation with 100 µL of N ,Obis (trimethylsilyl) trifluoroacetamide (BSTFA) at 37°C. The GC column was then injected with sample (≈2 µL). An Agilent 5975C mass spectrometer with electron impact ionization (70eV) coupled to an Agilent 7890 A series II gas chromatograph was used for the GC-MS analyses. Regarding the analytical conditions; the GC column used was an HP-5 MS (30 m × 0.25 mm × 0.25 µm) column, while the oven temperature was programmed by an initial column temperature of 150°C for 1 min that increased to 300°C at a rate of 10°C/min. The carrier gas was helium and the flow rate was of 0.9 mL/min and a 60:1 split ratio. The scanning range for the mass spectra was 70-700 mass-to-charge ratios and it was recorded at a rate of eight scans per second. The identification of compounds was based on mass fragmentation spectra and relative retention times of standards and by interrogation of NIST 2014 and WILLEY 2014 libraries.

Results and Discussion

Variation of total phenolic and flavonoid contents

Methanol solvent was chosen to extract the bioactive compounds from *C. sinensis* tissues due to its effectiveness for antioxidants extraction [15]. When compared to the fruit tissues, *C. sinensis* leaves exhibited 30 fold higher of phenolic content (160.61 mg GAE/g DW) (Figure 1). Similarly, higher flavonoid content (32.95 mg EQ/g DW) was displayed by leaves (Figure 1). The obtained total phenolic and flavonoid contents were higher than that reported for other citrus by-products [16,17]. Usually, the occurrence of high contents of phenolics and flavonoids in Citrus leaves sustain their nutritional value and would be a potential source of bioactive compounds for further exploitation.

Variation in total tannin contents

Plant-derived tannins have many pharmaceutical uses and were reported to be the basis of the tanning industry [18]. Moreover, tannins were used for the reduction of methane production from ruminants [19]. The results demonstrated that *C. sinensis* leaves exhibited by-far the highest condensed tannin contents (16.48 mg QE/g DW) when compared to the flavedo parts (0.2 mg QE/g DW) (Figure 1). In fact, these contents were higher than those reported in the literature for peels and for seeds [17,20]. Accordingly, *C. sinensis* leaves could be considered as rich and cheap sources of natural tannin which could be isolated and could be used in tannin industry for manufacture of leather, cosmetic industries as antimicrobial agents, and as additives in wine production to improve the astringency of wine.

Antioxidant activity

The *in vitro* antioxidant capacities of *C. sinensis* extracts and essential oils were evaluated using DPPH assays. The obtained results indicated that tissue could affect the antioxidant activity in *C. sinensis* species. In fact, the highest antioxidant capacity was reached for flavedo methanolic extracts with IC50 values of 0.7 mg/ml (Figure 2). However, for essential oils, leaf oils displayed the highest antioxidant activity with 7.8 mg/ml. What is more, for *C. sinensis* tissues, it was demonstrated that the methanolic extracts exhibited higher antioxidant activity than essential oils. Actually, in agreement with our finding, the antioxidant power of solvent extracts was previously reported [21,22].

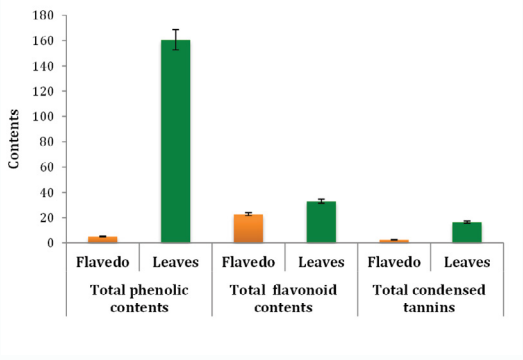

Figure 1: The total phenol (mg GAE/g DW), total flavonoid (mg CE/g DW) and condensed tannin (mg CE/g DW) contents in leaves and flavedo parts of *C. sinensis* species. The values are presented as the mean of three replicates ± the standard deviation. CE: Catechin Equivalent; DW: Dry Weight; GAE: Gallic Acid Equivalent

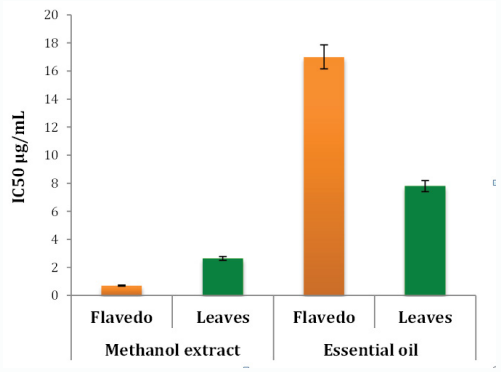

Figure 2: The antioxidant capacity of the methanolic extracts and essential oils of *C. sinensis* leaves and flavedo. IC50: the concentration at which DPPH radicals are scavenged by 50%. The values are presented as the mean of three replicates ± the standard deviation.

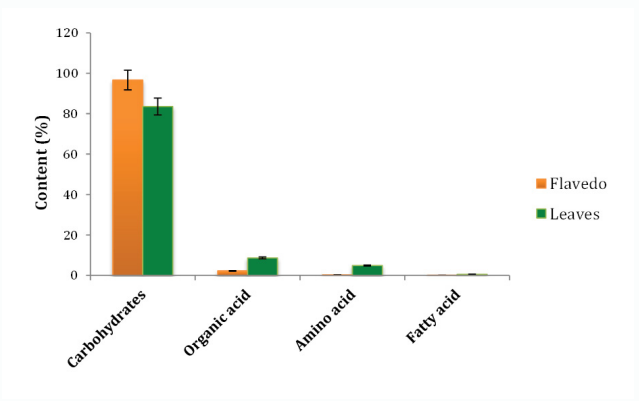

Figure 3: Percentage of chemical classes of methanolic extracts of *C. sinensis* parts. The values are presented the mean of three replicates ± the standard deviation.

Non-targeted metabolomics analysis in *C. sinensis* tissues

To assess and compare the methanol-soluble extracts between *C. sinensis* leaves and flavedo, a non-targeted based-GC-MS analysis was carried out. More than hundred metabolites were enumerated, of which 45 were confirmed using the National Institute of Standards and Technology (NIST) and Wiley libraries and covered mainly the primary metabolism pathways. Accordingly, 7 organic acids, 4 amino acids, 3 fatty acids, 24 carbohydrates, furans, indole and terpenes, a phytohormone and one tocopherol were detected (Table 1).

Carbohydrates: Methanol-soluble extracts were mainly conquered by sugar derivatives representing a high total percentage of 83.57% in the leaves and 96.65% in flavedo. Disaccharides (8 compounds) were found to be the predominant class of leaves where sucrose, known as a major source of energy for plant respiration, was the key compound (52.94%) (Figure 3). Sucrose, was the only disaccharide detected in *C. sinensis* fruit tissue, but in lower percentage (19.78%) (Table 1). Monosaccharide was the main sugar class representing 72.57% of Citrus fruit and where mannose (22.35%) and α-D-Glucopyranose (21.70%) were the predominant compounds. One trisaccharide; raffinose was just detectable for leaf methanol-extract with appreciable percentage (11.47%). Because of their diverse biological activities and uses as water retention agent, thickener, preservative, and film forming mediator to enhance foods' vividness and taste [23], carbohydrates have attracted much attention in latest years. In this sense, representing a rich source of carbohydrates, both *C. sinensis* tissues may have significant potential in pharmacological and nutritional aspect.

Organic acids, fatty acids and amino acids: Seven organic acids (7 in leaves and 4 in flavedo) and three fatty acids (3 in leaves and 1 in flavedo) were identified in the *C. sinensis* methanolic extracts (Table 1). Among organic acids, quinic acid was the predominant compound with a percentage of 7.53% and 1.87% in the leaves and in the fruit tissues, respectively. For these two tissues, succinic acid represented the second predominant compound (Table 1). It should be noted that some organic acids were specific to leaves (malonic acid, benzoic acid and malic acid), while the others were commonly detected in both tissues (Table 1). Not only they contribute to the organoleptic properties of the food items, the organic acids are known to have key role in various fundamental pathways involved in plant metabolism. Also, these metabolites are recognized to have antimicrobial, anti-tumorous, antiviral effect and antioxidant potentialities making them suitable for food processing and preservation [24,25]. Three fatty acids were detected in the methanolic extracts of leaves where α-Linolenic acid and palmitic acid were the predominant compounds. However, only palmitic acid was detected in the flavedo tissues (Table 1). Previous investigations showed that the most common feedstock's appropriate for biodiesel manufacture were enriched in the five most common C16-C18 fatty acids, specifically, palmitic, oleic, linoleic, stearic, and linolenic acids [26]. Accordingly, *C. sinensis* leaves could be suitable to be used as food additives, as acidulants, antioxidants or, as antimicrobial agent and co-crystallization agent in pharmaceuticals industries.

Amino acids are essential not only for human nutrient consumption but also they are the substrates for the biosynthesis of a wide range of secondary metabolites in plants. Higher content of total free amino acids were exhibited by leaves, where proline (4.41%) and serine (0.30%) were identified as the major acids. On the other hand, only two amino acids (alanine and proline) were detected in *C. sinensis* flavedo (Table 1).

Others: Small percentage of gibberellin A3 (0.48%), α-Tocopherol (0.08%), furanone (0.05%), phytol (0.24%) and β-Elemene (0.05%) were exclusively recorded in leaf tissues, while 1H-Indole-3-carboxaldehyde (0.4%) was specifically detected in *C. sinensis* flavedo.

Conclusion

As far as we know, this is the first report where an extensive metabolomic study has been undertaken to better understand tissue-metabolic specialization. This study revealed that in *C. sinensis* tissues contain nutritional antioxidants, including carbohydrates, fatty acids, amino acids, and metabolites with various bioactivities, which make them potential candidates for use in the nutraceutical and pharmaceutical industries. Therefore, we recommend here the potential health benefits of in *C. sinensis* tissues, especially leaves, based on the wide-ranging phytochemical characteristics. These results can represent a starting point for better understanding citrus species and can lead to discoveries regarding new mechanisms for plant-metabolic programming.

Acknowledgment

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Table 1: Composition of *C. sinensis* leaves and flavedo fractions assessed by GC-MS (% of total ion current).

Peak No	RT (min)	Metabolites	Categories of Metabolites	(%)	
				Leaves	Flavedo
1	5.044	Propanedioicacid (Malonicacid)	Organic acid	0.05	-
2	5.622	Propanoicacid (Propionicacid)	Organic acid	0.04	0.05
3	6.692	Alanine	Amino acid	0.11	0.04
4	10.285	Valine	Amino acid	0.08	-
5	11.04	Benzoicacid	Organic acid	0.07	-
6	13.095	Proline	Amino acid	4.41	0.31
7	15.89	Serine	Amino acid	0.3	-
8	16.059	Furanone	Furans	0.05	-
9	16.356	β-elemene	Terpenes	0.05	-
10	16.808	Malicacid	Organic acid	0.06	-
11	20.39	Butanedioicacid (Succinicacid)	Organic acid	0.83	0.24
12	21.369	Butanoic acid (Butyricacid)	Fattyacid	0.11	0.04
13	28.515	D-ERYTHROSE	Monosaccharides	0.26	0.23
14	28.618	D-Xylose	Monosaccharides	0.05	0.39
15	29.574	Glucose	Monosaccharides	0.11	0.19
16	29.917	D-(-)-Fructofuranose	Monosaccharides	0.57	2.63
17	30.17	D-Psicopyranose	Monosaccharides	-	14.68
18	30.999	D-(-)-Tagatofuranose	Monosaccharides	0.19	0.69
19	31.1	1H-Indole-3-carboxaldehyde	Indoles	-	0.4
20	31.325	QuinicAcid	Organic acid	7.53	1.87
21	31.846	d-(-)-Fructose	Monosaccharides	3.38	9.13
22	32.07	β-D-Glucopyranos	Monosaccharides	7.7	0.03
23	32.13	α-D-Glucopyranose	Monosaccharides	-	21.7
24	32.401	D-Galactose	monosaccharides	0.73	0.47
25	33.265	L-Ascorbicacid	Vitamin	0.14	0
26	33.631	Inositol	Sugar alcohol	2.8	4.33
27	33.8	D-(-)-Ribofuranose	Monosaccharides	0.13	-
28	34.47	Mannose	Monosaccharides	0.4	22.35
29	34.74	D-(-)-Ribofuranose	disaccharides	0.17	-
30	34.912	Palmiticacid	Fattyacid	0.25	0.05
31	35.445	α-D-(+)-Talopyranose	Monosaccharides	0.17	-
32	37.876	Phytol	Diterpene alcohol	0.24	-
33	38.552	Linoleicacid	Fattyacid	0.06	-
34	38.695	α-Linolenicacid	Fattyacid	0.3	-
35	45.801	β-Gentiobiose	Disaccharides	0.06	-
36	46.379	Arabinose	Monosaccharides	0.52	0.06
37	48.44	Sucrose	Disaccharides	52.94	19.78
38	55.38	α-Mannobiose	Disaccharides	0.24	-
39	55.48	D-Lactose	Disaccharides	0.07	-
40	55.63	D-(+)-Cellobiose	Disaccharides	0.24	-
41	56.513	Galactinol,	Disaccharides	0.17	-
42	57.783	α-Tocopherol	Tocopherol	0.08	-
43	59.008	Melibiose,	Disaccharides	1.08	-
44	61.079	Raffinose	Trisaccharides	11.47	-
45	62.99	Gibberellin A3	Phytohormones	0.48	-

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