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Detection and identification of phenolic compounds in the rinds of red and yellow watermelon rinds using UHPLC-Q Exactive HF Orbitrap

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Abstract

Watermelon (*Citrullus lanatus)* is a nutraceutical fruit that contain nutrients and many phytochemicals. Consumption of watermelon juice was known to be connected to reduced risk of certain cancers, obesity, diabetes, aging-related disorders and cardiovascular diseases. Research for bioactivity and nutritional composition of watermelon cultivars had always been on pulps and the seeds of the fruit. The rinds are always thrown as carcass after consumption. Little or no attention is given to the phytochemical and nutritional worth of the rinds. In this research bioactive phenolic compounds were investigated in rinds of red and yellow watermelon cultivars using Dionex UltiMate 3000 UHPLC system in tandem Q-Exactive HF Orbitrap. Sinapic acid glucoside, protocatechuic acid glucoside, p-coumaric acid glucoside, salicylic acid-*O*-hexoside, ferulic acid hexoside and 6-gengerol were among the putative phenolic compounds detected and identified, Major peaks on some of the compounds' MS/MS ions spectra were assigned their fragment ions via their molecular ions' fragmentation mechanisms. The findings from the research could reverse the views of people on the watermelon rinds and invigorate thought on how to use the fruit rinds for nutraceutical purpose.

Keywords: Phenolic compounds, MSMS spectra, glycosyl ring, decarboxylation, precursor ions and fragment ions

Introduction

Fruits and vegetables are generally sources of primary and secondary metabolites that are of great nutritional, nutraceutical, and pharmaceutical value [1]. In general, nutraceutical fruits aside from being nutritious, contain metabolites that are potential candidates for prophylactic and/or therapeutic effects of various diseases [2].

Watermelon (*Citrullus lanatus)* belongs to *Cucurbitaceae* family and is the third most popular fruit in the world [3] [4]. Watermelon fruits are found in several colours such as white, salmon yellow, orange, pale yellow, canary yellow, crimson red, and scarlet red and the size, shape, colour, fleshiness, texture, sweatiness, aroma and flavour are the regulatory criteria for the fruit's high-quality and medicinal robustness [5]. Many bioactive substances found in watermelon have been shown to have a range of positive health effects including lowering the risk of obesity,

diabetes, and aging-related disorders, as well as reducing the risk of cardiovascular problems [6] [7]. The presence of substantial phytochemicals with pharmaceutical effects including carotenoids, citrulline, and polyphenolic compounds is fundamental to the fruit's prophylactic and therapeutic effects [1]. The fruit primarily consists of flesh, seed, and rind.

Phenolic compounds consist of broad types of molecules that contain one or more aromatic rings with several hydroxyl groups to the rings. Phenolic compounds were reported to beneficially affect human health and well-being by reducing the risk of degenerative diseases like cardiovascular diseases and some classes of cancer through mechanisms such as free-radical neutralization, protection and regeneration of other dietary antioxidants example vitamin E and the chelating of pro-oxidant metal ions [8]. Special interest has since been given by the researchers' community to the area of research involving detection, characterization and isolation of bioactive phenolic compounds from various plant-derived foods.

Watermelon consists of the seeds, rinds and the pulps but researches for the many phytochemicals the fruit contains have always been on the pulps. Although, watermelon pulp is considered as the edible portion of the fruit because of its fleshiness, more sugar content and appealing colours however, not many people know that watermelon rind could also contain nutrients and phytochemicals that may worth its consumption. In this research, the rinds of a yellow and red watermelon cultivars were investigated for the presence of some bioactive phenolic compounds to reveal their possible medicinal potentials.

Materials and Methods

Chemicals and reagents

LCMS-grade water, was purchased from Merck Millipore (Darmstadt, Germany). All the reagents used for the analysis were Optima™ LC/MS Grade, Fisher Chemical™

Plant materials and biomass procurement

Fully grown red and yellow watermelon cultivars of 3 months of age were cultivated from a conventional farm in Jenderam Hulu, Sepang, Selangor, Malaysia in May 2019. The fruits were washed and rinsed thoroughly with tap water followed by distilled water. The rinds were removed and immediately ground with the blender (Panasonic, 500Ml tumbler. Model No. PSN-MXGM0501), transferred into zip-lock bags and froze in -80^{0}C. The frozen samples were then freeze-dried (lyophilised)

Extraction for LCMS analysis

To obtain the extract, 5g of freeze-dried samples were extracted each in 100ml Millipore water using ultra-sonication for 30min at less than 40^{0}C in three consecutive times. The pooled extracts were filtered through muslin cloth and the filtrate was obtained in a container and stored at -80^{0}C. Finally, the frozen aqueous filtrates were then freeze-dried and returned to -80^{0}C until analysis.

LCMS analysis

The chromatographic analysis was performed using Dionex UltiMate 3000 UHPLC system (Thermo Scientific, MA, USA) with a column temperature of 35°, and a flow rate of 0.4 mL/min. The samples (100 mg/mL) were reconstituted in LCM grade water and filtered through regenerated cellulose (Titan3™, Thermo Scientific™) before 10 uL each was chromatographed on Synchronis C18 column (Thermo Scientific, MA, USA) with a dimension of 2.1 mm ID x 100 mm L x 1.7 um particle size. The column temperature was set at 40°C.

All the reagents used were Optima™ LC/MS Grade, Fisher Chemical™. Mobile phase A composed of water with formic acid (0.1%) while mobile phase B was constituted of acetonitrile with formic acid (0.1%). The gradient solvent system was maintained at 0.4 mL/min flow rate, and programmed for B initially by running at 5% for 0 - 2 min, 95% for 2 -12 min, then 95 to 5% for 12 - 13.1 min, and finally with 5% was until 16 min.

MS analysis was carried out using Q-Exactive HF Orbitrap (Thermo Scientific, MA, USA). The analysis was done under negative ionization modes with a mass range of 60-900 m/z, sheath gas flow rate of 60, aux. gas flow rate maintained at 20, sweep gas flow rate at 1, and spray voltage of -3.00kV, capillary temperature was set at 300°C, S-lens RF level of 40 was used and aux. gas heater temperature was set at 370°C.

Results and Discussion

The base peak ion chromatograms of the two samples with the corresponding MS/MS ions spectra of each compound present in the samples were viewed in Thermo X-calibur 2.2 SP1.48 (Thermo Fisher Inc., MA, USA). The two chromatograms were overlaid to enable comparison of the peaks with regard to their retention times and peaks' intensities as shown in figure 1. Identification of each metabolite was done by matching the compound's experimental data: its retention time, mass-to-charge ratio (m/z) of the compound's parent molecular ion or precursor ion (M-H) and m/z of its corresponding MS/MS fragment ions with available data in the online libraries and literature.

The overlaid chromatograms depict the presence of peaks at different retention times and intensities which explained the variability of chemical composition between the rinds of the two watermelon cultivars.

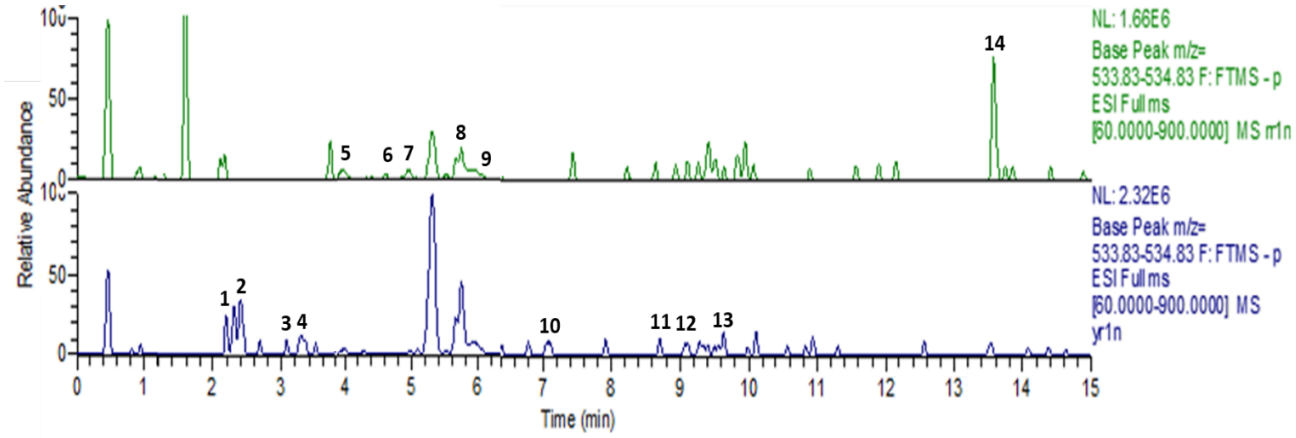


Figure 1: Overlaid ion peaks chromatograms for the rinds of red (green) and yellow (blue) watermelon's extracts.

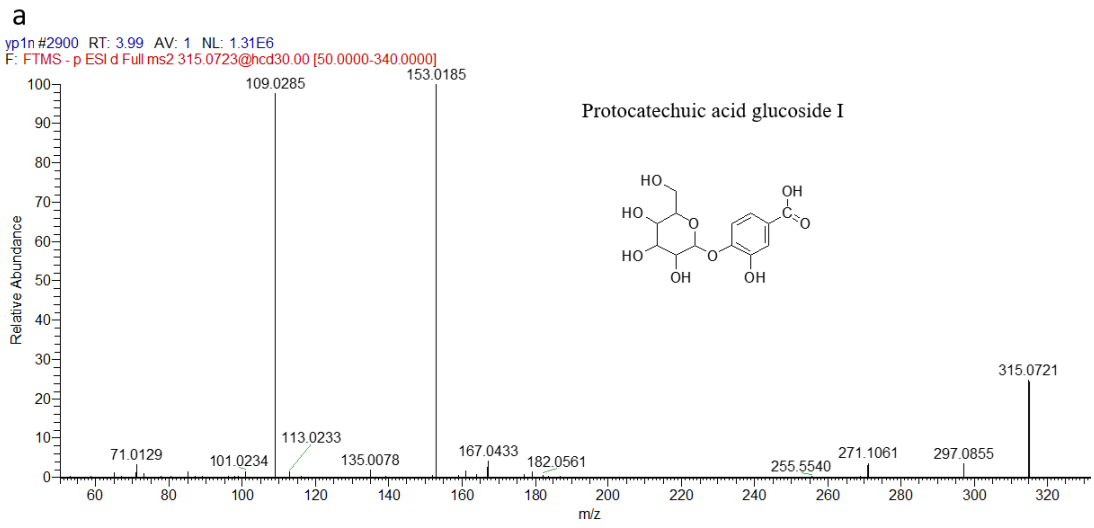
Table 1: Putative metabolites identified in spectra of polar samples of the red and yellow watermelon cultivars acquired by negative ionization mode of MS analysis

No	Putative metabolite	RT	M-H m/z	Major MS/Ms Fragments	Molecular formula	References
	Phenolic glucosides					
5	Protocatechuic acid glucoside I	3.99	315.0721	153.0184, 109.0284	$C_{13}H_{15}O_9$	[11]
6	p-Coumaric acid glucoside	4.73	325.0285	163.0393, 145.0285	$C_{15}H_{17}O_8$	[8]
9	Sinapic acid glucoside	6.07	385.1505	179.1068, 223.0970, 205.1063	$C_{17}H_{21}O_{10}$	[8]
10	Ferulic acid hexoside	7.10	355.1028	193.0500, 175.0392	$C_{16}H_{19}O_9$	[8] [13]
4	Salicylic acid-O-hexoside I	3.34	299.0788	137.0234	$C_{13}H_{15}O_8$	[10]
	Other phenolic compounds					
1	Hydroquinone glucuronide	2.18	285.0613	108.0208, 152.0105	$C_{12}H_{13}O_8$	[8]
2	Leachianol G	2.51	471.1455	355.1021, 193.0499	$C_{28}H_{23}O_7$	[8]
3	Phloroglucinol glucuronide	3.11	301.566	168.005, 125.0234	$C_{12}H_{13}O_9$	[8]
8	Ajugol	5.80	347.1716	3051605, 185.1172, 59.0129	$C_{15}H_{23}O_9$	[8]
13	Vanilloyl catalpol (Picroside) I	9.63	511.1455	349.0926, 193.0498	$C_{23}H_{27}O_{13}$	[9]
14	Obtusoside	13.56	455.2652	231.2111	$C_{21}H_{27}O_{11}$	[8]
12	Saligenin glucopyranoside	9.12	285.0632	123.0442	$C_{13}H1_7O_7$	[10]
7	Caffeoylhexose	4.97	341.0893	179.0553	$C_{15}H_{17}O_9$	[8] [13]
11	6-Gingerol	8.74	293.1756	236.1049, 221.1541, 192.1149	$C_{17}H_{26}O_4$	[8]

Phenolic glucosides

About 14 peaks were putatively assigned to phenolic compounds. Phenolic glucosides such as compound **4** sinapic acid glucoside, **5** protocatechuic acid glucoside, **6** p-coumaric acid glucoside, **9** salicylic acid-*O*-hexoside and **10** ferulic acid hexoside, were detected in this research. As organic compounds that have the bond link

between phenolic structure or aglycone and glycosyl moiety, the major fragments of phenolic acid glycosides may expectedly be due to the two structural portions with slight variation depending on the side bonds. The molecular ions ($M-H^+$) of compounds **4**, **5**, **6**, **9** and **10** : m/z 299.0788, m/z 315.0721, m/z 325.0921, m/z 385.1505 and m/z 355.1028 detected at RT 3.34, 3.99, 4.73 and 6.07 produced major MS/MS fragment ions that were attributable to sinapic acid glucoside, protocatechuic acid glucoside I, p-coumaric acid glucoside, salicylic acid-*O*-hexoside, and ferulic acid hexoside respectively. Protocatechuic acid glucoside occurs as isomers I and II [11], however, the major fragment ions produced by the precursor ion m/z 315.0721(M-H) of compound **5**, were characteristic of protocatechuic acid glucoside I isomer [11] whose peak was present in both chromatograms. The fragment ion peaks in the spectrum of the compound at m/z 297.0855 and m/z 271.0812 observed in the MS/MS spectrum was generated due to elimination of water molecules and CO_2 (dehydration and decarboxylation) from the parent ion respectively. The major fragment m/z 153.0185 may be due to loss of glycosyl moiety. A fragment m/z 109.0285 (benzene-1,2-diols cation) could due to further decarboxylation of fragment ion m/z 153.0185 while a fragment ion m/z 161.0445 with low intensity was probably due to removal of hydrogen from a glycosyl moiety (Figure 2b).



b

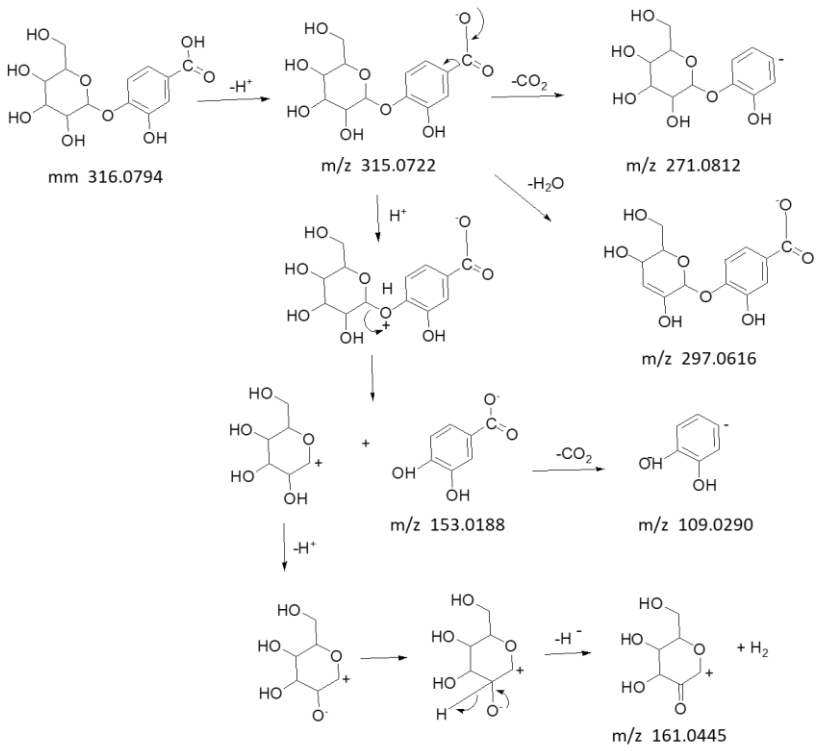


Figure 2 : a. MS/MS spectrum of Protocatechuic acid glucoside I and b. Its proposed mechanism of fragmentation pattern

The peak of compound **6** was only observed in the chromatogram of red watermelon's rind. Its deprotonated molecular ion ($M-H^+$) m/z 325.0921 produced fragment ions that were typical of p-coumaric acid glucoside [12], [13]. Its outstanding base peak at m/z 145.085 could be due to the loss of H_2 from α- and β unsaturation of carbon-carbon double bond of aglycone moiety which detached from the main molecular ion structure by the cleavage between benzene carbon and the oxygen bridge linking the glycosyl group and the benzene. A fragment peak at m/z 101. 0386 may be due to decarboxylation of the aglycone (m/z 145.0285) while m/z 71.0129 was likely due to carbon-carbon cleavage between benzene ring and the β-carbon of the precursor ion (Figure 3b). The removal of CO_2 from the glycosyl ring followed by deprotonation possibly generated an MS/MS ion at m/z 133.0495 and later m/z 89.0233 by carbon-carbon sigma bond dissociation as shown in (Figure 3b). On the other hand, the removal of glycosyl moiety by the cleavage of the bridge between the glycosyl ring and the oxygen atom probably led to the generation of the fragment ion at m/z 163.0393 which then decarboxylated to fragment at m/z 119.0493 whereas, as glucoside, the removal of neural hydrogen molecule from the glycosyl cation may in similar route, be responsible for the minor peak at m/z 161.0445.

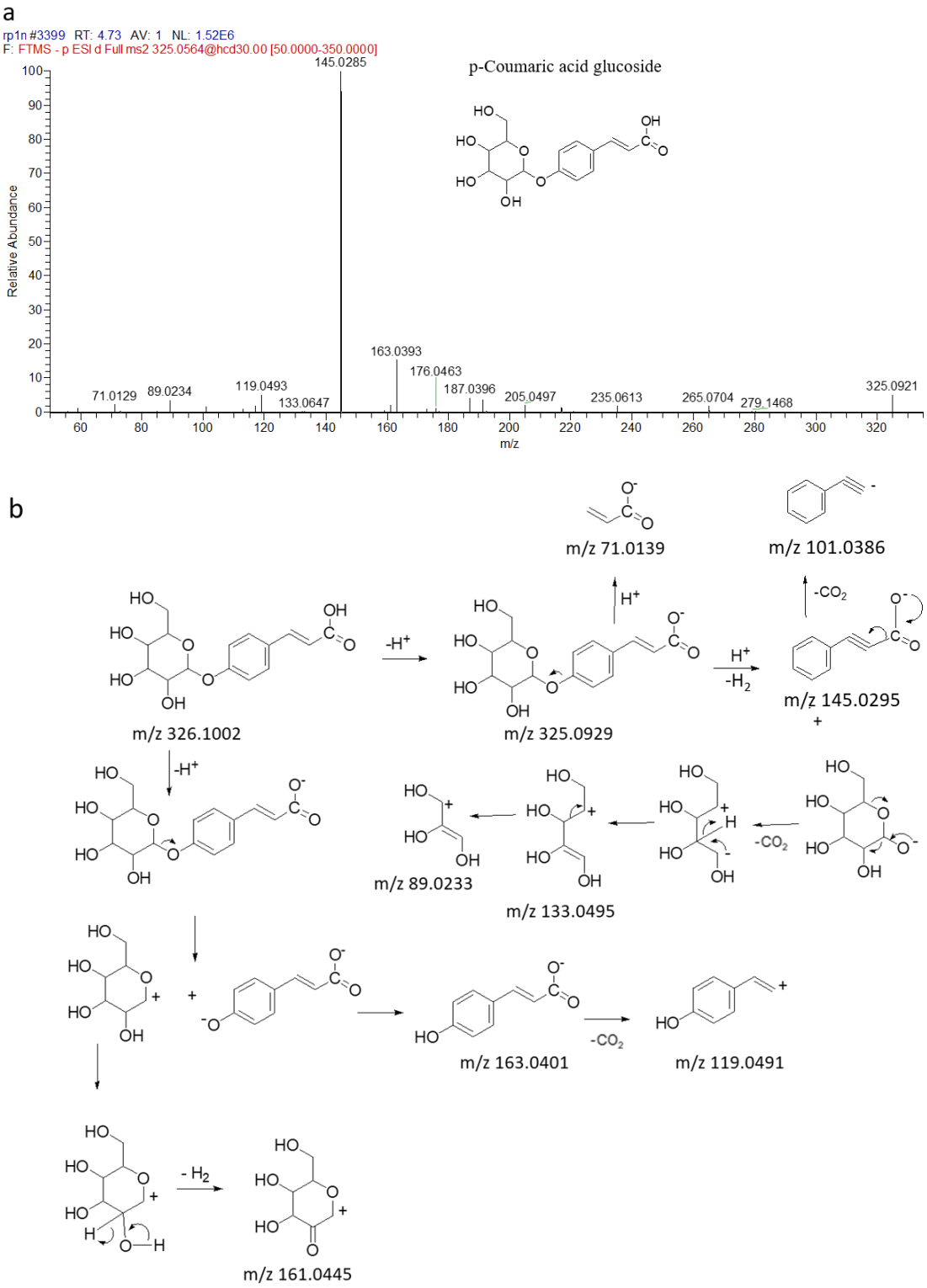


Figure Error! No text of specified style in document. : a. MS/MS spectrum and b. proposed mechanism of p-Coumaric acid glucoside fragmentation pattern

Compound **10** and **4** which both occur only in the rind of red watermelon's sample (Figure 3) were putatively identified as Salicylic acid-O-hexoside I [14] and Ferulic acid hexoside [12], [13]. Their precursor ions; m/z 299.0788 and m/z 355.1028 produced m/z 137.0234 and m/z 193.0500 respectively as their major fragments which were likely due to removal of glycosyl moiety from the precursor ions of both compounds (Figure 4).

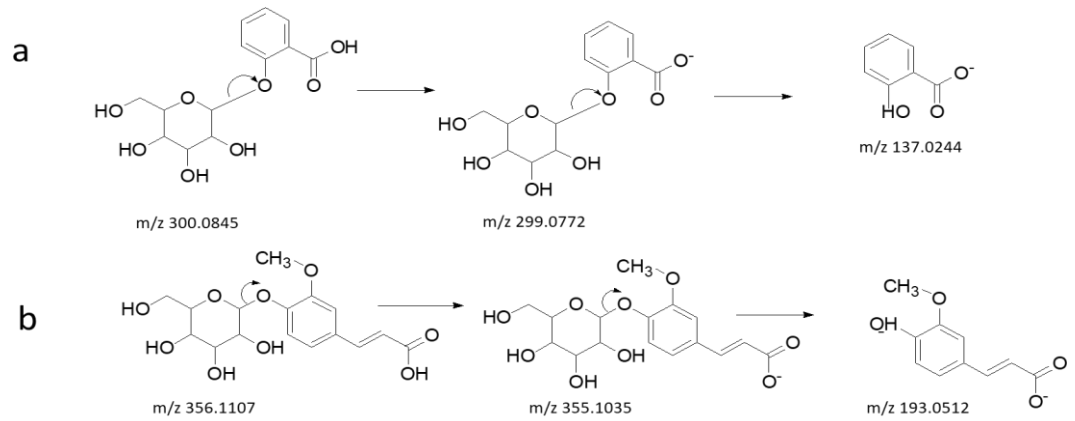


Figure 3: Proposed mechanism of a. Salicylic acid-O-hexoside I b. Ferulic acid hexoside fragmentation pattern

According to an MS/MS spectrum of compound **9** (Figure 5a), fragmentations of its precursor ion m/z 385.1505 produced m/z 179.1068 and m/z 223.0970 as major MS/MS peaks and were reportedly characteristic of sinapic acid glucoside [8]. The fragment ion at m/z 179.1068 was possibly a result of cleavage of the bond linking benzene carbon and oxygen bridge and the peak at m/z 135.1168 may be due to the removal of carbon dioxide from m/z 179.1068 which further produced m/z 89.0233 by neutral loss of ethene-1-ol. On the other hand, the fragments m/z 223.0970 and the glycosyl cation m/z 163.0601 were likely due to cleavage of the bond linking the same oxygen bridge and the glycosyl carbon as shown in Figure 5b. Dissociation between the β-carbon and the aromatic carbon of fragment m/z 223.0970 possibly led to m/z 71.0139 while the removal of hydrogen as the case with the other glucosides from m/z 163.0601produced a peak at 161.0445.

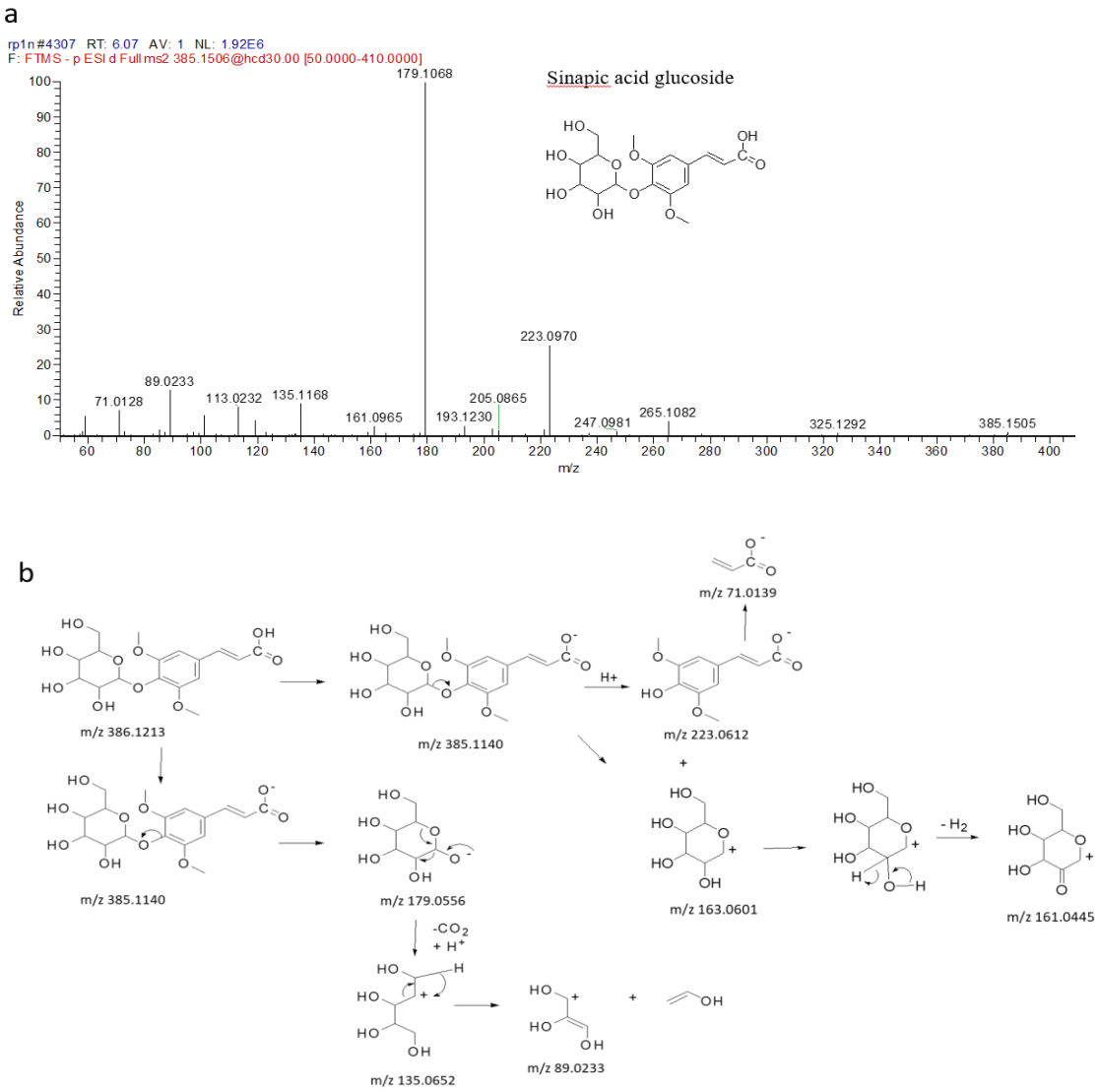


Figure 4 : a. MS/MS spectrum and b. proposed mechanism of Sinapic acid glucoside fragmentation pattern

Compound **8** was detected in both samples and its intense peaks in both samples' chromatograms denoted the compound's reasonable amount compared to other compounds detected. The compound was identified as ajugol. Ajugol is a bioactive, naturally occurring iridoid glycoside that was found in Rehmannia glutinosa's root [15]. It has been proven to be an effective inflammatory agent that inhibits type-2 high cytokine/ chemokine activity [15]. The presence of the compound is detected based on the MS/MS fragments produced by its precursor ion m/z 347.1716. Similar to other glycosides, the fragmentation of ajugol was initiated with the cleavage of the bond between bridging oxygen and the glycosyl carbon (Figure 3.6). The peak at m/z 59.0129 is the strongest as depicted in the MS/MS spectrum and may likely be due to the dissociation of the carbon-carbon bond of

a diol ion m/z 89.0491. A proposed detail mechanism of ajugol fragmentation is given (Figure 3.6). Like other glucosides, ajugol also produced a peak at m/z 161.0447 which is similarly due to dehydrogenation of glycosyl cation.

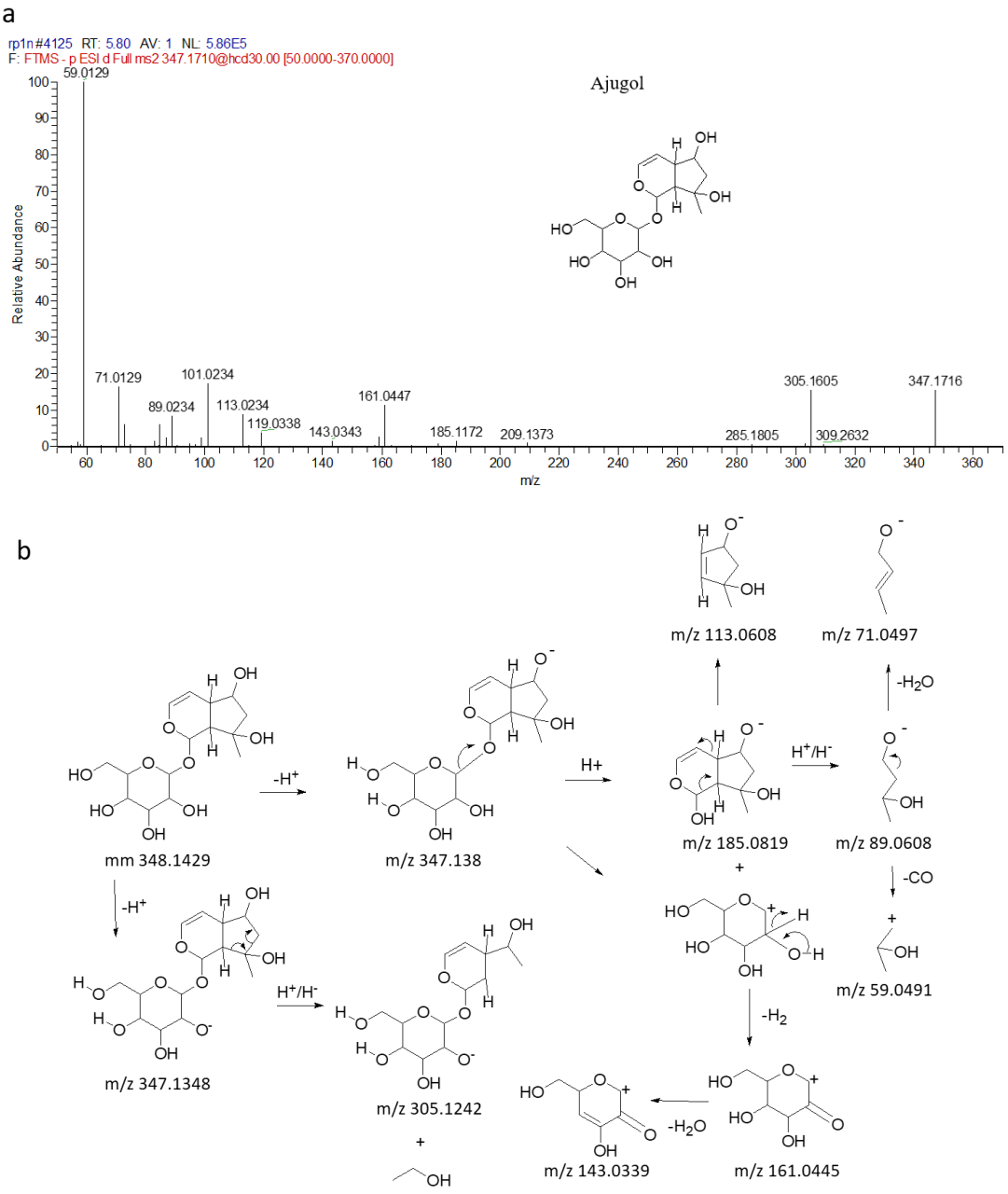
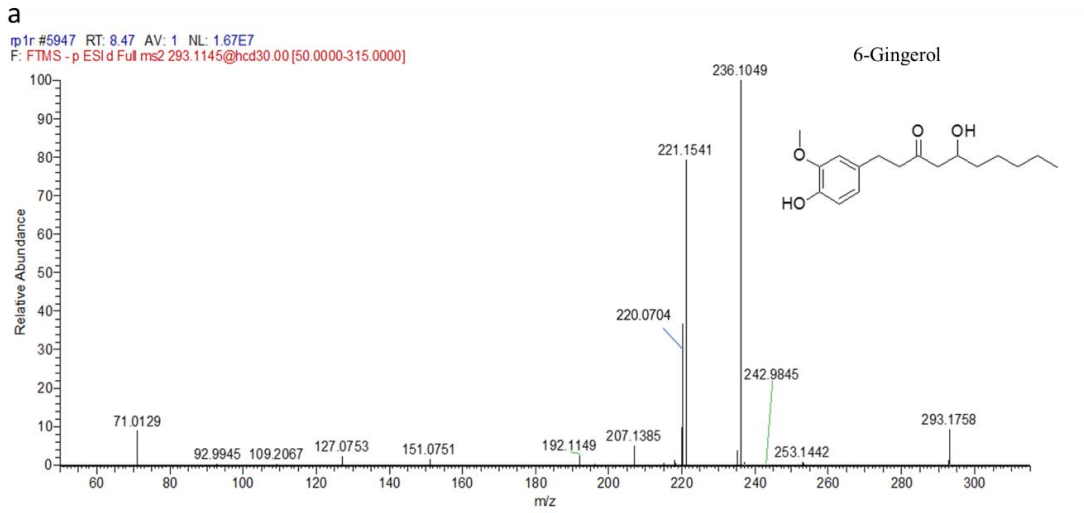


Figure 5 : a. MS/MS spectrum and b. proposed mechanism of the ajugol fragment ation pattern

Compound **11** was detected at RT 8.47 in both samples. Based on its fragmentation pattern, the compound was identified as another bioactive phenolic compound called 6-gingerol. The compound is commonly found in fresh ginger. An in-vivo and in-vitro analysis of 6-gingerol revealed the capacity of the compound as an anticancer, anti-inflammatory, anti-fungal [16], antioxidant, neuroprotective [17] and gastroprotective drug [18]. Attached to a benzene ring, 6-gingerol consists of a ten-membered carbon chain with ketonic and hydroxyl groups at 3 and 5 carbons respectively (Figure 7a). The strong peaks produced by the parent ion m/z 293.1758 included m/z 236.1049, m/z 221.1541and m/z 220.0704 and have been persistent with 6-gingerol [8]. The peak at m/z 236.1049 may have descended from carbon-carbon dissociation between 6 and 7 carbon of the chain while peak m/z 221.1541 likely appeared due to dehydrogenation of fragment ion m/z 223. 0970 that is broken from the parent ion between 5 and 6 (Figure 7).

a



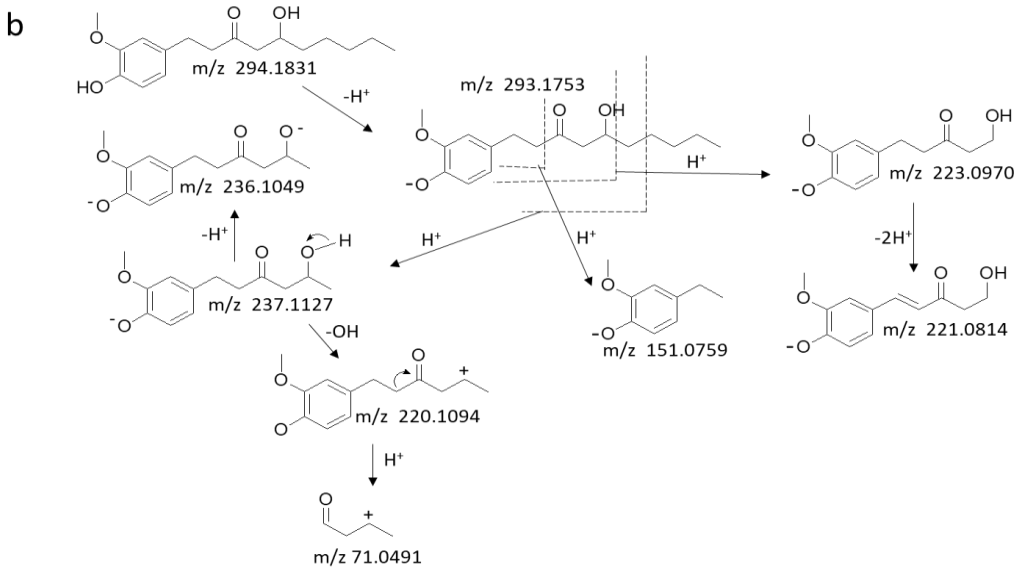


Figure 6: a. MS/MS spectrum and b. proposed mechanism of the 6-gingerol fragmentation pattern

Conclusion

The result revealed the bioactive potential of watermelon rinds and that the fruit is rich in phenolic compounds. It also showed that the phytochemical compounds present in watermelon rinds are distributed based on the watermelon cultivar as some of the phenolic compounds occur in both red and yellow watermelon rinds while other phenolic compounds are present in one and absent in the other one. The findings, in addition, revealed the presence of common peak m/z 161 in the MS/MS spectra of phenolic glucosides which was shown to emanates from glycosyl ring that can stand additional information for the identification of glycosides.

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