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FULL PAPER

Flavonoid and main aroma-active compounds identification of Taiwan *Citrus depressa* Hayata's peels

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Abstract

Citrus peels are a known source of flavonoids and pleasant aroma compounds. Flavonoid compounds in peels of Taiwanese *Citrus depressa* Hayata (shiikuwasha) were extracted using microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE). Different solvents like distilled water, 50% aqueous ethanol, and methanol were used. The qualitative analysis was carried out by observing UV/Vis and FTIR-ATR spectra, and then quantitatively by High Performance Liquid Chromatography – Diode Array Detector (HPLC-DAD). The composition of volatile aroma components was analyzed using GC-HS-SPME (Gas Chromatography, Head Space Solid Phase Microextraction). UV/Vis analysis showed highest intensity at samples that treated by MAE in 50% aqueous ethanol. HPLC DAD quantitatively confirmed UV results by showing that MAE with ethanol showed higher amounts of quercetin, myricetin, rutin, and kaempferol (670.00, 0.36, 8282.52, 7307.51 µg/g), compared to UAE (336.17, 0.20, 5000.62, 4469.15 µg/g), respectively. FTIR-ATR (Fourier-Transform Infrared Spectroscopy - Attenuated total reflectance) showed typical vibrations at phenolic compounds fingerprint area. Unlike quercetin as flavonoid standard, shiikuwasha peels extract showed peak at polymethoxyflavones (PMF) area (2829 – 2835 cm^{-1}). Fresh and dried unripe peels mostly contained limonene (53.01 – 54.79%), γ-terpinene (27.67 – 29.41%), pinene, myricene and cymene (1.61 – 3.20%) as aroma-active compounds.

1. Introduction

Citrus depressa Hayata or shiikuwasha is a popular citrus fruit cultivated in Japan and Taiwan. Shiikuwasha is cultivated to produce beverage products for an abundant source of ascorbic acid and flavonoids. After juice extraction, the peel that constitutes almost 50% of fruit mass is the main fruit waste. Nowadays, food waste is considered a social, economic, and environmental problem due to susceptibility to microbial spoilage because of high moisture content. However, this by-product is a rich source of high value for human health. Citrus peel can be dried and fermented to produce valuable products such as biogas, bioethanol, volatile flavoring compounds, or purified bioactive compounds to develop healthy products. Citrus peels contain more bioactive compounds than pulp. The peels of lemons, oranges, and grapefruit contain higher phenolic and flavonoid compounds than the juice (M'hiri *et al*., 2014; Ferrentino *et al*., 2016; Shiu *et al*., 2016; Papoutsis *et al*., 2017).

Flavonoids are widely distributed in plants as secondary metabolites responsible for pigments, aroma, and flavor of flowers and fruits and protect plants from environmental stresses. Flavonoids are classified into flavanones, flavones, flavonols, flavans, isoflavones, and anthocyanidins, depending upon their chemical structures. Flavonoids have broad health-promoting properties such as antioxidative, anti-inflammatory, anti-mutagenic, anti-carcinogenic associated with various diseases such as cancer, Alzheimer's disease (AD), atherosclerosis, and obesity. Thereby, flavonoids have applications in nutraceuticals, pharmaceuticals, and cosmetics. Citrus fruits are rich in flavanones, flavones, and flavonols (Ledesma-Escobar *et al*., 2016; Panche *et al*., 2016; Zou *et al*., 2016).

A study on bioactive compounds of Taiwan *Citrus depressa* Hayata has already been done to extract particular flavonoids such as nobiletin and tangeretin (Lee *et al*., 2010) and the leaf extract effect in

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aquaculture (Shiu *et al*., 2016). However, identification of other flavonoid and volatile compounds in Taiwanese shiikuwasha using microwave-assisted extraction has not yet been reported. The extraction of phenolic compounds from *C. depressa* is performed using several techniques and solvents depending on their nature and distribution in the plant samples. Extraction solvents and extraction techniques were two main factors that affect the yield of flavonoid extract. Different solubility of each phenolic compound in a particular solvent also affects the nature and bioactivity of the phytochemicals extract (Liau *et al*., 2017; Ngo *et al*., 2017; Dirar *et al*., 2019).

Both microwave and ultrasound-assisted extraction (MAE and UAE) are simple, efficient, inexpensive, and favorable under atmospheric conditions. Microwaves have electric and magnetic fields that cause dipolar rotation and ionic conduction and generate heat and internal pressure. This change could accelerate cell rupture by modification of the physical properties of biological tissues. This allows solvent to diffuse across the sample matrix and collect the phenolic compounds. MAE is relatively new and recognized as a green technology for reducing organic solvent consumption (Lou *et al*., 2014; M'hiri *et al*., 2014; Ferrentino *et al*., 2016; Orsat and Routray, 2017; Papoutsis *et al*., 2017; Macedo *et al*., 2021). Ultrasonication uses ultrasonic waves to interact with subjected plant material and create cavitation bubbles on the cell wall. The cavitational effect increases the release of extractable compounds. However, using UAE at high temperature and high ultrasonic power could degrade the phenolic compounds (M'hiri *et al*., 2014; Nipornram *et al*., 2018).

The present study aimed to discover favorable solvent (distilled water, ethanol, methanol) and microwave-assisted extraction method for flavonoid extraction in shiikuwasha, compared to specific UAE method. For preliminary research, flavonoid extraction on different shiikuwasha particle sizes, parts, and fruit ripeness using UAE was also analyzed. To minimize the use of chemicals for analysis, total phenolic compounds were identified qualitatively by observing UV spectra intensity from extracted samples diluted in the solvent. Sample with the highest UV spectra intensity was recorded then qualitative and quantitative analysis by FTIR and HPLC DAD. For additional data, volatile compounds in shiikuwasha peels were identified by Gas Chromatography, Head Space Solid Phase Microextraction (GC-HS-SPME).

2. Materials and methods

2.1 Sample preparation

Fruits from *Citrus depressa* Hayata plants grown in Pingtung, Taiwan, were collected in summer 2020. Fresh fruits were extracted immediately or stored at 4°C. Most of the samples were dried at 45°C to constant moisture content. The pulps and peels were pulverized in a domestic blender, sieved into 20 and 50 mesh, sealed, and stored at ambient temperature in desiccators until further processing.

2.2 Extraction methods

2.2.1 Microwave-assisted extraction

Microwave treatments were performed by the method of Macedo *et al*. (2021) with modification. Dried samples (0.2 g) was suspended in 20 mL of distilled water, 50% aqueous ethanol, methanol (Sigma Aldrich St. Louis, MD, USA). Distilled water was purified using a Sartorius arium pro (Göttingen, Germany). The mixture was heated in CEM MARS 6™ Microwave Digestion and Extraction System (CEM Corporation, Matthews, NC, USA). The microwave temperature, time, and power setting variations were 50°C, 10 mins, 600 W; 60°C, 20 mins, 800 W; 70°C, 30 mins, 900 W. Extracts were filtered and concentrated using a rotary evaporator.

2.2.2 Ultrasonic-assisted extraction

Ultrasonic treatments were performed by the method of Lee *et al.* (2010). Dried sample (0.2 g) was suspended with 20 mL distilled water, 50% aqueous ethanol, and methanol (Sigma Aldrich St. Louis, MD, USA) by ultrasonication (Ultrasonic water bath Delta DC400) for 4 hrs at 40°C. The extract was filtered and concentrated using a rotary evaporator.

2.3 Flavonoid extract characterization

2.3.1 UV spectra analysis

UV spectra of *Citrus depressa* H. extract were analyzed by the method of Cordenonsi *et al*. (2017). Briefly, 100 µL extract was diluted in 5 mL solvent (dH_2O, ethanol, methanol) and the spectra observed from 190 – 1100 nm wavelength using UV/Vis Perkin Elmer Lambda 265, and a quartz cuvette with 1 cm optical path. Quercetin standard (0.01 mg/mL) in solvents was used to identify flavonoid in the extract.

2.3.2 Infrared spectroscopy

Dried samples were analyzed using FTIR-ATR spectrometer Perkin Elmer. Dried peels extract (0.5 mg) and quercetin standard was poured on the ATR crystal. The reading was performed in the region of 4000 – 600 cm^{-1} spectrum. After each sample, the crystal was rinsed with acetone and then wiped and dried with a soft tissue.

2.3.3 HPLC DAD analysis

The phenolic profile of the extracts was evaluated by

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the method of Macedo *et al*. (2021) with modification. High-performance liquid chromatography system coupled with a UV/Vis photo diode-array detector (HPLC-DAD, Shimadzu Prominence-I, LC 2030C 3D plus) and an SGE C18G column (5 µm, 4.6 mm × 250 mm) at a flow rate of 1.0 mL/min. The mobile phase consisted of 2.5% formic acid (Merck Company, Darmstadt, Germany) in water (v/v, phase A) and acetonitrile as phase B (Merck Company, Darmstadt, Germany). The elution gradient consisted of: 5% B at 0 min; 5% B at 10 mins; 15% B at 35 mins; 30% B at 70 mins; 95% B at 71 mins 95% B at 95 mins; 5% B at 96 mins; 5% B at 120 mins. The injection volume was 20 µL, and DAD signals were recorded at 360 nm. Stock solutions of individual compounds (Myricetin, quercetin, kaempferol and rutin were purchased from Sigma-Aldrich Company St. Louis, MO, USA) were prepared in methanol and used to generate a standard mixture in methanol. Using this mixture, a five-point linear calibration curve in methanol:water 1:1 (v/v) was developed. Identification of flavonoids was carried out by comparing retention times and spectral data of the separated peaks with those of authentic standards.

2.3.4 GC-HS-SPME analysis

Aromatic compounds of *Citrus depressa* H. peels were identified using GC-HS-SPME by the method of Łyczko *et al*. (2019). Briefly, 2 g of fresh and dried shiikuwasha peels were enclosed in the sample vials, and the PDMS/DVB (Polydimethylsiloxane/Divinylbenzene) fiber was exposed to the vial headspace for 30 mins. The sample temperature was kept at 50°C during the equilibration and extraction steps, using a heated circulating bath. The extracted analytes were immediately desorbed in the injection port of the GC–FID at 250°C; the fiber was kept in the GC injector for 15 mins to ensure total desorption and to avoid inter-run carryover.

3. Results and discussion

The preliminary research found that dried peel samples with 50 mesh (300 µm) particle sizes are more favorable compared to 20 mesh (841 µm) particle size for phenolic compound extraction (data not shown). In several earlier studies, drying of the samples was done to stabilize the raw material and optimize the extractable phenolics. Excess amount of water left in the sample could lead to biochemical and microbiological degradation (M'hiri *et al*., 2014; Alara *et al*., 2019; Dobrinčić *et al*., 2020). The amount of extracted flavonoid was analyzed qualitatively by observing the UV/Vis spectra at 190 – 1100 nm wavelength compared to standard quercetin spectra. Quercetin is abundant in fruit (especially citrus), green vegetables, nuts, and berries, that is used as a flavonoid standard in research on medicinal plants (Duan, 2014; Anand David *et al*., 2016; Dhawan and Gupta, 2016; Wulandari *et al*., 2016). To minimize the use of chemicals for analysis, total flavonoid compounds were identified qualitatively by observing UV spectra intensity of extracted samples diluted in the solvent and compare the absorption pattern to flavonoid standard (quercetin). Figure 1 shows that quercetin spectra in ethanol, methanol, and distilled water have the same pattern and are visible at 200 – 400 nm wavelength. This result is in conformity with the research by Duan (2014) and Bancirova (2015). Flavonoids usually showed three distinctive peaks between 200 – 450 nm wavelength. One peak between 300 – 380 nm, attributed to the B-ring (with lambda max around 350–370 nm), A–C benzoyl structure on the range 240 – 280 nm, and a weak peak with an absorption maximum around 300 nm attributed to the C-ring (Bancirova, 2015).

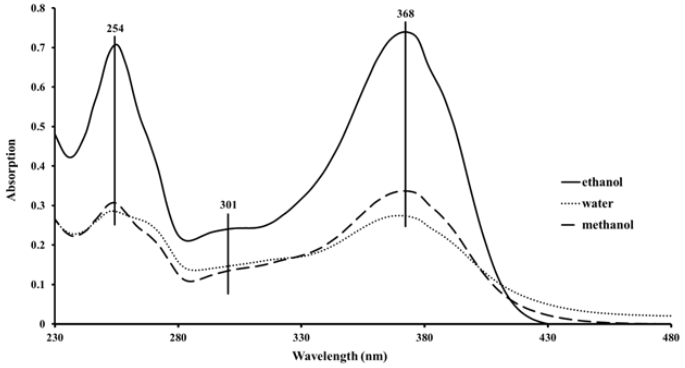

Figure 1. Spectra of quercetin standard compound diluted in ethanol, distilled water and methanol

Pulps extract showed weak UV absorption intensity compared to peels extract (Figure 2). Plants produce phenolic compounds as a defense mechanism for biotic and abiotic stresses. Some phenolic compounds such as polymethoxylated flavonoid were only found in the citrus peels. Therefore, genotype and environmental conditions are also considered as the important factors for the accumulation of phytochemicals. Reports on gooseberry, apples, plums, lemon, grapefruit, kiwi fruit,

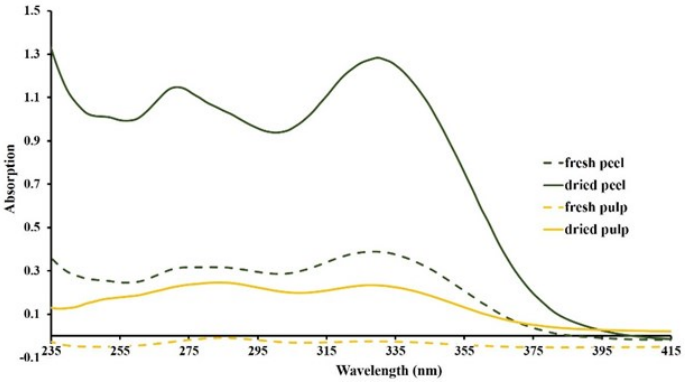

Figure 2. UV/Vis spectra of *Citrus depressa* H. fresh and dried peels and pulps

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melon, orange, grapes also showed that natural antioxidants were found more in the peels (Mansour, 2019; Multari *et al*., 2020).

Because peels showed better result, and pulps are not considered as a waste in beverage industry, except for the pomace, further research discussed mainly about the peels.

3.1 Microwave-assisted extraction

Peels from ripe and unripe fruits (orange and green) were dried to constant moisture content, sieved to 50 mesh particle size, and then extracted using CEM MARS 6™ Microwave Digestion. The present research did not analyze a single factor but tested ascending treatment sets (temperature, time, and power). The treatment sets were 50°C, 10 mins, 600 W; 60°C, 20 mins, 800 W; 70°C, 30 mins, 900 W. Figure 3 shows the different UV spectra intensity of ripe and unripe peels extract in different solvents (distilled water, 50% aqueous ethanol, and methanol).

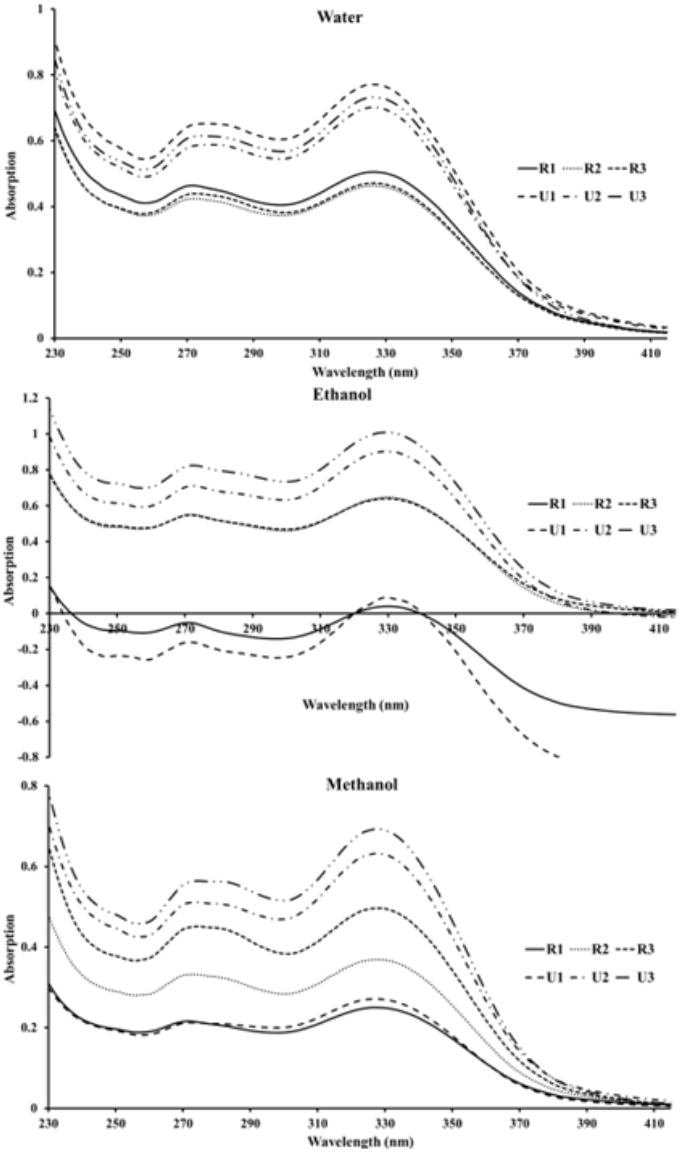

Figure 3. UV Spectra of ripe (R) and unripe (U) peels extracted by different solvents and MAE sets of temperature, time and power ((1) 50°C, 10 mins, 600 W, (2) 60°C, 20 mins, 800 W, (3) 70°C, 30 mins, 900 W).

3.1.1 Effect of solvents

UV spectra intensity of samples in 50% aqueous ethanol and methanol showed the same trend. The highest intensity was in unripe peels that were treated at 70°C, 30 mins, 900 W, followed by unripe peels at 60°C, 20 mins, 800 W; ripe peels at 70°C, 30 mins, 900 W; ripe peels at 60°C, 20 mins, 800 W. The last were ripe and unripe peels treated with the lowest set of microwave temperature, time and power. Otherwise, in water, the UV spectra intensity showed a random trend. The highest intensity was recorded with ripe and unripe peels treated with the lowest set of microwave temperature, time, and power (50°C, 10 mins, 600 W) followed by 70°C, 30 mins, 900 W, and 60°C, 20 mins, 800 W. From the absorption intensity, ethanol extraction showed higher absorption compared to water and methanol.

These results are similar to the extraction of phenolic compounds from *Salacia chinensis* root, macadamia skin, *Limnophila aromatica* and *Cucurbita maxima* pulps and peels where aqueous ethanol extracts had more phenolic compounds than methanol or distilled water (Ngo *et al*., 2017; Rakass *et al*., 2018). However, *M. longifolia* subsp. *typhoides* var. typhoides (Ozen *et al*., 2018) and *Datura metel* leaf (Dhawan and Gupta, 2016) showed that phenolic compounds of were found more in methanol than ethanol or distilled water extract.

The efficiency of MAE is affected by the properties of the solvents and flavonoid compounds. The higher dielectric constant of the solvent related to polarity induces higher absorbance of heat energy's electrical energy dissipation. The dielectric constant of water is the highest compared to other solvents and is referred to as “universal solvent”. Lower absorption of water extract and decreased intensity at higher MAE set possibly showed that prolonged microwave irradiation could cause some phenolic compounds' degradation. Since polarity affects flavonoids' solubility in various solvents, further research on the water as a solvent for flavonoids in shiikuwasha needs to be done. The less polar flavonoid in citruses such as flavanones, methylated flavones (nobiletin, tangeretin), and flavonols (quercetin, kaempferol) were extracted using non-polar solvents such as chloroform, dichloromethane, diethyl ether, or ethyl acetate. Moderately polar flavonoids usually found in citrus such as hesperidin, naringin, hesperetin, rutin, narirutin, diosmin were extracted using alcohols and aqueous alcohol solutions (Gattuso *et al*., 2007; Mohsen-Nia *et al*., 2010; Asikin *et al*., 2012; Orsat and Routray, 2017).

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3.1.2 Effect of fruit ripeness

Figure 3 shows that unripe peels in all extraction treatments have the highest absorption than ripe peels. These results are similar to the reports on pomelo (*Citrus maxima*), lemon (*Citrus limon* L. Burm. f), and sweet orange (*Citrus sinensis* (L.) Osbeck), red raspberries (*Rubus ideaus* L.), and *Citrus aurantium* where unripe fruits had more bioactive compounds than the ripe ones. Flavonoids increase at an early stage of fruit development and decrease during cell enlargement has been attributed to the action of polyphenol oxidase (Wang *et al*., 2009; Mphahlele *et al*., 2014; Lado *et al*., 2018; Mansour, 2019; Multari *et al*., 2020).

3.1.2 Effect of increasing time, temperature and power

Increasing temperature, time, and microwave power in the present research increased the UV absorption of samples. The increased power generates more pressure and temperature in the bio cellular matrix that causes more disintegration of the cell. A higher temperature accelerates intermolecular interactions and motion, which increases the solubility of the solute into the solvent. When lower power is applied, a prolonged microwave time was needed. Decreased flavonoid yield in *Melastoma sanguineum* fruit after extraction using MAE for more than 45 mins showed that sample exposure to a specific temperature for a more extended period is not suitable for thermolabile flavonoids (Orsat and Routray, 2017; Zhao *et al*., 2018). However, a report by Yu *et al*. (2017) on phenolic extraction in *Osmanthus fragrans* flowers showed that higher microwave power only slightly promoted flavonoid compounds dissolution.

3.2 Ultrasonic-assisted extraction

Ultrasonication uses acoustic energy (mechanical energy transmitted through the medium but not absorbed by molecules) to produce cavitation and go through a liquid containing solid materials, caused compression and expansion on the plant cells that enhance cell disintegration and solvent penetration (Yu *et al*., 2017). Figure 4 shows UV spectra absorption of ripe and unripe shiikuwasha peels extracted using UAE in different solvents (distilled water, 50% aqueous ethanol, methanol). The highest absorption intensity was observed in unripe peels extracted using 50% aqueous methanol, followed by aqueous ethanol and water. Similar to MAE, ripe and unripe shiikuwasha peels extracted using distilled water produced lower absorption intensity than other organic solvents. Except for unripe peels extracted using MAE in 50% aqueous ethanol at 70°C, 30 mins, 900 W, the results showed that the absorption intensity of samples extracted using UAE was higher than MAE (Figure 3). This result contrasts with a previous study on *Citrus sinensis* peels by Nayak *et al*. (2015), who obtained more phenolic and flavonoid compounds using MAE. Longer extraction time using UAE could be the possible explanation for this result. Further, the present results conform with a study on *Osmanthus fragrans* flowers, olive leaves (*Olea europaea* L.), and *Opuntia engelmannii*, where phenolic and flavonoid compounds obtained in *Opuntia engelmannii* by UAE were 43% higher than MAE (Yu *et al*., 2017; Melgar *et al*., 2019; Dobrinčić *et al*., 2020).

Although UAE results in higher phenolic and flavonoid compounds, the technique has certain limitations compared to MAE, such as low penetration depth, uncontrolled heating, long extraction time, more solvent consumption, and lower recovery of purified product solvent extraction (Yu *et al*., 2017).

Despite showing the highest absorption intensity, methanol is not preferred as solvent in food and cosmetics uses because of its toxicity and lead to environmental problems. Ethanol was chosen over distilled water because of its efficiency (M'hiri *et al*., 2014). Unripe peels extracted using MAE (50% aqueous ethanol, 900 W, 70°C, 30 mins) and UAE (50% ethanol, 40°C, 3 hrs) were then qualitatively confirmed by FTIR and quantitatively analyzed using HPLC DAD.

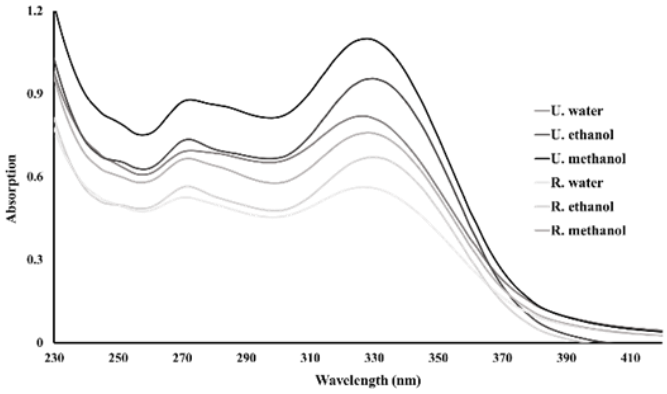

Figure 4. UV Spectra of ripe (R) and unripe (U) peels extracted by UAE at 60°C for 3 hrs using different solvents.

3.3 HPLC-DAD analysis

UV/Vis spectra of unripe peels extracted using MAE at 900 W, 70°C for 30 mins showed higher absorption intensity than unripe peels extracted by UAE. HPLC DAD was done to confirm if the UV spectra result were congruent to the flavonoid quantity (Figure 5). Myricetin, quercetin, kaempferol and rutin were used as standards to make standard calibration curve for flavonoids quantifcation in shiikuwasha. Common flavonoid compounds in citrus peels are flavanones, flavones, flavonols, and PMFs such as quercetin and rutin, narirutin, naringin gallic acid, hesperidin, sinensetin, tangeritin, nobiletin, tetra-O-

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methylscutellarein, heptamethoxy flavone. A flavonoid found in citrus has a wide range of biological effects, such as inhibition of key enzymes in mitochondrial respiration, protection against coronary heart disease, and anti-spasmolytic, anti-inflammatory, antioxidative, anticancer, and antimicrobial activities (Wang *et al*., 2007; Asikin *et al*., 2012; Ahmed *et al*., 2019).

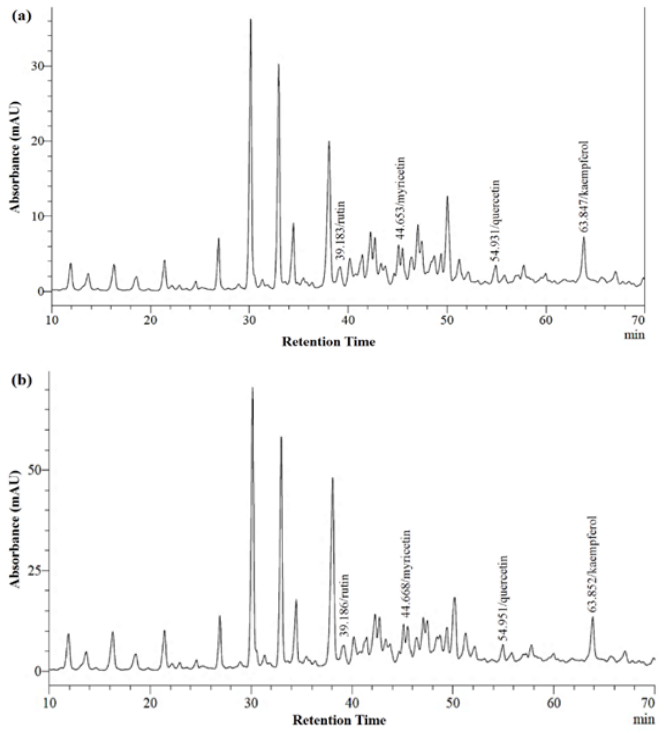

Figure 5. Chromatogram of unripe shiikuwasha peels extracted by (a) MAE at 900 W, 70°C, 30 mins; (b) UAE at 60°C, 3 hrs

Table 1 shows the amount of myricetin, quercetin, kaempferol and rutin calculated by the linear equation of standard calibration curves. Furthermore, Table 1 shows that the amount of flavonoid in unripe peels extracted by MAE is higher than in UAE. The quantification confirmed that UV/Vis spectra intensities are a depiction of the flavonoid content in the extract. Putnik *et al*. (2017) reported that the maximum total polyphenols in dry orange peel extracts were in the range of 9100 – 49200 mg/kg GAE (gallic acid equivalent) and 2000 – 30000 mg/kg QE (quercetin equivalent). Petrotos *et al*. (2021) extracted 1909.27 mg/2 kg total phenolic and flavonoid from orange pomace waste using vacuum MAE.

UAE were commonly showed better result than MAE in previous studies on phenolic and flavonoid extraction. Nayak *et al.* (2015) reported ultrasonic extraction were able to extract 982.79 µg/g rutin from *Citrus sinensis* peels, while MAE could extract only 589.13 µg/g. Ramos *et al.* (2017) and Nishad *et al.* (2019) also reported higher flavonoid compounds using UAE than other methods. Solubility of particular flavonoids in various solvents affects the composition of the extract. Standardized solvent composition for all the flavonoid classes was not available (Orsat and Routray, 2017). Present results showed that, contrary from previous studies to different samples, microwave treatment is more efficient for flavonoid extraction in *Citrus depressa* H. MAE is considered more efficient because it requires less time and energy and results in a higher yield. Moreover, MAE is easy to operate, economical, and suitable for industrial-scale production (Yu *et al*., 2017).

3.4 FTIR spectroscopy

Quercetin was used as a standard compound to compare the IR spectra of the samples. IR pattern between quercetin and sample is almost similar. Only in the sample, the peaks were not separated completely (Figure 6). The extract was not purified since it contains other phenolic and flavonoid compounds. Typical vibration phenolic molecules at fingerprint region (1800 – 900 cm^{-1}) were the stretching band of carbonyl (C=O) groups (1712 – 1704 cm^{-1}) and C=C stretching bands on aromatic molecules (1609 – 1608 and 1519 – 1516 cm^{-1}), antisymmetric in-plane bending of $–CH_3$ or phenyl nuclei (C=C bonds), there are also bands of deformation of $–CH_2–$ groups at 1448–1444 cm^{-1}, symmetric in-plane bending of $–CH_3$ at 1376 – 1373 cm^{-1}, CH bending and CH_2 wagging at 1340–1339 cm^{-1}, in-plane bending of O–H at 1281–1278 cm^{-1}, stretching vibration of C–O at 1207, 1110 – 1107, 1068 – 1062 cm^{-1} (Silva *et al*., 2014).

IR vibration of quercetin was carbonyl C=O stretching prominent band at 1663 and 1606 cm^{-1}, NO_2 bending at 1520 – 1500 cm^{-1}, C–O vibration at 1449 – 1400 cm^{-1}, C–O–C of ester for quercetin compound at 1260 – 1200 cm^{-1}, plane bending of C–H at 819 and 800 cm^{-1}. *Citrus depressa* also rich in polymethoxyflavones

Table 1. Flavonoid compounds (µg/g) identified in unripe shiikuwasha peels extracts obtained by Microwave-Assisted Extraction (MAE) and Ultrasonic-Assisted Extraction (UAE).

No	Standards	MAE		UAE	
		RT (min)	Concentration (µg/g)	RT (mins)	Concentration (µg/g)
1	Rutin	39.18	8282.52±10.03	39.19	5000.62±8.23
2	Myricetin	44.65	0.36±0.04	44.67	0.20±0.00
3	Quercetin	54.93	670.00±2.55	54.95	336.17±3.12
4	Kaempferol	63.85	7307.51±6.74	63.85	4469.15±6.55

RT : retention time, Concentration values are means of two replications

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(PMF), which the assigned IR spectra were the phenyl ring (C=C) and mixed alkyl/aryl methyl ether (C-O-C) vibrations of the much simpler molecule, anisole (phenylmethyl ether). The asymmetric methoxy (C-H) stretch occurs at 2829 – 2835 cm^{-1} (Manthey, 2006; Asikin *et al*., 2012; Patle *et al*., 2020). Identified polymethoxyflavone (PMF) IR spectra in the sample showed that the PMFs also need further analysis.

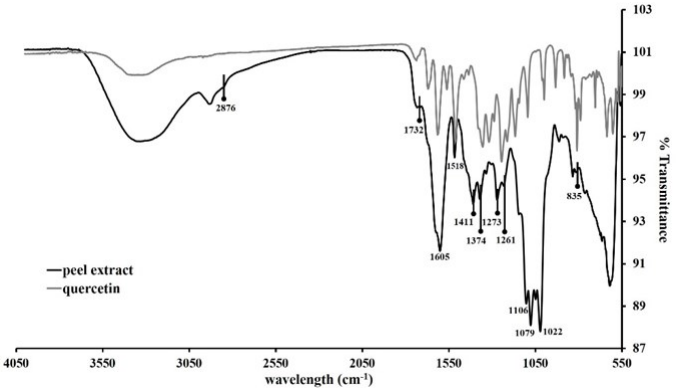

Figure 6. IR spectra of quercetin standard and unripe peel extract

3.5 Aroma active compound

The fragrance reflects the characteristics and one of the quality factors of fruit. The unique aroma of citrus fruits depends on the aroma-active volatile content. Citrus essential oil could be used as a flavor or fragrance (Xiao *et al*., 2017; Zhang *et al*., 2019). Volatile compounds of shiikuwasha peels were analyzed using GC-HS-SPME to identify the compounds that generate the strong characteristic aroma. The volatile compounds between fresh and dried unripe peels were compared to observe the change of composition after drying treatment. Moreover, only ten highest peaks were identified in the present study (Figure 7).

These results are almost similar to the previous study on Japanese shiikuwasha peel (var. izumi kugani, katsuyama kugani, kaachi, and ogimi kugani). The main volatile aroma compounds were limonene (46.52 − 68.26%), γ-terpinene (21.48 − 30.52%), and p-cymene (0.57−8.98%) (Tables 2 and 3). Several compounds in moderate amounts (1.02−2.36%) such as myrcene, terpinolene, and pinene (α-pinene and β-pinene) were also identified (Asikin *et al*., 2012). In the present study, p-cymene was not found in unripe peels (fresh and dried) but in ripe shiikuwasha peels (data not shown). From the analysis of several volatile compounds in fresh and dried shiikuwasha's peels samples, limonene was found to be the main aroma-active compound in comparable amount, which means that drying does not affect the amount of volatile compounds. Further analysis in different temperature treatment need to be done. However, different varieties showed different volatile compounds composition. The main volatile compound in lemon and kumquat other than limonene were β-myrcene, cadinene, linalool, geraniol acetate, and α-farnesene (Zhang *et al*., 2019). While in *Citrus reticulata* (mandarin), the main aroma compounds were ethyl acetate, myrcene, γ-terpinene, α-pinene, 3-methyl-1-butanol, and ethyl-2-methyl propanoate (Xiao *et al*., 2017).

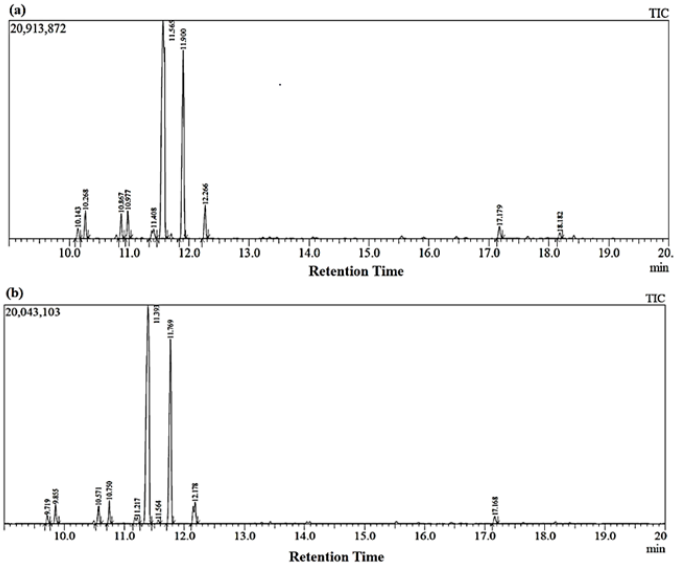

Figure 7. Gas Chromatogram of (a) fresh and (b) dried unripe shiikuwasha peels

Table 2. Volatile compounds identified in fresh unripe shiikuwasha peels

Peak	Fresh unripe shikuwasa peels		
	RT	Concentration (%)	Name
1	11.57	53.01±1.10	D-limonene
2	11.91	27.67±0.82	γ-terpinene
3	12.27	4.69±0.01	1,6-octadien-3-ol, 3,7-dimethyl-, formate
4	10.27	3.20±0.00	α-pinene
5	10.98	2.87±0.00	β-myricene
6	10.87	2.71±0.01	bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methyl
7	11.41	2.07±0.00	o-cymene
8	17.18	1.83±0.00	Caryophyllene
9	10.14	1.19±0.02	bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methyl)
10	18.18	0.76±0.00	(1S.2E.6E.10R)-3,7,11,11,-tetramethylbicyclo

RT : retention time, Concentration values are means of two replications

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Table 3. Volatile compounds identified in dried unripe shiikuwasha peels

Peak	Dried unripe shikuwasa peels		
	RT	Concentration (%)	Name
1	11.39	54.79±1.90	D-limonene
2	11.77	29.41±2.11	γ-terpinene
3	12.18	4.18±0.01	1.6-octadiene-3-ol, 3,7-dimethyl-, formate
4	9.86	2.54±0.01	α-pinene
5	10.75	2.59±0.00	β-myricene
6	10.57	2.23±0.00	bicyclo[3.1.1]heptane, 6.6-dimethyl-2-methyl
7	11.22	1.61±0.02	o-cymene
8	17.17	1.14±0.17	Caryophyllene
9	9.72	1.07±0.00	bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(-1-methyl)
10	11.56	0.45±0.00	1,3,6-octatriene, 3,7-dimethyl-(Z)-

RT : retention time, Concentration values are means of two replications

4. Conclusion

In conclusion, this study showed that Taiwanese shiikuwasha contain flavonoid such as kaempferol, myricetin, rutin and quercetin. FTIR-ATR analysis showed peak at polymethoxyflavones (PMF) that need further research. Microwave Assisted Extraction using ethanol as solvent is better method to extract these flavonoid compounds than ultrasonication. Several aroma-active compounds also found in shiikuwasha's peels such as limonene, γ-terpinene, pinene, myricene and cymene.

Conflict of interest

The authors declare no conflict of interest.

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