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Research Paper

Valorization of Taiwan's *Citrus depressa* Hayata peels as a source of nobiletin and tangeretin using simple ultrasonic-assisted extraction

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ABSTRACT

As the highest yield crop worldwide, citrus peels that possess bioactive compounds were discarded as a futile by-product. Ultrasonication with environmentally friendly solvent (50% ethanol and ddH_2O) were used in the present study to extract flavonoids from *Citrus depressa* Hayata peels with extraction period and fruit maturity as other variables. DPPH scavenging activity was investigated. Qualitative flavonoid content analysis was done by UV/Vis and FTIR-ATR spectra. Quantification of flavonoid using LC-MS/MS found that solvent type, fruit maturity, and ultrasonication period significantly affect the extracted flavonoid yield ($p < 0.05$). Extraction using 50% ethanol showed a higher yield than ddH_2O. Flavonoid content was also higher in unripe than ripe samples. Nobiletin, tangeretin, and rutin were dominant among the identified compounds in all sample treatments. Flavonoid content in *Citrus depressa* Hayata extract was found to negatively correlate to DPPH scavenging activity, which needs further research to identify other bioactivities of these flavonoids.

1. Introduction

Citrus, which consists of several important commercial fruits such as oranges, tangerines, mandarins, grapefruit, lemons, and limes, are the most consumed fruit and became the third most important fruit crop grown in tropical and subtropical regions of the world. Citruses are a fruit with the highest yield crop worldwide. US Food and Agriculture Organization report on 2017 mentioned that the annual production of citrus fruits is approximately 124 million tonnes and more than 100 million tons in 2018/9, with 30% destined for industrial purposes. Citrus processing industries play an important role in the agro-industrial system (Fakayode and Abobi, 2018; Saini et al., 2019; Uckoo et al., 2015; Victor et al., 2021; Zhang et al., 2019).

Citrus depressa Hayata, also known as shiikuwasha, is a popular citrus fruit in Okinawa - Japan, and Taiwan. It has small yellowish-green fruits, a very sour taste, and a strong, unique aroma. The average annual commercial production of shiikuwasha fruits was around 3000 tons from 2007 to 2009. Shiikuwasha is commonly consumed as fresh fruit or processed into several food products, such as beverages, confectionery items, food additives, and livestock feed. Shiikuwasha juice is a rich source of bioactive compounds such as ascorbic acid and flavonoids, flavone glycoside, and polymethoxylated flavones (PMFs) (Asikin et al., 2012; Shiu et al., 2016).

Citrus industries worldwide in the last decades have been continuously growing (Gómez-Mejía et al., 2019). Besides the food industries, citrus is also widely used in cosmetic ingredients and pharmaceutical industries (Tuz Zohra et al., 2020). The side effects of this productive activity of the juice sector industry and high domestic consumption of citruses are the discarded part. Most people use only the orange pulp and dispose the peel because it tastes bitter. Since the yield of orange juice is 50% of the wet fruit mass, a massive amount of orange by-product wastes are generated annually without proper processing (Fakayode and Abobi, 2018; Gómez-Mejía et al., 2019; Zhang et al., 2019). These vast amounts of by-products may contain high levels of bioactive compounds. Phytochemical investigations into *Citrus* species have shown that the peel contains flavonoids, limonoids, coumarins, vitamins, and other phenolic compounds that have antioxidant activity. However, these residues are mainly used as animal feed or directly discarded as waste to the environment. With the development of public awareness of zero waste, exploitation of by-products has become the main challenge of juice factories. This also necessarily involves an environmentally friendly process to recover and recycle the bioactive compounds from citrus waste (García et al., 2015; Gómez-Mejía et al., 2019; Mitani et al., 2021).

In some countries, citrus peels have been utilized as a traditional medicine for stomachache, cough, skin inflammation, muscle pain,

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ringworm infections, and lowering blood pressure (Li et al., 2009).

Citrus peels contain pleasant flavor compounds and significant amounts of biologically active substances such as polyphenols, specifically phenolic acids, limonoids, and flavonoids, that benefit human health. Phenolic compounds are secondary metabolites produced by plants, found in various fruits and vegetables, and play an important role in defending against infections, injuries, and plant growth and development (Keshavarz and Rezaei, 2020). These compounds have exhibited important antioxidant, anti-inflammatory, anticancer, antiobesity, antiproliferative, anti-allergic, antiviral, anticarcinogenic, anti-hyperglycemia, anti-neuroinflammation, neuroprotective, antimicrobial, and insulin resistance properties. Common flavonoid compounds in citrus peels are flavanones, flavones, polymethoxyflavones (PMFs), and flavanone glycosides (Asikin et al., 2012; Gómez-Mejía et al., 2019; Kim and Lim, 2020; Tuz Zohra et al., 2020). Recently, polymethoxyflavones (PMFs) such as nobiletin and tangeretin that exist abundantly in bitter orange has drawn particular interest in food and pharmaceutical industries due to the protective effects against memory impairment in dementia, Alzheimer's disease and used in Parkinson's disease treatment (Mitani et al., 2021; Stuetz et al., 2010; Tuz Zohra et al., 2020; Uckoo et al., 2015). Moreover (Asikin et al., 2012), and (Lee et al., 2010) were successfully extracted nobiletin and tangeretin as the abundant PMF in *Citrus depressa* Hayata peels.

Because of the potential application of flavonoids, researchers have studied specific techniques and conditions for flavonoids extraction from natural products. (Chaves et al., 2020). Different heating processes such as boiling, frying, baking, steaming, and microwaving cause oxidation, thermal degradation, and leaching of bioactive compounds from fresh vegetables, resulting in different levels of flavonoids reduction. Ineffective flavonoid extraction from citrus peels results in environmental pollution (Londoño-Londoño et al., 2010). Efficient methods were expected to be useful for foods either for analytical, preparative, or industrial purposes. The efficiency is influenced by compounds' chemical nature, sample particle size, as well as the presence of interfering substances (Corell et al., 2018).

There are several options of bioactive compounds extraction method. Traditional methods, including water-extracted, maceration extraction and solid-phase microextraction are very slow, costly and with low efficiency. More sophisticated methods such as microwave-assisted extraction, pressurized liquid extraction, and supercritical fluid extraction were considered high energy costs due to the operation under high pressure. Ultrasound-assisted extraction (UAE) has the potential for high recovery of active compounds and low-cost extraction method. UAE, categorized as "green extraction" techniques for reducing the use of organic solvents, uses fewer steps to extract in the preparation and extraction process while achieving high yields in short times. This method is easy to use, versatile, flexible and requires low investment compared to other extraction techniques. Moreover, UAE is applicable in laboratories and on an industrial scale (Chaves et al., 2020; Damak et al., 2019; Irakli et al., 2018; Saini et al., 2019; Tiwari, 2015; Yu et al., 2019).

Solvent selection plays a crucial role in achieving optimum recovery of bioactive polyphenols. Due to the solubility of phenolic compounds, different solvents lead to various compositions of extracted phenolic compounds that would affect the bioactivity. The previous experiment showed that aqueous ethanol is an effective solvent to extract flavonoid from *Citrus depressa* Hayata peels. Water is also used for efficiency, lower cost, toxicity and safety concerns (Chaves et al., 2020; Gómez-Mejía et al., 2019; Lou et al., 2014). Apart from the instrument conditions, the plant nature also affects the yield of extracted phenolic compounds. A steady decrease or increase of phenolic content were two distinct phenomena during fruit ripening (Hwang et al., 2020; Mokhtar et al., 2021).

This study aims to validate *Citrus depressa* Hayata peels as a source of nobiletin and tangeretin by environmentally friendly solvent using UAE. Solvent type, extraction time and fruit maturity effect on the yield of flavonoid is examined. Previous study showed that ultrasonication for several minutes in *Citrus depressa* Hayata peels pretreatment results in higher amount of total flavonoid compared to microwave pretreatment and combination of both methods. Hence, in present study, ultrasonication were studied further to get better result of flavonoid compounds and to discover the effect to antioxidant activity.

2. Materials and methods

2.1. Citrus sample preparation

Citrus depressa Hayata from Pingtung region (North Taiwan), were purchased at ripe (yellow peels) and unripe (dark green peels and firm fruit) maturity. Fruits were carefully cleaned with distilled water to remove dirt, dust, microflora, and pesticide residue on the surface. Peels were separated by hand and air dried at 45 °C until constant moisture content. Dried peels were pulverized by domestic blender, sieved 50 mesh and stored in a container with desiccant until use.

2.2. Chemicals

All chemicals and solvents were of analytical grade. Myricetin, quercetin, rutin and kaempferol from Sigma-Aldrich Company (St. Louis, MO, USA). Nobiletin (purity 98%), tangeretin (purity 97%) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Toronto Research Chemicals (Canada). 95% ethanol from Echo chemical (Taiwan). 98% methanol as mobile phase were the HPLC-grade reagents from Merck Company (Darmstadt, Germany). Water was distilled using a Sartorius arium pro (Göttingen, Germany).

2.3. Ultrasonic-assisted extraction (UAE)

Three independent variables were studied by completely randomized design (CRD). The variables were extraction time (10′, 20′, 30′, 40′ and 50′), solvent type (ddH_2O and 50% aqueous ethanol) and fruit maturity (ripe and unripe). Sample (0.2 g) dissolved in 20 mL solvent and ultrasonicated (Ultrasonic water bath Delta DC400) at ambient temperature for a pre-determined time. The extract was filtered through filter paper and 2 mL were taken for UV spectra characteristics and DPPH scavenging activity analysis. The remaining extract was concentrated using a rotary evaporator and dried for FTIR and flavonoid compounds analysis using LC MS/MS.

2.4. UV Spectra analysis (Bancirova, 2015)

0.1 mL sample were diluted to 5 mL with the extraction solvent (50% ethanol or ddH_2O), vortexed, put in the quartz cuvette (1 cm optical path) and the absorbance were observed at 190–1100 nm wavelength by UV/Vis PerkinElmer Lambda 265.

2.5. FTIR analysis (Lucarini et al., 2019)

The FTIR spectra were recorded on a PerkinElmer FTIR spectrometer equipped with a diamond crystal cell for attenuated total reflection (ATR) operation. The spectra were acquired (32 scans per sample or background) in the range of 4000–600 cm^{-1} at a nominal resolution of 4 cm^{-1}. Background spectrum of air were used to correct the spectra. The analysis was carried out at room temperature. For a measurement, dried samples were placed on the surface of the ATR crystal. Before acquiring a spectrum from different sample, the ATR crystal was carefully cleaned and checked spectrally.

2.6. DPPH scavenging activity

Free radical-scavenging capacity of polyphenols in *Citrus depressa* Hayata was tested as the bleaching phenomena of the stable radical DPPH. The DPPH assay was done according to the reported method

(Mokhtar et al., 2021). The maximum absorbance of DPPH is 517 nm; conversion of DPPH to DPPH-H turns the deep violet color to colorless. A volume of 0.1 mL of the tested samples were added to 1 mL of DPPH. After incubation for 30 min in a dark room, the absorbance was measured at 517 nm. DPPH scavenging activity (in percentage) was calculated by following equation:

$$\text{DPPH scavenging activity } (\%) = \frac{(A_0 - A_s)}{A_0} \times 100$$

where A_0 is the absorbance of the blank (methanol) and A_s is the absorbance of the sample.

2.7. LC MS/MS analysis of flavonoid compositions

Agilent 1260 infinity binary LC system coupled with an Agilent 6470 Triple Quadrupole MS system equipped with ESI ionization source with Agilent Jet Stream were used in the analysis (Waters, Milford, MA, USA). All compounds were separated by a gradient elution program with distilled water used as the mobile phase A and 95% methanol used as the mobile phase B. The gradient elution program was performed as follows: 0.0–7.0 min, 90% B; 7.1–10.5 min, 90% B; 10.6–15 min, 5% B, and the total run time was 15 min. The flow rate was set at 0.350 mL/min, the column chamber and sample chamber were kept at constant temperatures of 40 °C and 10 °C, respectively. The sample injection volume was 1.0 μL. The drying gas temperature and the sheath gas temperature were 345 and 350 °C. Also, the flow rate was 10 L/min, respectively. Experimental data were collected and processed by MassHunter software (Agilent Corp., Milford, MA, USA).

For sample preparation, 1 mg of dried peel extracts were dissolved in 1 mL of 98% methanol by vortex and 10 min ultrasonication. The solution then centrifuged at 14000 rpm for 10 min, 100 μL supernatant were put in the vial and injected to LCMS/MS. Six flavonoids used as standards were rutin, quercetin, kaempferol, myricetin, nobiletin, and tangeretin.

Linear ranges (n = 8) were set at concentrations between 15 and 2000 μg/kg for all standards. The linearity of chromatographic peak areas was evaluated with R^2 values. Calibration curve equations and the slope (S) were developed to quantify the Limit of Detection (LOD) and Limit of Quantitation (LOQ) of each standard. Standard deviations (SD) of each compounds were acquired and LOD were calculated as 3.3 SD/S while LOQs as 10 SD/S.

2.8. Statistical analysis

Three replicates analyses were performed. Data means presented with standard deviation (SD) and statistical analyses were performed using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL). Differences among groups were detected by ANOVA and Duncan's multiple comparison post-test. Univariate regression analysis was performed to evaluate the confounding impacts of extraction time, solvent type, and fruit ripeness on flavonoid content. Statistical significance consideration were obtained when p-value less than 0.05 ($p < 0.05$). The correlation coefficient between antioxidant activity and total flavonoids was done by Pearson's correlation coefficient (r) option.

3. Results and discussions

3.1. Ultrasonic-assisted extraction (UAE)

Extraction method is a crucial factor that affect the biological activity of the extracted compound. Due to the diverse structure, degree of polymerization, and solubility, there is no universal extraction procedure for structurally phenolic compounds. Conventional method requires high temperatures and long extraction times that lead to low yield, product quality, high energy input, and finally create safety hazards and environmental risks. Ultrasonication has become a preferred alternative method to accelerate pollutant-free phenolics extract from a plant in the past few years. UAE provides a broader range of solvent selection so that toxic solvent could be replaced by non-toxic (environmentally friendly) solvent. High purity product, lesser use of solvent, extraction time and effectivity compared to conventional methods are the main reason of the preference. Extraction efficiency could be enhanced by the moderate increase in temperature during ultrasonication. However, this increased temperature also has a negative effect on the bioactivity of the extract (Rao et al., 2021; Rodsamran and Sothornvit, 2019; Saini et al., 2019; Vasantha Rupasinghe et al., 2011).

Ultrasonication generates acoustic cavitation in the biological matrix that develops shock waves, microjets, shear force and turbulence matrix fragmentation which cause modification of plant matrices such as cell erosion, pore formation, or increased absorption. This mechanism provides better contact of the active ingredient between the solid phase and the liquid phase. Cell wall fragmentation increases the solubility of bioactive compounds in the solvent. UAE is an economical, rapid, green, and developing technology appropriate for improving the efficiency of bioactive compound extraction (Ranjha et al., 2021; Rao et al., 2021; Yu et al., 2019).

3.2. UV spectra qualitative analysis

To reduce the use of toxic chemicals, phenolic compounds were qualitatively analyzed by observing the UV spectra pattern and intensity of extracted sample in the solvent (ddH_2O and 50% aqueous ethanol). Phenolic compounds strongly absorb UV light, and the natural color of certain compounds such as carotene or anthocyanin leads to absorption in the visible range. Moreover, the π type molecular orbitals electronic transitions containing polyphenols would provide the UV–visible spectrum. Despite the cumbersome result satisfactory and highly material dependent, UV/vis spectroscopy appears to be the appropriate method to analyze phenolic compounds (Luis Aleixandre-Tudo and du Toit, 2019; Stalikas, 2007).

Previous experiment proved that this qualitative UV spectra pattern and intensity analysis represents the amount of phenolic compounds. Therefore, this analysis is carried out as a prelude to further analysis to indicate if the phenolic compounds were actually extracted from the samples. Both samples in different solvents showed a similar UV/Vis absorption pattern. Fruit ripeness and solvent type affect the UV absorbance intensity, where unripe samples and extraction by 50% aqueous ethanol showed higher absorbance intensity than ripe samples and ddH_2O extraction.

Phenolic compounds are secondary metabolites that are produced and accumulated by plants for growth, reproduction, and protection against environmental signals biotic or abiotic stresses. Two groups of phenolic compounds are classified as non-flavonoids and flavonoids that contain at least one aromatic ring and several hydroxyl groups. Non-flavonoid groups including hydroxycinnamic, hydroxybenzoic acids and stilbenes, while flavonoids including flavone, flavanone, flavonol, isoflavonoid, anthocyanidin, and chalcones. Flavonoids such as quercetin, rutin, naringin, hesperidin, and didymin share a common C3–C6–C3 structure (Luis Aleixandre-Tudo and du Toit, 2019). Flavonoids exhibit two distinctive bands in a broad range between 240 and 400 nm. Band I, covering the range 300–380 nm, is attributed to the B-ring (with lmax around 350–370 nm), while band II, covering the range of 240–280 nm (with lmax around 260–270 nm) attributed to the A–C benzoyl system; and the C-ring was also detected with a weak band at around 300 nm. Polymethoxyflavones (PMFs) such as tangeretin, nobiletin and sinensetin showed absorption in the 320–385 nm range corresponds to the B ring of flavonoids, and the absorptions in the 240–280 nm range correspond to the A ring portion. Moreover, tangeretin shows maximum responses at wavelengths λ = 210, 250 270 and 334 nm while nobiletin at approximately λ = 210, 271 and 324 nm (Sharma et al., 2019). The result showed that all samples showed peaks

closer to PMFs top peaks, which were 272, 325, and 330 nm. These results implied that samples were likely to contain more PMFs than other flavonoid types. A shifting top peaks occurred at 330 nm in 50% aqueous ethanol extract to 325 nm in ddH_2O extract. Although the mechanism is not absolutely clear, this result showed that the surrounding solvent could modify the chemical and biological properties of the compounds (Bancirova, 2015; Duan, 2014) (see Fig. 1).

3.3. FTIR-ATR analysis

Infrared radiation is absorbed or some were transmitted by the sample, that create molecular fingerprint of the sample. Molecular vibrations, including stretching, bending, and torsions of the chemical bonds generate characteristic signature of the chemical or biochemical substances present in the and the signal were captured by FTIR instrument and presented as spectrum (Lucarini et al., 2019). The averaged spectra from the shiikuwasha peels extract are shown in Fig. 2. The infrared spectra were interpreted according to the literature, to confirm the dominant flavonoid type in ripe and unripe peels of *Citrus depressa* Hayata that showed in UV/Vis result.

The results showed that the signal from samples extracted by 50% aqueous ethanol was stronger than ddH_2O. Moreover, all extracts showed similar peaks at approximately 1641–1599 cm^{-1} attributed to carbonyl stretch, 1514, 1455, and 1407 cm^{-1} related to C═C vibration of the phenyl ring, C–O–C asymmetric stretch mixed phenyl/methyl ether at 1373-1172 cm^{-1}. Bands at 1111-839 cm^{-1} could be attributed to C–H in plane. Specific IR spectra of PMFs were phenyl ring (C═C), mixed alkyl methyl ether (C–O–C), and anisole (phenyl/methyl ether). Asymmetric methoxy (C–H) in anisole were assigned in spectra at 2829-2848 cm^{-1}. Furthermore, mixed phenyl/methyl ether linkages of anisole occur at 1019–1155 and 1210-1264 cm^{-1} (Luque-Alcaraz et al., 2012; Manthey, 2006; Wang et al., 2022).

A different peak showed in samples extracted by ethanol. Ethanol extract showed a peak at 1747 cm^{-1}. This also occurred in a result by (Patle et al., 2020) at 1748 cm^{-1} which attributed to C═O stretching vibration. This peak shows the maximum extraction efficiency of different phytochemical species such as gallic acid, quercetin, rutin, and tannic acid with ethanol as solvent.

Infrared vibrations of nobiletin and quercetin are shown in Table 1. A comparison showed that the vibrations in the samples were similar to the FTIR spectrum of nobiletin. This result showed that PMFs are possibly the dominant flavonoid type in *Citrus depressa* Hayata peel extract, especially in samples that extracted using 50% ethanol.

3.4. Determination of flavonoid content by LC MS/MS

Mass spectrometry is an analytical technique that is useful in determining nutraceutical substances such as phenolic compounds. Big molecules were fragmented into smaller ones by electric and magnetic fields from the instrument, and then ions were separated according to their mass-to-charge ratio (m/z). Retention time in LC MS/MS was affected by the structure of the saccharide residues and methylation of the compounds. For flavonoid compounds, flavanones were eluted first, followed by flavonols and flavones. Flavonoid glycosides' moiety positions also have significant effects on the retention time (Sharma et al., 2019).

The UV/Vis absorption intensity trend qualitatively represents flavonoid content in samples. UV/Vis spectra intensity showed that 50% aqueous ethanol extract more phenolic compounds than aquadest and unripe samples contain more phenolic compounds than ripe samples. Moreover, the trend also showed only a slight difference between unripe samples extracted using ddH_2O and ripe samples extracted using aqueous ethanol. A similar trend from quantitative LC-MS/MS analysis also showed in the total identified flavonoid compounds in the samples. Standards used in this research were quercetin, kaempferol, myricetin, rutin, nobiletin, and tangeretin. Several flavonoid standards were also analyzed to identify other potential flavonoids abundant in *Citrus depressa* Hayata peels. As can be observed in Table 2, good linearity was observed for all analytes at concentrations within the tested intervals, with determination coefficients (R^2) higher than 0.9920 in all standards.

Individual flavonoid compounds in the citrus peel extracts including kaempferol, myricetin, rutin, quercetin, nobiletin and tangeretin are shown in Table 3. The results showed that the amount of kaempferol, myricetin and quercetin were detected in minuscule amount below the Limit of Detection (<LOD), while rutin, nobiletin and tangeretin were found to be the dominant flavonoid. The weak signals of kaempferol, quercetin and myricetin were possibly because of the incompatible form between standards and flavonoids in nature. These flavonoids were usually in the glycoside or uronic acid conjugate form in the nature (Bouderias et al., 2020; DuPont et al., 2004; Esselen and Barth, 2014; Taheri et al., 2020; Wang et al., 2018).

Polymethoxyflavones (PMFs) are a unique class of flavonoids with more than two methoxyl groups on their chemical skeletons and have been found to exclusively exist in citrus peels. The most abundant PMFs in high-yielding citruses such as *Citrus sinensis* and *Citrus aurantium,* are also nobiletin and tangeretin. Interest in PMFs has developed in recent years due to their various biological activities, including anticarcinogenic, anti-inflammatory, antioxidant, anticancer, neurotrophic activity, antiobesity, anti-atherogenic and antiviral activity. Moreover, in vivo analysis showed that PMFs could improve cognitive memory deficits and seems to be applicable to Alzheimer's and Parkinson's diseases. Hence, nobiletin and tangeretin have become popular in the food, pharmaceutical, and cosmetic industries (Bakoyiannis et al., 2019; Lai et al., 2013; Li et al., 2012; Mitani et al., 2021; Tung et al., 2019; Zhang et al., 2019). Total nobiletin and tangeretin yield in this study were 6.87–15.34 mg/g dried peels. The maximum yield was achieved at 30 min of ultrasonication in 50% aqueous ethanol. These results are similar to *Citrus depressa* Hayata extraction by Lee et al. (2010) using supercritical CO_2 for 80 min with 85% ethanol which produced 12.92–14.68 mg/g PMF from dried peels. Asikin et al. (2012) used ultrasonication

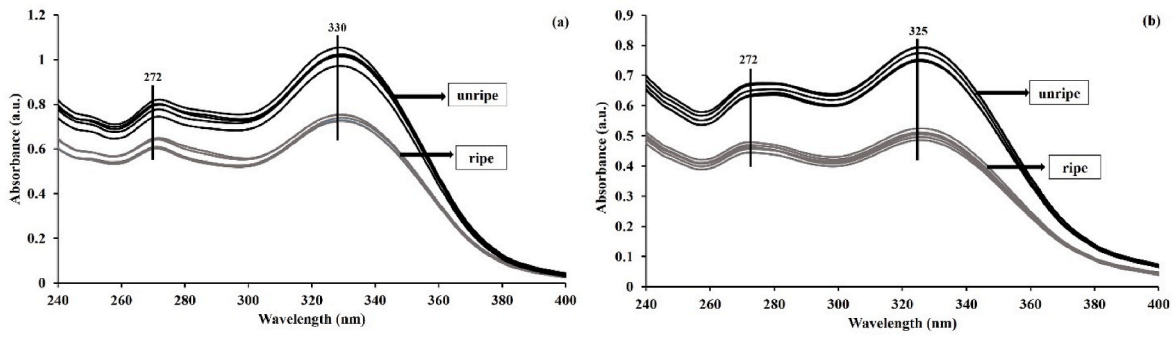

Fig. 1. UV Spectra of samples extracted by (a) 50% aqueous ethanol (b) ddH_2O.

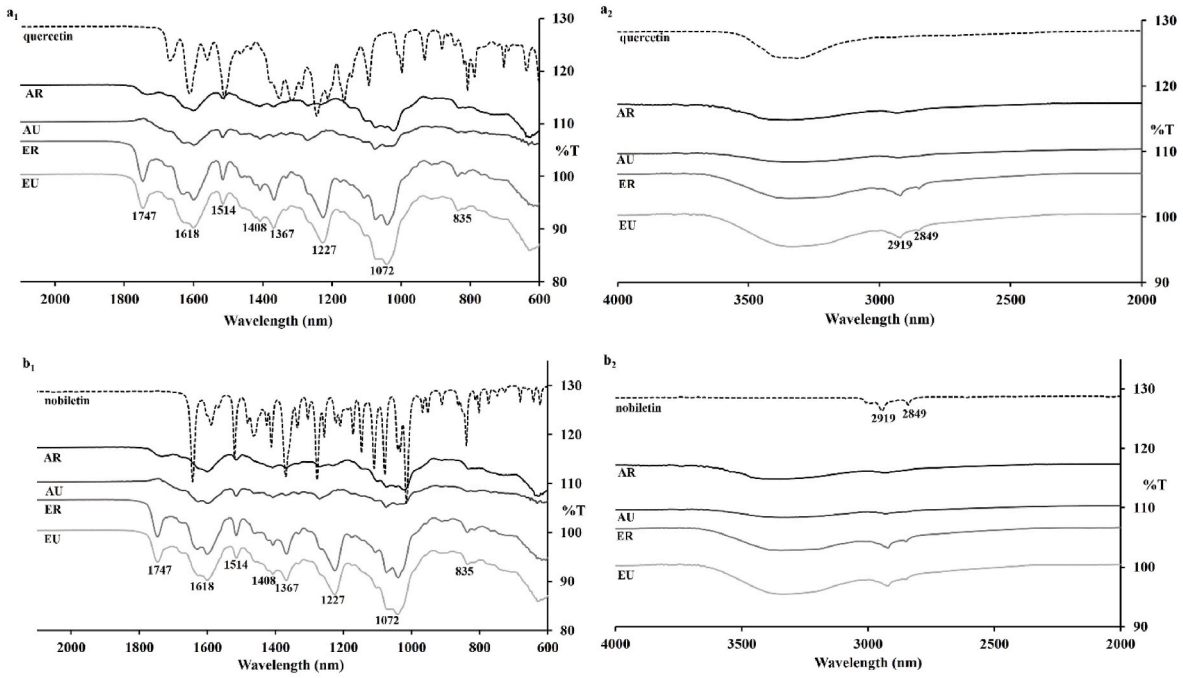

Fig. 2. IR spectra of samples compared to quercetin (a_1 and a_2) and nobiletin (b_1 and b_2) standards.

Table 1
IR peak frequencies and functional groups present in nobiletin and quercetin.

No	Nobiletin		Quercetin	
	Band (cm^{-1})	Group	Band (cm^{-1})	Group
1	3500–3200	Hydrogen bond	3406, 3283	OH stretching
2	2829	Asymmetric methoxy C–H stretch	1666	C=O aryl ketonic stretch
3	1210, 1150	C–O–C asymmetric stretch mixed phenyl/ methyl ether	1610, 1560, 1510	C=C aromatic ring stretch
4	1590, 1516, 1408	C=C vibration of the phenyl ring	1379	OH bending of phenol
5	1612, 1645, 1652	Carbonyl stretch	1317	C–H aromatic hydrocarbon bending
6	1622	C=O	1263	C–O stretching in aryl ether ring
7	1107, 1076, 1040, 1012, 972	C–H in plane	1200	C–O stretching in phenol
8	1588, 1519	C=C aromatic rings	1165	C–CO–C stretching and bending in ketone
9			933, 820, 679, 600	Out-of-plane bending

(Catauro et al., 2015; Luque-Alcaraz et al., 2012; Manthey, 2006; Wang et al., 2022).

Table 2
Analytical parameters of the LC MS/MS employed for determination of flavonoids.

Compound	LOD (mg/kg)	LOQ (mg/kg)	Calibration equation y = ax + b		
			a	B	R^2
Kaempferol	0.219	0.663	0.0011	- 0.0191	0.9922
Myricetin	0.197	0.596	0.001	- 0.0097	0.9937
Rutin	0.130	0.395	0.0011	- 0.0114	0.9972
Quercetin	0.123	0.373	0.001	- 0.0079	0.9975
Nobiletin	0.053	0.160	0.0009	0.0208	0.9995
Tangeretin	0.061	0.187	0.0009	0.0178	0.9994

with dimethylsulfoxide (DMSO) and methanol as solvent and extracted 200–300 mg/100g nobiletin and tangeretin from fresh *Citrus depressa* Hayata peels. The present study showed that simple ultrasonication methods with moderate ethanol concentrations could extract comparable amounts of nobiletin and tangeretin from *Citrus depressa* Hayata peels than other complicated methods with toxic solvent.

Beside nobiletin and tangeretin, rutin was also found to be the most abundant identified flavonoid (Fig. 3). Rutin (quercetin-3-rutinoside) is a vital nutritional component of plants and found abundantly in oranges, lemons, grapes, limes, berries, and peaches. Similar to nobiletin and tangeretin, rutin also reported to have neuroprotective effects due to high antioxidant activity. The chemical structure of rutin can directly scavenge reactive oxygen species (ROS) and also inhibit xanthine oxidase that generates ROS (Enogieru et al., 2018). Rutin also exhibited antibacterial activity against *Staphylococcus aureus, Staphylococcus glurance, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Escherichia coli* (Ganeshpurkar and Saluja, 2017; Gullón et al., 2017). This result showed that *Citrus depressa* Hayata peels contain an abundant amount of neuroprotective compounds.

Myricetin, quercetin and kaempferol also exhibit antioxidant properties and free radical-scavenging effects. These activities seem to support a wide range of nutraceutical activity such as including, antiplatelet aggregation, antidiabetic, antihypertensive, immunomodulatory, anti-inflammatory, anti-allergic, analgesic, anticancer actions, have a positive influence on cardiovascular diseases, antidiabetic, hepatoprotective, neurodegenerative disease, and antibacterial activity (Alam et al., 2020; Anand David et al., 2016; Semwal et al., 2016; Taheri et al., 2020; Wang et al., 2016, 2018).

3.4.1. Fruit maturity

Chromatographic data were also analyzed using multi-factorial analysis of variance to confirm the effect of independent variables (extraction time, solvent, and fruit ripeness) on individual extraction of phenolic compounds. Multi-factorial ANOVA test showed significant differences in flavonoid content for the different ripeness. The effect of

Table 3
Individual flavonoid compounds (mg/g dried peels) in *Citrus depressa* Hayata at different ripeness, solvent (ddH_2O and 50% aqueous ethanol), and five different ultrasonication time.

Ripeness	Solvent	US time (min.)	Kaempferol	Myricetin	Rutin	Quercetin	Nobiletin	Tangeretin
Unripe	ddH_2O	10	< LOD	< LOD	4.132 ± 0.245^{ab}	< LOD	8.784 ± 0.110^{ef}	4.358 ± 0.052^{e}
		20			2.828 ± 0.146^{d}		6.509 ± 0.040^{de}	3.108 ± 0.075^{d}
		30			2.228 ± 0.303^{e}		10.374 ± 0.178^{i}	4.952 ± 0.132^{i}
		40			4.172 ± 0.199^{ef}		8.930 ± 0.385^{h}	4.626 ± 0.143^{f}
		50			4.475 ± 0.167^{c}		8.937 ± 0.352^{g}	5.011 ± 0.116^{e}
	50% ethanol	10	< LOD	< LOD	5.785 ± 0.344^{i}	< LOD	12.298 ± 0.15^{j}	6.101 ± 0.073^{k}
		20			4.524 ± 0.233^{h}		10.415 ± 0.06^{f}	4.973 ± 0.119^{g}
		30			2.673 ± 0.363^{d}		12.449 ± 0.21^{j}	5.910 ± 0.159^{j}
		40			5.841 ± 0.279^{i}		12.502 ± 0.54^{j}	6.476 ± 0.201^{m}
		50			6.265 ± 0.233^{j}		12.511 ± 0.49^{j}	7.016 ± 0.163^{n}
Ripe	ddH_2O	10	< LOD	< LOD	1.021 ± 0.136^{ab}	< LOD	6.025 ± 0.105^{b}	2.414 ± 0.047^{a}
		20			1.061 ± 0.140^{b}		4.832 ± 0.113^{a}	2.041 ± 0.019^{a}
		30			1.059 ± 0.162^{a}		7.627 ± 0.087^{def}	3.062 ± 0.056^{b}
		40			1.631 ± 0.205^{cd}		6.341 ± 0.101^{cd}	2.727 ± 0.013^{c}
		50			1.036 ± 0.057^{ab}		6.150 ± 0.074^{bc}	2.590 ± 0.040^{b}
	50% ethanol	10	< LOD	< LOD	2.944 ± 0.267^{fg}	< LOD	7.651 ± 0.083^{h}	3.678 ± 0.070^{h}
		20			2.551 ± 0.335^{fg}		5.942 ± 0.076^{f}	2.930 ± 0.102^{f}
		30			2.789 ± 0.340^{e}		10.292 ± 0.243^{j}	5.054 ± 0.079^{k}
		40			5.982 ± 0.223^{k}		8.332 ± 0.332^{i}	4.487 ± 0.037^{l}
		50			3.033 ± 0.229^{gh}		7.439 ± 0.141^{h}	3.569 ± 0.033^{g}

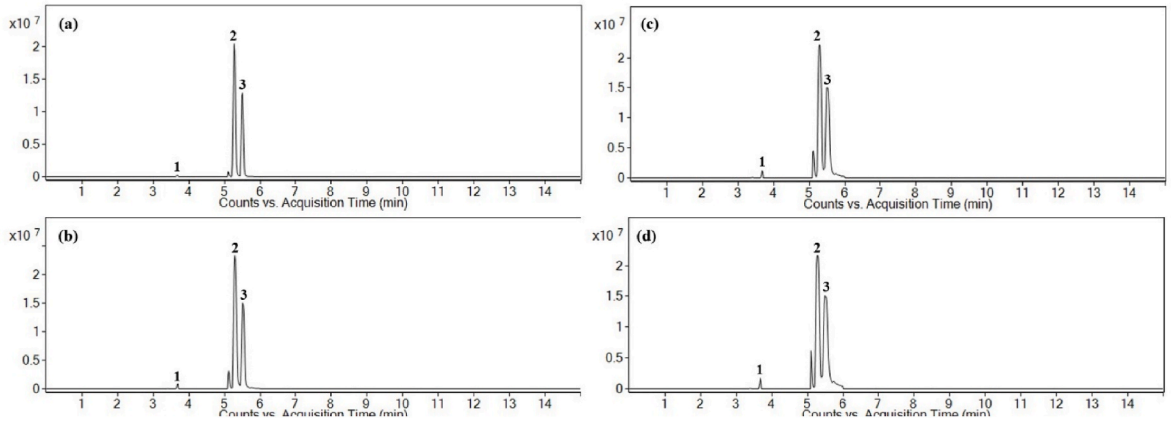

Fig. 3. LC MS/MS chromatogram of identified flavonoids (1. Rutin; 2. Nobiletin; 3. Tangeretin) in *Citrus depressa* Hayata extract (a. ripe, ddH_2O; b. unripe, ddH_2O; c. ripe, 50%ethanol; d. unripe, 50%ethanol).

fruit ripeness was significantly influential at a confidence level of 95% to total identified flavonoid amount and all individual flavonoids. Significantly ($p < 0.05$), higher flavonoid contents were achieved from unripe than ripe peels extracts (Fig. 4). *Citrus depressa* Hayata peels yield the highest content of nobiletin, tangeretin, and rutin, while the amount of kaempferol, quercetin and myricetin extracted from all samples were quite similar. Ethylene (phytohormone) released during fruit ripening induces the genes responsible for the synthesis of enzymes. These enzymes degrade the phytochemicals involved in the ripening process (Mansour, 2019).

Flavonoid composition appeared to vary significantly among fruits depending on their genetic origin, differential expression of genes in

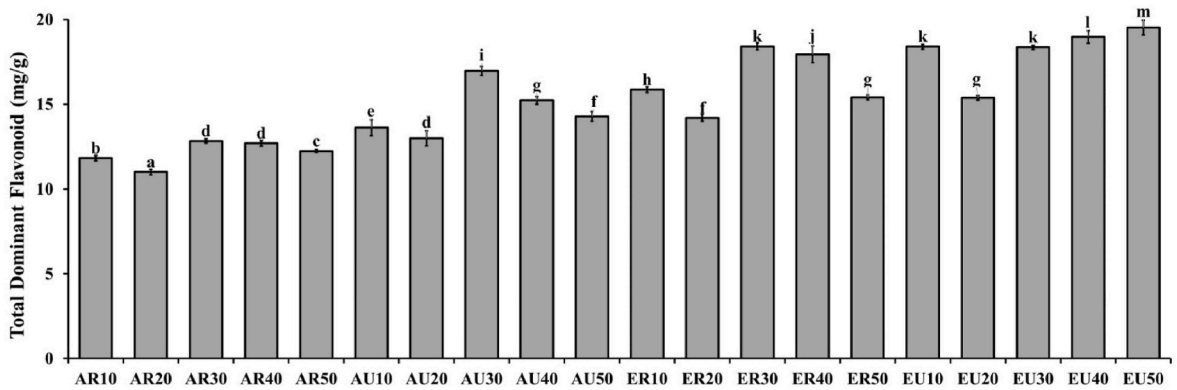

Fig. 4. Total dominant flavonoids in *Citrus depressa* Hayata peels.

flavonoid biosynthesis, time of fruit harvesting, and part of fruits (peel and edible parts). Peels may contain higher concentrations of phenolic compounds because it acts as protective agents against UV lights, pathogens and predators in fruits and vegetables. Phenolics accumulate in the outer part of the fruit exposed to light, which stimulates the synthesis (Asikin et al., 2012; Mansour, 2019). Citrus by-products (peel) also represent a rich source of naturally occurring flavonoids. The peels, which represents almost one-half of the fruit mass, contain the highest concentrations of flavonoids in the citrus fruits (Mansour, 2019). Flavonoids are important secondary metabolites present at relatively high concentrations in citrus fruit, mainly as conjugated molecules (Lado et al., 2018). Accumulation of flavonoid during maturity were different in some fruits. Pomegranate showed increasing amount during fruit maturity and no significant difference between ripe or unripe fruit (Mphahlele et al., 2014). Meanwhile, phenolic compounds in apple juice gala and lis gala varieties showed decreasing amounts (Silva et al., 2019). Similar to *Citrus depressa* Hayata in present study, flavonoid concentration of *Citrus aurantium* (Mansour, 2019), sour or bitter orange (Nawaz et al., 2020), ponkan, huyou (Xu et al., 2008b), Lane Late oranges and Delta oranges (Lado et al., 2018) also decreased during maturity process. Accumulation of many nutrients and phytochemicals in the fruit are influenced by factors such as genetic, environmental, agronomic and cultural practices. Moreover, fruit type and growing environmental conditions influence the phenolic composition. Flavonoid content is highly dependent on fruit ripening (Lado et al., 2018).

3.4.2. Solvent type

Due to the ease of use and broad applicability, solvent extraction is one of the most commonly used methods to extract bioactive compounds from plant materials. The solubility of flavonoids is dependent on the chemical nature of the sample and polarity of the solvents. Solvent polarity is a crucial factor in ultrasonic-assisted extraction that primarily affects the solubility of targeted bioactive compounds. Moreover, changes in the solvents used during treatment will affect the ultrasonic efficiency and the stability of flavonoids (Chaves et al., 2020; Rao et al., 2021; Sharma et al., 2019). Acidity, temperature, sample to solvent volume ratio, and the number and time intervals of individual extraction steps, also play a crucial role in the extraction procedure. Organic solvents such as methanol, ethanol, acetonitrile, petroleum ether, acetone, isopropanol, or mixtures with water, are generally used for flavonoids extraction using UAE in plant matrices samples (Chaves et al., 2020; Ngo et al., 2017; Stalikas, 2007). Hydrophobicity of phenolic compounds in the sample is also an important factor in investigating and identifying the optimal extraction solvent (Muñiz-Márquez et al., 2013).

Previous study determined the selection of ethanol and water over methanol, acetone, and hexane due to less toxicity and high extraction yield. The results in the present study showed that the effect of solvent selection was significantly influential at a confidence level of 95% to total identified flavonoids and all individual flavonoid. Significantly higher ($p < 0.05$) flavonoid contents were achieved from extraction by 50% aqueous ethanol than ddH_2O (Sembiring et al., 2017). and (Muñiz-Márquez et al., 2013) reported that alcohol-water mixture were preferred to optimize phenolic and flavonoid extraction from medicinal plants. Water ability to enhance swelling of plant material is favorable to increase contact surface area between plant matrix and solvent extraction, which would increase the bioactive yield (Muñiz-Márquez et al., 2013).

This result is in accordance to a study by Ngo et al. (2017) who found that 50% aqueous ethanol extracted more phenolic compounds from the root of *Salacia chinensis* L. than water. Moreover, Dhawan and Gupta (2016) also found that alcohol extracted more phenolic compounds from Datura metel plant leaf. Vasantha Rupasinghe et al. (2011) reported that highly polar (water) and non-polar (chloroform) solvents were not effective in extracting flavonols from apple peels.

3.4.3. Extraction time

Varying ultrasonic-assisted extraction duration effect on the extraction yield of bioactives has been extensively investigated. Prolonged sonication time initially escalates the yield, yet after certain time limits, the phytoconstituents may start degrading. During ultrasonication, cavitation enhances the swelling, hydration, fragmentation, and enhancing the permeability of the tissues that leads to the release of bioactive into the solvent. Increased ultrasound duration would cause intense structural damage to the solutes, inter-bubble collisions, and saturation effect that reduce bioactive extraction yield. Furthermore, degradation of heat-sensitive substances that extracted earlier might occur due to prolonged cavitation and thermal effect of ultrasound. A more extended time may lead to an inefficient extraction (Nishad et al., 2019; Ranjha et al., 2021; Rao et al., 2021; Yu et al., 2019).

The present study showed that extraction time was significantly influential at a confidence level of 95% to total identified flavonoids and all individual flavonoids. Ultrasonication duration were tested from 10 to 50 min. Significant fluctuation ($p < 0.05$) of extracted flavonoids were achieved during different extraction time. Different treatment variations showed different duration to produce the highest yield of flavonoid. Highest flavonoid extraction of ripe and unripe samples by ddH_2O and ripe samples in 50% aqueous ethanol were found at 30 min ultrasonication, while flavonoid extraction in unripe peels in 50% aqueous ethanol was highest at 50 min of treatment (Fig. 4).

Optimum ultrasonication period were differed between samples, depend on the nature and biological characteristics of the plant material (Muñiz-Márquez et al., 2013). Similar to flavonoid extraction in unripe *Citrus depressa* Hayata peels in 50% aqueous ethanol, phyllyrin extraction from *Forsythia suspensa* showed a gradual increase of phyllyrin from 0 to 60 min sonication (Xia et al., 2011). Study by Vasantha Rupasinghe et al. (2011) on apple peels reported that 15 min was the optimum period to extract quercetin, quercetin-3-*O*-galactoside, and quercetin-3-*O*-rhamnoside using 80% and 100% methanol. Nipornram et al. (2018) found that prolonged extraction time to 40 min leads to decreasing yield of phenolic extract in *Citrus reticulata* Blanco cv. Sainampueng. Meanwhile, flavonoid extraction by Yu et al. (2019) in *Crinum asiaticum* tested at10 to 80 min showed that extraction rate were increased rapidly at 50 min. Phenolic compounds of *Citrus reticulata* (kinnow) peel were extracted optimally at 33.71 min (Saini et al., 2021).

3.5. DPPH scavenging activity

There are several different methods recommended to evaluate antioxidant activity due to the complexity of some plant extracts. DPPH (1,1-diphenyl-2-picrylhydrazyl) assay is the common method to measure the ability of compounds to scavenge the DPPH radical. Delocalization of the spare electron over the molecule caused DPPH to become a free radical that exhibit deep violet color. When a substance can donate a hydrogen atom, hydrazine is obtained, with a change in color from violet to yellow, and the amount of reduced DPPH was visible in absorbance at 517 nm (Formagio et al., 2014; Sembiring et al., 2017).

DPPH scavenging activity of *Citrus depressa* Hayata peels that was extracted using 50% aqueous ethanol and ddH_2O showed different patterns through different ultrasonication periods. Unripe samples extracted by ethanol showed highest DPPH scavenging activity at 40 min yet at 30 min when extracted by ddH_2O. Meanwhile, contrary to unripe samples, highest DPPH scavenging activity of ripe samples in ethanol were found at 30 min and 40 min at ddH_2O (Fig. 5). Ngo et al. (2017) reported that antioxidant capacity is significantly varied, correspond to solvents used in a study on extraction of phenolic compounds in *Salacia chinensis* L., where samples extracted by acetone showed higher activity than water and ethanol. The polarity and hydrophobicity of bioactive groups extracted by the different solvents lead to different impacts of solvents on the bioactive compounds. Different bioactive groups exhibit different antioxidant power. Compared to other citruses, DPPH scavenging activity of pasteurized *Citrus sinensis* juice were

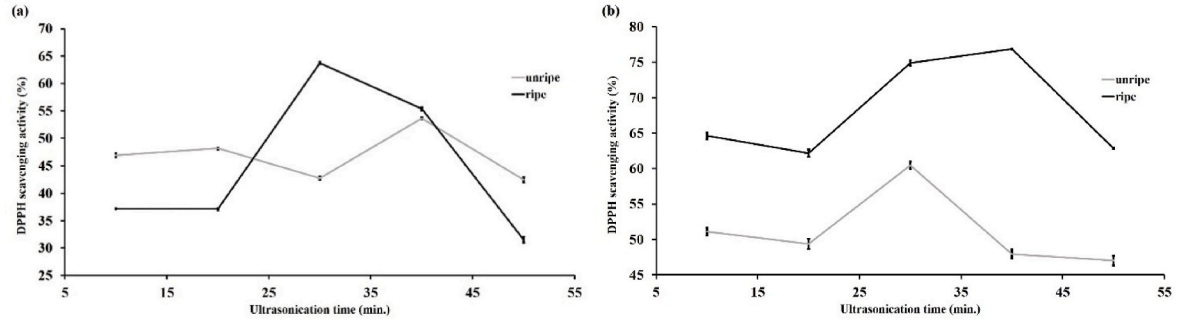

Fig. 5. Fluctuation of DPPH scavenging activity of samples extracted by (a) 50% ethanol and (b) ddH_2O at a different time of ultrasonication.

approximately 1.1–41.8% (Brasili et al., 2017), while *C. hystrix* ± 90%, *C. aurantifolia* ± 80%, and *C. microcarpa* ± 50% (Ghafar et al., 2010). These values are similar to the antioxidant capacity of several citrus varieties in China, which were 24.50–61.62% (Xu et al., 2008a).

Moreover, unlike some studies where higher phenolic content lead to higher antioxidant activity (Londoño-Londoño et al., 2010; Muflihah et al., 2021; Muñiz-Márquez et al., 2013), 50% ethanol extract of *C. depressa* Hayata peels that possess higher flavonoid, exhibit lower antioxidant activity (31.53–63.71%) than ddH_2O extract that possess lower flavonoid content (47–76.86%). Correlation analysis (Pearson's *r*) showed a moderate (r = - 0.43) and insignificant negative correlation ($p > 0.05$) between DPPH scavenging activity and total identified flavonoid (Fig. 6), which means that flavonoids were the components in the *Citrus depressa* Hayata extracts that in some condition moderately contribute to the decrease of antioxidant activities. Individual dominant flavonoids also showed negative correlation to DPPH scavenging activity, nobiletin (r = - 0.362), tangeretin (r = - 0.430) and rutin (r = - 0.422). This result is in accordance with studies by Dhawan and Gupta (2016) on Datura metel plants, Abarca-Vargas et al. (2016) on *Bougainvillea x buttiana*, *Capsicum chinense* (Herrera-Pool et al., 2021) and (Rahman et al., 2018) on *Citrus grandis* L. where negative correlation between phenolic compounds content to antioxidant activity occurred.

The chemical structure of flavonoids affects the antioxidant capacity. The presence of the free 3-hydroxyl group and the 4′-OH moiety (catechol structure) of the B-ring are crucial factors in the antioxidant capability. The total number of hydroxyl groups defines the antioxidant activity of the compounds (Baranowska and Bajkacz, 2018; Jakhar et al., 2014). LC MS/MS analysis showed that *Citrus depressa* Hayata peels contain more polymethoxyflavonoid (nobiletin and tangeretin) than other flavonoid types. Methyl groups occupying pivotal hydroxyl groups at 3′,4′ position at the flavonoid backbone in PMFs, lead to decreased of antioxidant activity (Jakhar et al., 2014; Londoño-Londoño et al., 2010). Furthermore (Gedikoğlu et al., 2021), stated that not all phenolic compounds have good DPPH scavenging activity.

4. Conclusions

In the present study, the effect of solvent types, extraction period and fruit maturity to extraction of several flavonoid compounds in *Citrus depressa* Hayata using ultrasonication were examined. These three factors were found to be significantly affect the flavonoid extraction yield. *Citrus depressa* Hayata contain nobiletin, tangeretin, and rutin as dominant flavonoids, where unripe peels extracted by ethanol exhibit the highest amount. A negative correlation occurred between flavonoid

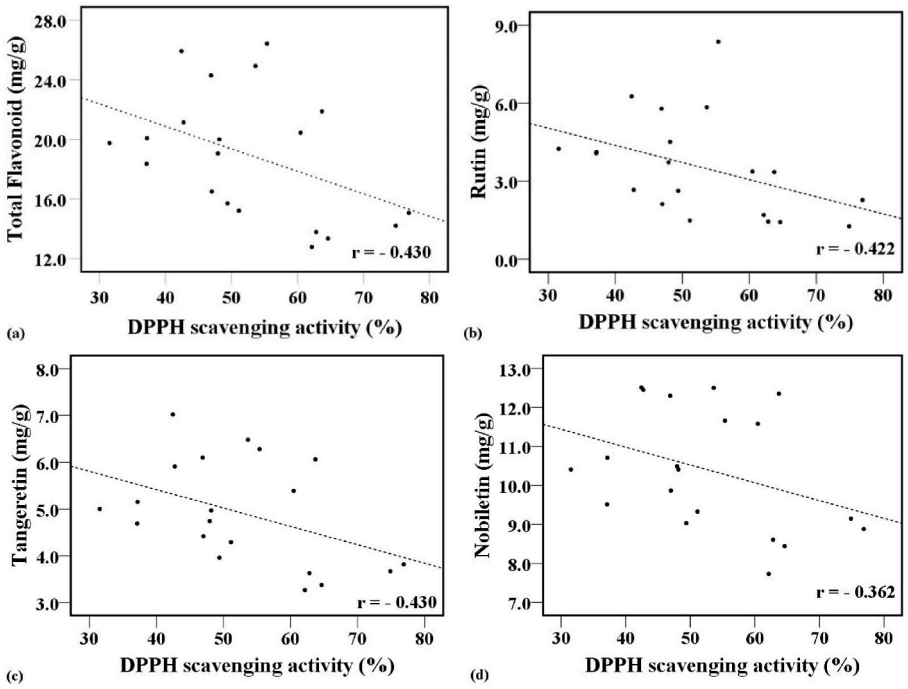

Fig. 6. Pearson's (r) Correlation analysis between DPPH scavenging activity and (a) total identified flavonoids; (b) rutin; (c) tangeretin (d) nobiletin of *Citrus depressa* Hayata.

amount and DPPH scavenging activity, which needs further research on the optimization process and other bioactivity of these flavonoids.

CRediT authorship contribution statement

Wei-Jyun Chien: Funding acquisition, Supervision, Resources, Conceptualization, Writing – review & editing. **Dinar S. Saputri:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft. **Hung-Yu Lin:** Methodology, Data curation, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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