

## Untargeted Metabolite Profiling of Philippine-grown *Crescentia cujete* and its Commercial Fruit Juice using GC-MS and UPLC-HRMS

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Calabash fruit (*Crescentia cujete* L.) juice has gained traction in Southern Philippines for its miraculous effect against stroke, diabetes, and cancer. Metabolite profile of local *C. cujete* fruit pulp and the commercial fruit juice were established using gas chromatography mass spectrometry (GC-MS) and ultra-high performance liquid chromatography high-resolution mass spectrometry (UPLC-HRMS). Putative hits to the NIST Mass Spectral Library included methyl salicylate, (+)- $\delta$ -cadinene, benzene and its derivatives such as toluene and *o*-xylene. Toluene and *o*-xylene are known air pollutants. Results indicated that the fruit has the potential to bioaccumulate small organic molecules. Volatile organic compounds detected in the fruit pulp were absent from the processed commercial juice. Other benzenoid compounds – thiazole and (+)- $\delta$ -cadinene, which are important biosynthetic precursors – were identified from the juice sample. Molecular networking analysis of the tandem MS data of the ethanol extract of the juice putatively identified the presence of 1-kestose and sucrose. 1-Kestose is considered as an essential prebiotic compound associated with boosting metabolism and immunity. Other kestose isomers were also indicated to be present in the juice based on the elution profile and MS/MS data. Preliminary activity tested for both samples yielded positive result against *Candida albicans* using disc diffusion assay. Only the juice sample yielded significant activity against *Escherichia coli*.

Key words: calabash, *Crescentia cujete* L, GC-MS, LC-MS/MS, metabolite profiling, untargeted metabolomics

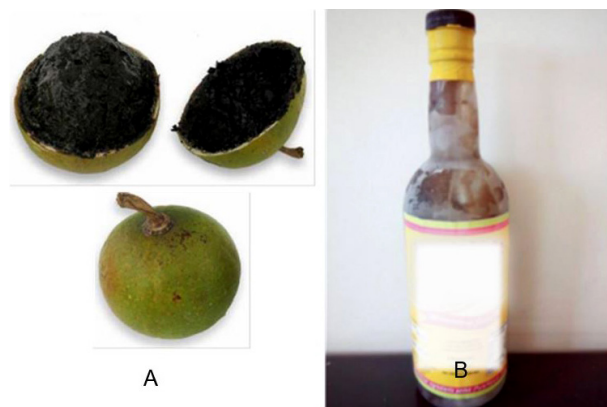
### INTRODUCTION

*Crescentia cujete* L.(Bignoniaceae) or calabash tree (Figure 1) is a species of flowering plants native to South and Central America, but has become widely distributed in some parts of Africa and Southeast Asia (Arbonier 2004). In these areas, the fruit from the plant has gained an extraordinary repute as a remedy for respiratory ailments such as coughs, colds, and even asthma (Morton 1968).

Leaves are also used to reduce blood pressure while decoction from tree bark is consumed for its antiseptic and anti-inflammatory properties (Parvin *et al.* 2015).

A phytochemical study of the African variety of the fruit revealed the presence of flavonoids, saponins, cyanogenic glycoside (as HCN), phenolic compounds, and tannins – with mineral elements (Na, K, Ca, P, and Mg) and vitamins (A, C, E, thiamin, riboflavin, and niacin) (Ogbaugu 2008). Iridoids, iridoid glycosides (Kaneko *et al.* 1998), as well as *n*-alkyl glycosides and *p*-hydroxybenzoyloxy glucose

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**Figure 1.** *C. cujete* fruit collected from DOST-PTRI compound in Bicutan, Taguig City (A), with deep-purple pulp after prolonged exposure to air, and a bottle of the ‘miracle fruit’ juice from Davao City (B).

were isolated from methanol extracts of *C. cujete* fruits from Vietnam (Kaneko *et al.* 1997). In several cities in Southern Philippines, *C. cujete* grew in popularity as a tonic against diabetes (DOST-PCHRD) and as a final alternative remedy for late-stage cancer cases due to its supposed chemo-preventive activity (Tacio 2015). *C. cujete* is known as the ‘miracle fruit’, a local sobriquet that might have been inspired by word-of-mouth. Online testimonials (Tacio 2015, World Ngayon) narrating recovery stories from serious diseases after consuming its fruit juice contributed to its local popularity, in addition to the diverse use of the plant in ethno-medicine.

*C. cujete* fruit is globular with a smooth, hard, and woody exterior and green coloring. Traditionally, syrup is made from the fleshy pulp and sometimes mixed with either liquorice drops, boiled milk with nutmeg, or aloe vera pulp with brown sugar and cinnamon (Morton 1968). Locally, the juice is extracted from the fruit by heating sliced pulp in a large vat without the addition of other ingredients. After cooling, the juice is bottled for sale or consumed immediately (Tacio 2015). The sale and consumption for this product in the Philippines is based solely on its alleged effects and unverified testimonials.

There is little published information on the secondary metabolites produced by the Philippine variety of *C. cujete*. In order to fill the knowledge gap, this research aimed to investigate the secondary metabolites from local *C. cujete* to complement the vast array of hypothesis-driven studies that continuously explored its medicinal properties. To achieve this, gas chromatography-mass spectrometry (GC-MS) analysis was used to obtain the untargeted metabolite profile of the volatile organic compounds extracted from the fruit pulp and the commercially available juice. Additionally, ultra-high performance liquid chromatography high-resolution mass

spectrometry (UPLC-HRMS) analysis was done to profile the ethanol extract from the fruit juice.

## MATERIALS AND METHODS

### Sample Preparation

*C. cujete* fruit samples were obtained from Sasa, Davao City (7.1273°N, 125.6531°E) and from the DOST-PTRI compound in Bicutan, Taguig City (14.4908°N, 121.0493°E). Commercial fruit juice purchased from Davao City was also analyzed for its metabolite content. Steam distillation was done to extract volatile organic compounds from all samples and subsequently followed by extraction with *n*-hexane (JT Baker) in 1:1 volume ratio. The organic layer was collected and concentrated *in vacuo* and then subjected to GC-MS analysis.

The fruit juice from Davao City was filtered with Whatman® Grade 3 (6 µm pore size) filter paper, followed by 0.4 µm syringe filter. This step was necessary because the juice contained a lot of residue. HPLC-UV grade absolute ethanol (Merck) was added to the filtered juice in a 1:1 volume ratio. The set-up was allowed to equilibrate for 30 min before *in vacuo* concentration. Dried extracts were resuspended in 100% LC-MS grade methanol (Merck LiChrosolv) to a concentration of 1.0 mg/mL.

### Gas Chromatography Mass Spectrometry (GC-MS)

A Varian 450 GC-240 MS (ion trap mass analyzer) system with a Zebtron ZB-WAXplus (length: 30 m; internal diameter: 0.25 mm; film thickness: 0.25 µm) column was used to establish the metabolite profile of *C. cujete* fruit. GC parameters were set as follows: injector temperature at 240 °C, injection volume of 5.0 µL, and a split ratio of 20. Initial column temperature was at 50 °C held for 2.0 min, increased to 150 °C at a rate of 20 °C/min and held for 5.0 min, then increased to 200 °C at 40 °C/min and held for 10 min, and finally increased to 250 °C at 60 °C/min and held for 10 min. The total analysis time for each run is 45 min with a column flow rate of 1.0 mL/min. For the MS parameters, scan range was set from 50 m/e to 500 m/e, with 70 eV ionization at 0.41 s per scan.

Data acquisition and analysis were done through Varian MS Workstation software version 6.9.1 (2008, Varian Inc.) via MS Data Review, Automated Mass Spectral Deconvolution and Identification System (AMDIS) II. The software was equipped with a National Institute of Standards and Technology Mass Spectral Search Program (NIST MS Search) version 2.0f (NIST Scientific Instrument Services, Inc., NJ, USA).

### LC-MS and MS/MS Analysis

Untargeted LC-MS/MS analysis of the fruit juice was performed using a Waters Acquity UPLC® H-Class System with a Xevo® G2-XS Quadrupole Time-of-Flight (QToF) high-resolution mass spectrometer. For LC separation, an Acquity UPLC® CSH Fluoro-Phenyl column was used (particle size 1.7 µm, 2.1 x 50 mm in length). LC parameters were set as follows: temperatures for the column and the sample manager were set at 40 °C and 15 °C, respectively. Injection volume was 2 µL and a flow rate of 0.35 mL/min was maintained throughout the run. LC gradient at 0–1 min is 75% water: 25% methanol and maintained for 3 min. It was raised to 50% water: 50% methanol for 1 min and sustained for another minute for a total run time of 5 min. Both solvents were spiked with 0.1% formic acid to enhance ionization.

MS/MS analysis was done in positive data dependent acquisition (DDA) mode. Analyzer was operated in sensitivity mode. Capillary voltage was set to 3.0 kV sampling cone to 88 V and source offset to 80 V. Temperatures for desolvation and source were optimized to 500 °C and 150 °C, respectively. Desolvation gas flow rate was 1000 L/h. The low mass collision energy ramp started at 8 eV and ended at 15 eV; for the high mass, the ramp began at 24 eV and ended at 50 eV. Energy ramps were optimized according to the sample.

### Preliminary Assessment of Antimicrobial Activity

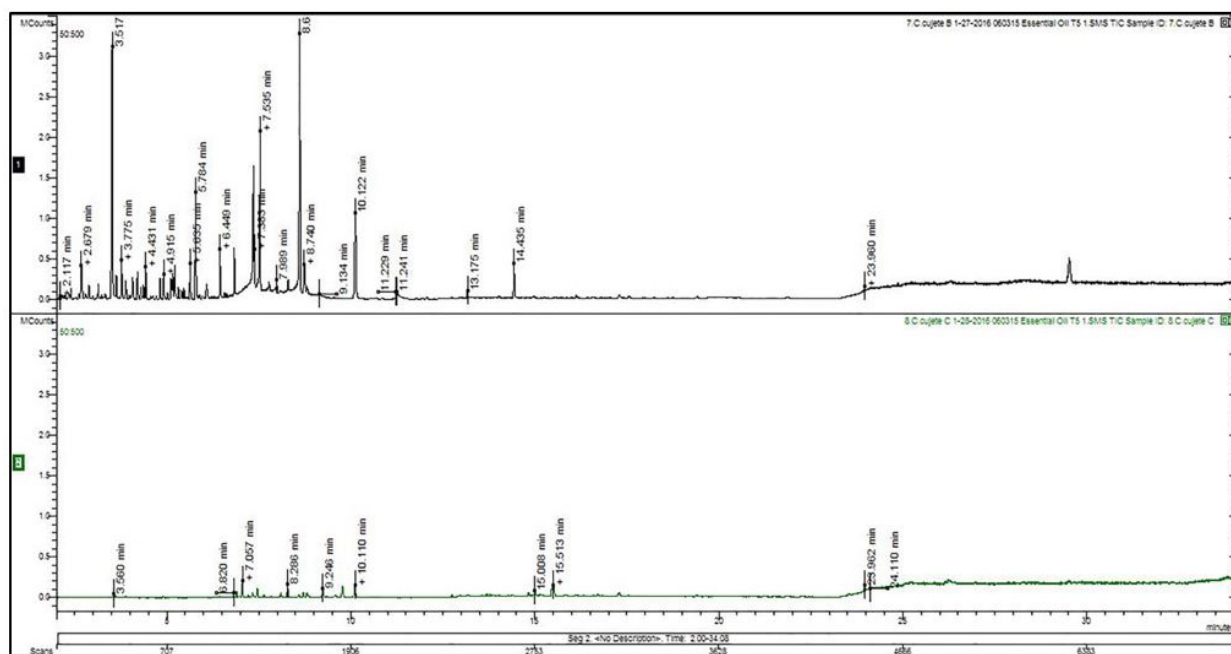
Hexane extracts of *C. cujete* distillates were submitted to the Microbial Research and Services Laboratory of the University of the Philippines – Natural Sciences Research Institute (UP-NSRI) for antimicrobial assay. Pre-poured plates for bacteria (nutrient agar) and yeast (glucose yeast peptone) were inoculated with test microorganisms *Escherichia coli* UPCC 1195, *Staphylococcus aureus* UPCC 1143, and *Candida albicans* UPCC 2168. Three (3) equidistant wells 10 mm in diameter were made in each plate and loaded with 200 µL sample. The inoculated plates were incubated at 35 °C and observed after 24 h. Antimicrobial activity was based on the antimicrobial index (AI) obtained using the equation below.

$$AI = \frac{\text{clearing zone diameter (in mm)} - \text{well diameter (in mm)}}{\text{well diameter}} \quad (1)$$

## RESULTS AND DISCUSSION

### Results

Optimized run method for GC-MS with ion trap mass analyzer produced the best chromatograms shown in Figure 2. A comparison between the chromatograms with normalized intensities showed that more compounds in higher intensities were present in the fruit pulp than in the commercial juice. This could be due to the processing done by the manufacturer,



**Figure 2.** GC-MS Profiles of *C. cujete* samples. Stacked chromatograms of the organic extracts of the fruit pulp (A) and the commercially available fruit juice (B). More volatile metabolites were detected in the fruit pulp versus the juice due to the pre-processing done before bottling of the commercial sample.

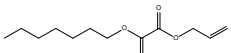


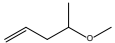
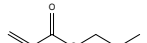







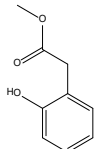
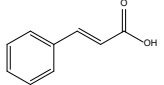
which may have removed majority of the volatile compounds seen in the fruit pulp. Identities of constituents detected were assigned based on the NIST Spectral Library following manual peak deconvolution by AMDIS.

To determine the identity of the compound that corresponds to the peak of interest, NIST MS Search hit list reports a match factor and a reverse match factor (MF and RMF, respectively) – both of which have values that ranged from zero to 999, with 999 being a perfect match. For the purposes of this study, only compounds with RMF values




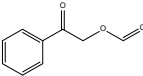
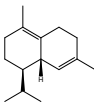
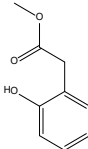
850 or greater were considered as probable identities of the compounds (Clement & Taguchi 1991) (see Table 1 and Table 2).

The chromatogram for the full scan MS optimized for the sample (Figure 3) showed a few peaks eluting before 1 min at 75% water gradient, indicating that most of the compounds are highly polar. Peaks eluted at 0.47 min and 0.53 min were not baseline resolved but both with  $m/z$  527.164, indicating the presence of structural isomers. Based on the full scan MS of these unresolved peaks

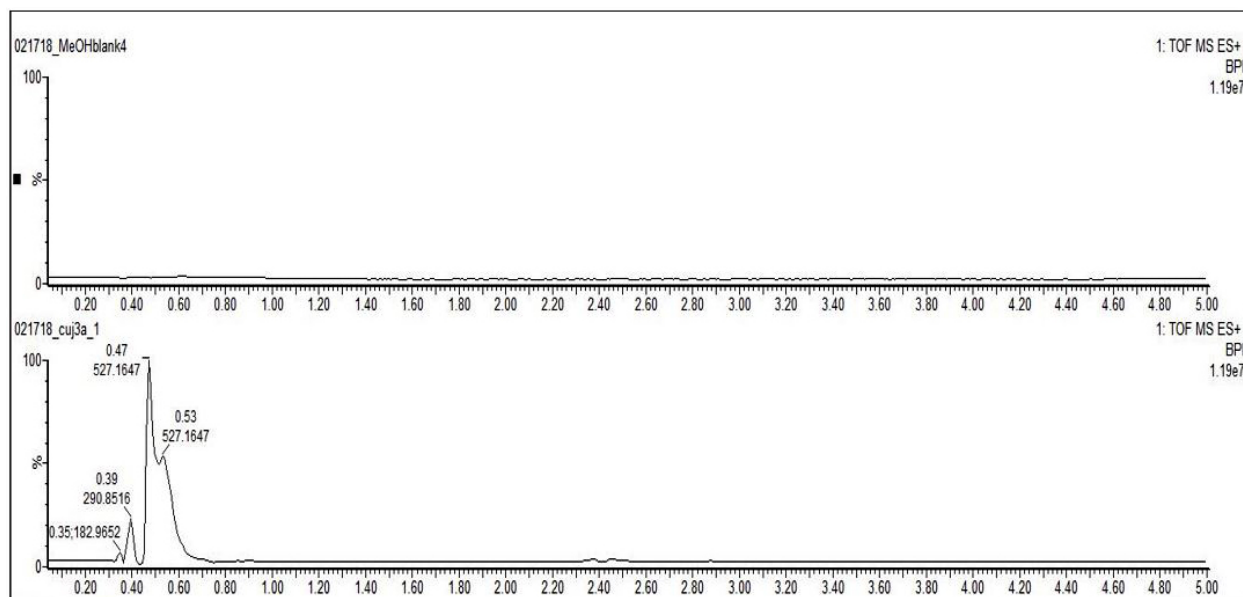
**Table 1.** Identities of compounds from *C. cujete* fruit pulp.

RT (min)	ID	RMF <sup>a</sup>	Structure	% Composition
2.398	oxalic acid, allyl heptyl ester	851		0.013
2.678	benzene	899		2.23
3.561	toluene	903		16.134
4.071	4-methoxy-1-pentene	925		1.412
4.206	<i>n</i> -propylacrylate	906		1.585
4.423	1,3-dimethylbenzene	898		0.668
4.817	<i>o</i> -xylene	907		1.139
5.160	1-ethyl-2-methylbenzene	902		1.585
5.214	1-methylcyclopentanol	887		1.554
5.326	1,3,5-trimethylbenzene	881		0.521
5.635	1,2,3-trimethylbenzene	880		1.719
8.301	2-hydroxy-1-phenylethanone	929		0.572
10.121	methyl salicylate	918		8.541
14.436	3-phenyl-2-propenoic acid	911		2.168

**Table 2.** Identities of compounds from *C. cujete* fruit juice.

RT (min)	ID	RMF <sup>a</sup>	Structure	% Composition
3.890	thiazole	923		1.99
7.462	phenylglyoxal	901		5.962
8.286	2-hydroxy-1-phenylethanone	930		9.66
8.594	2-(formyloxy)-1-phenylethanone	947		8.953
9.778	(+)- $\delta$ -cadinene	851		12.869
10.111	methyl salicylate	930		13.091

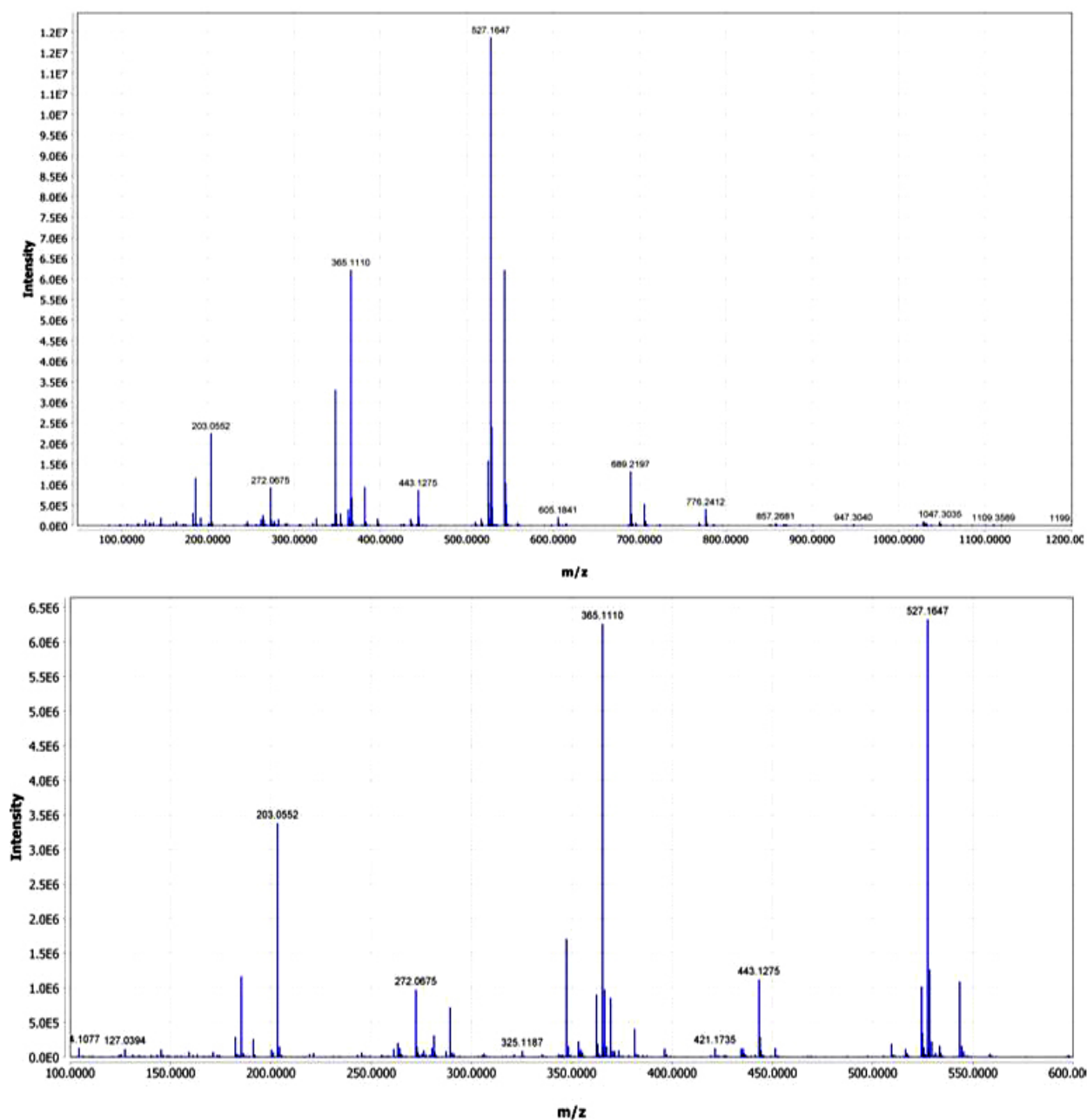
<sup>a</sup>Reverse Match Factor was used since it disregards the peaks present in the sample spectrum that are not in the library spectrum, ensuring that the mass spectrum of the peak of interest correlates only to a single compound (Clement & Taguchi 1991). Reverse search is preferred when analyzing complex mixtures containing significant background noise and where individual components are not completely separated by the GC column (Clement & Taguchi 1991). In reverse search, a compound is identified through a residual spectrum that results from the subtraction of the reference spectrum from the unknown. This results to noise reduction and improvement of the signal-to-noise ratio of the target peak (Clement & Taguchi 1991).



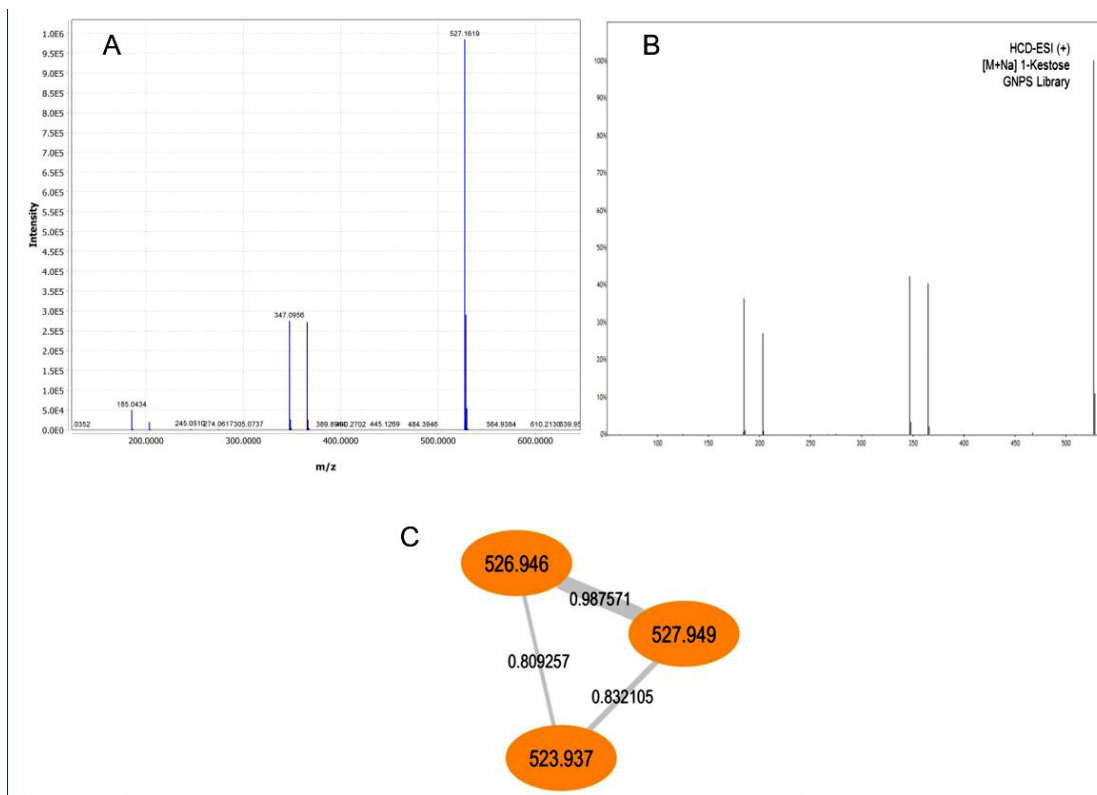
**Figure 3.** Chromatographic profile of the solvent blank (top) and ethanol extract of *C. cujete* juice (bottom). Profile shown above indicated the presence of few ethanol-soluble metabolites in the juice sample. Peaks with  $m/z$  527.165 at 0.47 min and 0.53 min are probable structural isomers of 1-kestose based on the MS/MS fragmentation pattern.

(Figure 4), there are several other compounds co-eluting with the isomers but present at lower intensities. Tandem MS or MS/MS data generated from a data-dependent analysis (DDA) was used to identify the presence of two putative compounds in the *C. cujete* juice. Analysis of the tandem MS spectra through the GNPS Molecular Networking Platform utilized fragmentation pattern of the compounds in the juice, and were matched to those of two reference carbohydrates in the database. 1-Kestose and sucrose were putatively identified to be present in

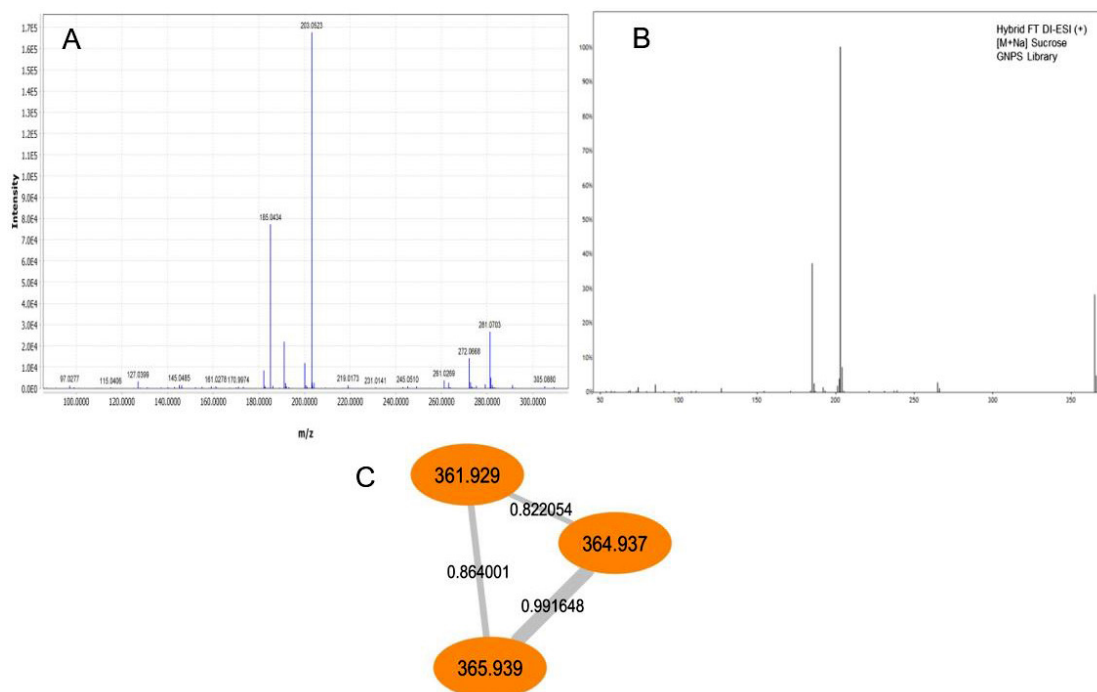
ethanol extract of the fruit juice. Alignment of the MS/MS peaks from the sample with that of the reference spectra results in a molecular network. Within the network, one node corresponds to one shared MS/MS spectrum. This similarity is empirically determined with a cosine or similarity score ranging from 0 (no match) to 1 (same spectrum). Examples of visualization of the molecular network for both 1-kestose and sucrose – which used the fragmentation pattern generated by MS/MS – are shown in Figure 5 and 6, respectively. In the nodes are the precursor



**Figure 4.** Full scan MS of compounds eluting at 0.47 min (top) and 0.53 min (bottom). Intensities are relatively higher for ion peaks detected earlier than later. Similar peaks with different intensities based on the elution profile indicated the presence of structural isomers of 1-kestose. Most of the peaks corresponds to the mSacNa<sup>+</sup> complexes together with the microhydrated form mSacNa<sup>+</sup>(H<sub>2</sub>O).



**Figure 5.** Comparison of 1-kestose MS/MS profiles of the juice (A) and the reference (B) with a sample visualization of a molecular network (C). Parent ion at m/z 527.162 (1KesNa<sup>+</sup>) with product ions m/z 365.110 [SucNa<sup>+</sup>(H<sub>2</sub>O)], 347.096 (SucNa<sup>+</sup>), 203.052 (GluNa<sup>+</sup>), and 185.043 (Glu-18.010)Na<sup>+</sup>. The cosine score between the sample and the GNPS reference spectrum is 0.91.



**Figure 6.** Comparison of sucrose MS/MS profiles of the juice (A) and the reference (B) with a sample visualization of a molecular network (C). Parent ion at m/z 365.110 [SucNa<sup>+</sup>(H<sub>2</sub>O)] with products ions m/z 203.052 (GluNa<sup>+</sup>) and 185.043 [(Glu-18.010)Na<sup>+</sup>]. The cosine score between the sample with the GNPS reference spectrum is of 0.94.

masses detected from the MS/MS spectra of the sample, while the edges or connections between nodes (gray lines with thickness relative to the cosine score) represent the similarity between the precursor ions. Cosine scores have values ranging from 0 to 1, with 1 pertaining to absolute similarity (Yang *et al.* 2013). For the putatively identified 1-kestose (0.91) and sucrose (0.94), the cosine scores indicated very high similarity.

Distillates of *C. cujete* fruit pulp and juice showed activity against *Candida albicans* in an antimicrobial disk diffusion assay (Table 3). From Table 3, the commercial juice from *C. cujete* showed antimicrobial activity against all test organisms compared to the distillate from the fruit pulp. Moreover, the fruit juice distillate showed more potent inhibition of Gram-positive *S. aureus* and Gram-negative *E. coli*. However, the fruit pulp distillate was more effective against *C. albicans*. Comparisons between the two samples were based on the antimicrobial index (AI), which is a ratio of the difference between the clearing zone diameter and the well diameter divided by the well diameter. As the degree of clearing is taken as proportional to inhibition, higher AI values indicate good inhibition. Moreover, it is possible to exceed the AI value of the standard, which would then indicate that the sample exhibits better inhibition than the standard compound tested.

## DISCUSSION

Compounds found to be common between fruit pulp and juice samples were 2-hydroxy-1-phenylethanone and methyl salicylate. For the *C. cujete* fruit pulp, the most abundant peaks identified with high confidence included toluene and methyl salicylate. Benzene derivatives and acid esters were also identified. Analysis of the juice

revealed the presence of compounds structurally related to those from the fruit pulp. Aside from benzenoids (phenylethanone and methyl salicylate), naturally occurring compounds – such as the bicyclic sesquiterpene, (+)- $\delta$ -cadinene, and thiazole – was also found in the fruit juice sample.

Most of the compounds in Tables 1 and 2 have reported bioactivities. Oxalic acid derivatives and esters are by-products of plant and animal metabolism. These compounds contribute to the aroma of plants and essences while, at the same time, helping maintain visual quality and antioxidant potential of fruits after long-term storage (Sayyari *et al.* 2010). Naturally occurring oxalic acid and its derivatives also play a role in antifungal and disease suppression as was observed in *P. resinosa* seedlings (Duchesne *et al.* 1989). As a natural antioxidant, oxalic acid also reduces lipid peroxidation *in vitro* (Kayashima & Katayama 2002) and acts as a detoxification mechanism for plants with high aluminum resistance such as buckwheat – enabling them to thrive in acidic soils (Zheng *et al.* 1998).

Benzene and its derivatives such as toluene and *o*-xylene are known contaminants in industrial areas, although natural benzene and benzenoids are important precursors in the synthesis of secondary metabolites. Exposure to benzene and its derivative compounds is associated with a range of acute and long-term adverse effects, which includes narcosis, decreased host resistance to infection, cancer, and aplastic anemia (WHO 2010, IARC 2009). Uptake of aromatic hydrocarbons by plants had been documented and studies on this particular phenomenon are important in identifying detoxification mechanisms of plants, including the metabolism of natural benzene derivatives (Ugrekheldze *et al.* 1997). The ability of hypostomatous leaves to take up benzene and toluene from the air, and transform them into non-volatile metabolites

**Table 3.** Results of the antibacterial and antifungal disk diffusion assay.

Test Organism	Sample	Average Clearing Zone (mm)	Antimicrobial Index
<i>Escherichia coli</i>	<i>C. cujete</i> fruit pulp T1	12.0 ± 0.8	0.2
	<i>C. cujete</i> fruit juice T2	20.0 ± 0.0	1.0
	Chloramphenicol disc <sup>A</sup>	27.0 ± 0.0	3.5
<i>Staphylococcus aureus</i>	<i>C. cujete</i> fruit pulp T1	0.0	0.0
	<i>C. cujete</i> fruit juice T2	11.0 ± 0.0	0.1
	Ofloxacin disc <sup>B</sup>	29.0 ± 0.0	3.8
<i>Candida albicans</i>	<i>C. cujete</i> fruit pulp T1	31.7 ± 2.5	2.2
	<i>C. cujete</i> fruit juice T2	29.3 ± 0.9	1.9
	Canesten solution (100 $\mu$ L) <sup>C</sup>	35.0 ± 0.0	2.5

<sup>A</sup>contains 30  $\mu$ g chloramphenicol, 6 mm in diameter

<sup>B</sup>contains 5  $\mu$ g ofloxacin, 6mm in diameter

<sup>C</sup>contains 1% clotrimazole



indicates that leaf cuticle is permeable to aromatic hydrocarbons (Ugrekheldize *et al.* 1997). However, absorption of air-borne hydrocarbons is not isolated to the leaves as accumulation of monocyclic aromatic hydrocarbons in fruits has also been recorded (Górna-Binkul *et al.* 1996). Low concentrations of benzene were detected in apples, kiwifruit, and oranges. Citrus fruits such as grapefruit, lemon, and mandarin – as well as vegetables (cabbages, Brussels sprouts, parsley, and paprika) – were found to have toluene and xylenes in varying concentrations (Górna-Binkul *et al.* 1996).

Accumulation of aromatic hydrocarbons in *C. cujete* can be attributed to exposure to air-borne pollutants during its growth and also during transport, storage, and sale. It must be noted as well that the samples used in this study were obtained from highly populated areas (Taguig City and Davao City), both of which are sites of rapid urbanization and industrialization. This result indicated that *C. cujete* fruit has the potential to bioaccumulate small organic pollutants and serve as air purifier for areas with high concentration of air pollution. Disappearance of these monocyclic aromatic hydrocarbons from the commercial juice can be linked to the pre-processing done to the product by the manufacturer prior to the laboratory-based distillation for GC-MS analysis. The distinct pungent and stinky odor of the fruit pulp during the distillation process is proof of the abundance of volatiles contributing to the aroma of the fruit, and can be perceived as an indication of the presence of benzenoid compounds. However, to identify the specific compounds contributing to the odor of *C. cujete* fruit pulp sample, a targeted approach to profiling must be applied. Because of the low boiling points of these compounds, removal can easily be done by heating the samples before the collection of the fruit juice. This process could attribute to the sweet smell of the commercial juice. Moreover, it was observed that the pulp of the freshly-cut fruit is white but – upon prolonged exposure to atmosphere and room conditions – the pulp visibly darkens with a deep violet hue. This drastic change of color is probably due oxidative processes. Most volatile constituents from plants are prone to oxidation and degradation in room conditions (Grosch 2007), as well as by the action of polyphenol oxidase. This enzyme is present in most fruits that causes the browning of fruit flesh (Araji *et al.* 2014), although the specific metabolite or pigment molecule affected by this oxidation reaction remains unknown.

Other compounds of biological importance detected were thiazole, methyl salicylate, and (+)- $\delta$ -cadinene. Thiazole serves as precursor to the biosynthesis of thiamine along with a pyrimidine heterocycle. Both precursors form separately and then couple to form the co-factor (Raschke *et al.* 2007). Plants, prokaryotes, and fungi

are the main organisms capable of producing thiamine, with plants being the major source of Vitamin B1 in the human diet (Raschke *et al.* 2007). Thiamine in its active form (thiamine diphosphate) has been implicated in tolerance to DNA damage (Machado *et al.* 1996) and as activator of disease resistance in plants (Ahn *et al.* 2005). Methyl salicylate, on the otherhand, is considered to be a common essential oil constituent. This volatile methyl ester is synthesized from the methylation of the free carboxyl of benzoic acid, a reaction catalyzed by benzoid carboxylmethyltransferase (Wildermuth 2006). Methyl salicylate is a major volatile ester from wintergreen, *Gaetheria procumbens* L. (96.90%) (Nikolić *et al.* 2013) and the flower *Viola trusca* (96.00%) (Flamini *et al.* 2002). Because of the minty aroma of methyl salicylate, it has been used as one of the active ingredients of commercial mouthwash (Vlachojannis *et al.* 2015). (+)- $\delta$ -cadinene, meanwhile, is the precursor for phytoalexin production catalyzed by  $\delta$ -cadinene synthase in response to pathogens (Chen *et al.* 1995).  $\delta$ -cadinene is also associated to antibacterial activity against *B. subtilis*, *S. aureus*, *E. cloaceae*, and *K. pneumoniae* (Chen *et al.* 1995).

LC-MS profile of the juice ethanol extract is shown in Figure 3. Few observed chromatographic peaks indicated the low number of ethanol-soluble metabolites present in the juice sample. Full scan MS showed the presence of several metabolites co-eluting, with higher peak intensities for the more abundant ions formed (Figure 4). Each peak in the mass spectrum can correspond to a single molecule or to a fragment of a molecule. Fragment residues can be detected in full scan MS due to in-source fragmentation. Chromatographic peaks at 0.47 min and 0.53 min shared similar mass peaks ( $m/z$  527.162, 356.110, 347.096, 203.146, and 185.043) but with different intensities. Based on information from the elution profile and its correlation with the mass spectra, the data suggested that structural isomers are present in the sample. The use of the UPLC platform with PFP-modified C18 column enabled the partial resolution of these compounds owing to the ionic interaction, hydrogen bonding, dipole-dipole, and  $\pi$ - $\pi$  interactions afforded by the stationary phase. Although the peaks were not baseline resolved, the interaction mentioned above allowed for the observation of the presence of the isomers with  $m/z$  527.162. From the MS/MS spectra generated from the DDA mode, the detected metabolites were putatively identified through GNPS molecular networking. Fragmentation pattern analysis putatively identified the most abundant compound at 0.47 min as 1-kestose, with the equally abundant compound at 0.53 min ( $m/z$  365.110) as sucrose.

1-Kestose differs only from sucrose by one fructose unit. MS/MS fragmentation of 1-kestose (Figure 5) gave the

product ions  $m/z$  365.110, 347.096, 203.052, and 185.043. Sucrose parent ion valued at  $m/z$  365.110, with MS/MS product ions at  $m/z$  203.052 and 185.043 (Figure 6). Fragmentation residues of the parent ion resulted from the cleavage at the glycosidic bonds, forming non-covalent monosaccharide-sodium complexes ( $m\text{SacNa}^+$ ). The presence of microhydrated complexes was also detected. Fragment residue of  $m/z$  347.096 corresponds to sodiated sucrose ( $\text{SucNa}^+$ ), and  $m/z$  203.052 corresponds to the sodiated glucose ( $\text{GluNa}^+$ ). The  $m/z$  365.110 corresponds to the microhydrated complex,  $\text{SucNa}^+(\text{H}_2\text{O})$ . Sodiated fragment due to loss of water from glucose is detected at  $m/z$  185.043. Literature search on kestose revealed that there are three isomeric forms of this molecules found in sugar beets – 1-kestose, 6-kestose, and neokestose – which only differ by the fructose linkage to the sucrose (Draycott 2006). In a previous study (Sims *et al.* 1992), all three isomers were detected from the leaves of *L. temulentum*, with 1-kestose and neokestose as the most abundant and 6-kestose present at lower abundance. Additional five novel kestose isomers were also recently reported to be present in sugar beet molasses (Shiomi *et al.* 2016). Based on the relative abundance of the ions at different retention times, the presence of either one of these isomers can be asserted. However, additional structural analysis is needed to verify the identity of the kestose isomers present.

Fructooligosaccharides like 1-kestose are considered prebiotics (Campbell *et al.* 1997) and, as such, are often used as supplements to promote the growth of a healthy digestive microbiota. Fruits – particularly apples, bananas, and berries – and several onion varieties like shallots and red onions have been reported to contain a large amount of fructooligosaccharides (1-kestose, nystose, and 1- $\beta$ -fructofuranosylnystose) (Campbell *et al.* 1997). Grain-based feedstuff also showed higher fructooligosaccharide content compared to forage-based feeds (Campbell *et al.* 1997). The potential of 1-kestose as a dietary supplement was observed in rats fed with 0.5% to 5% 1-kestose diets (Tochio *et al.* 2016). 1-Kestose induced cecal hypertrophy and alterations in the cecal microbiota composition, including an increase in the cell number of *Bifidobacterium* spp. (Tochio *et al.* 2016). These changes were associated with significant increases in acetate and lactate, and a marked increase in butyrate in cecal contents. Rats fed with 2.5–5% 1-kestose diet also showed remarkable decrease in serum insulin concentration. (Tochio *et al.* 2016). These findings suggest that 1-kestose has the potential to improve the metabolism of the host.

Aside from metabolism, immune response associated with immunoglobulin A (IgA) were observed to increase with BALB/c mice fed with a 5% 1-kestose diet during pregnancy and lactation (Jinno *et al.* 2014). A significant positive correlation was found between the mean count

of *Bacteroides* spp. in maternal feces and the total IgA concentration in maternal milk, suggesting a potential link between the gut and the mammary gland immune system (Jinno *et al.* 2014). Neokestose was reported to have chemopreventive and antineoplastic effect on colorectal cancer cells (Lee *et al.* 2015). Neokestose also inhibits cyclooxygenase-2, an enzyme important in tumorigenesis in colorectal carcinoma cells, which showcases its prospective use as a dietary chemopreventive agent (Lee *et al.* 2015).

Sucrose, on the other hand, is naturally present in fruits and may have contributed to the sweet-tangy taste and smell of the commercial juice.

## CONCLUSION

In summary, this study has shown that volatiles from *C. cujete* fruit juice and pulp vary in both metabolite constituents and bioactivity. Fruit pulp metabolites include methyl salicylate, benzene, benzene derivatives such as *o*-xylene and *p*-xylene, as well as oxalic acid esters. Benzene derivatives were found in the fruit juice as well, in addition to thiazole and (+)- $\delta$ -cadinene, both of which are biologically significant as they serve as precursors in the synthesis of compounds associated with disease and pathogen response. Only 2-hydroxy-1-phenylethanone and methyl salicylate were found to be common between the two samples. These findings offer an interesting insight on the effect of preprocessing step during the manufacture of the fruit juice, as aromatic hydrocarbons such as benzene were not identified from the fruit juice sample. Moreover, the juice distillate revealed significant inhibition against *E. coli*, *S. aureus*, and *C. albicans* while the pulp sample was bioactive only to the latter. This variation in bioactivity can be attributed to the presence of the pathogen response agent, (+)- $\delta$ -cadinene, in the fruit juice sample. UPLC-HRMS with MS/MS analysis of the fruit juice putatively identified two sugars, 1-kestose and sucrose, both of which may be contributory to the aroma and taste of the juice. 1-Kestose is a known prebiotic that has been cited as metabolism and immunity booster. The presence of kestose isomers was also asserted for the juice. A kestose isomer, neo-kestose, was reported to have chemopreventive activity. However, further structural analysis is needed to elucidate the identity of the isomers present in the juice.

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## STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflicting interests.

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