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Evaluation of different extraction methods on the polyphenols yield, flavonoids yield, and antioxidant activity of the pomelo flavedo extract from Da Xanh (*Citrus maxima [burm]* merr.) variety

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Abstract

Pomelo peels have been well known as a valuable source of phenolic compounds mainly flavonoids. In this study, the influences of extraction conditions of three extraction methods on the polyphenols yield, flavonoids yield, and antioxidant activity of resulting pomelo flavedo extract (PFE) were evaluated. As a result, the suitable process parameters for the ultrasound assisted extraction were selected at an applied power of 120 W, the temperature of 60 °C for 20 min. Meanwhile, the microwave assisted extraction was operated at an applied power of 150 W for 25 min and the Soxhlet extraction was carried out up to 4 reflux cycles (35 min per cycle) at 80 °C. Among three methods, the microwave assisted extraction was considered as the most efficient method to obtain the high yield of polyphenols (80.56%) and flavonoids (86.58%). Naringin and hesperidin determined by high-performance liquid chromatography showed the value of 64.42 ± 2.90 mg/g DW and 0.97 ± 0.02 mg/g DW, respectively. The PFE extracted by the microwave assisted extraction could be a potent nutraceutical in further application on food or pharmaceutical industries.

Keywords: pomelo flavedo extract; ultrasound assisted extraction; microwave assisted extraction; soxhlet extraction; antioxidant activity.

Practical Application: This study suggested the potential of the microwave-assisted extraction method in achieving the highest yield of polyphenols and flavonoids in the pomelo flavedo extract. Besides, naringin and hesperidin characterized in the pomelo flavedo extract can be a potent nutraceutical which is needed to be purified for further application in the food and pharmaceutical industries.

1 Introduction

Solvent extraction process has been considered a crucial step for the use of the extract to further apply in food or pharmaceutical industries (Luengo et al., 2013). The variation of process parameters in the extraction process showed a great influence on the extraction yield (Contini et al., 2008). Around 120 million tons of citrus peels, which wastes valuable resources, have been annually discarded, whereas the orientation of "green recovery" is one of the most promising solutions (Mahato et al., 2020). To date, studies in phenolics enriched extract have been drawing attention of researchers to optimize the extraction process and to enhance its potential in multi-field applications (Carrasquero, 2018). The terminology "phytochemical" has gained attention of health-conscious customers due to its health-beneficial effects. These bioactive compounds, which serve a role in anti-inflammation, anti-cancer, anti-carcinogenicity, and anti-aging, have been widely found in citrus species (Liu, 2004; Nayak et al., 2015; Proestos et al., 2006).

Pomelo (*Citrus maxima (burm*) merr) peels are mostly composed of water, cellulose, hemicellulose, soluble sugars, essential oils (mainly D-limonene), and polyphenols (mainly flavonoids) (Tocmo et al., 2020). Besides, pomelo peel also contains a certain amount of pectin, amylase, peroxidase, vitamin A, and vitamin C (130-170 mg/100 g fresh matter) (Nhi et al., 2020; Yadav et al., 2009). Citrus flavonoids, mainly flavanones including naringenin, hesperetin and its glycosides (naringin, hesperidin, narirutin, and neohesperidin), have been demonstrated to exhibit antihypertensive and anti-atherosclerotic effects, and cardiovascular protection activities (Yi et al., 2017). Naringenin and naringin extracted from citrus peels, they were reported to have anti-tumor potential in breast cancer by modulating the estrogen signaling and inhibiting aromatase (El-Kersh et al., 2021). Naringin, a predominant flavanone in the pomelo flavedo, has shown a wide range of biological activities including antioxidant, antimicrobial, antiviral, anticarcinogenic, and hypolipidemic activities (Almeida et al., 2012; Zhang et al., 2011). It was also reported to protect vascular smooth muscle cells by enhancing

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the strength and resistance of blood vessels, leading to a reduction of the atherogenic effects (Kim et al., 2003).

Normally, the maceration method shows economic efficiency but requires an extended extraction time of 72 h according to a previous report of Chollakup et al. (2020). Flavonoids extraction assisted with enzymatic and ultrasound treatment from the pomelo peel showed the increment in naringin and hesperidin contents by 5.70% and 1.20% compared to conventional method (Anh et al., 2021). Supercritical CO_2 extraction has been considered an advanced technology for the fragrant oil extraction from pomelo flower but may show the limited applicability due to high cost production (Huiming et al., 2005). Polyphenols from pomelo peels extracted using liquid-phase pulsed discharge at a high voltage of 12 kV showed an improvement in the extraction efficiency (Xi et al., 2021). In summary, most of studies were carried out to better yield the extraction efficiency. Besides, the phytochemical content and its biological activities are mostly dependent on noticeable variables such as variety, cultivation, environmental conditions (Toh et al., 2013; Zhang et al., 2014). Each cultivar from different regions may possess different phytochemical constituents.

In Vietnam, a vast number of pomelo peels from Da Xanh variety are annually discarded without perceiving its valuable source of phytochemical compounds. Hence, we, in this study, aimed to investigate the effects of process parameters from different extraction methods (Soxhlet extraction, ultrasound assisted extraction, and microwave assisted extraction) on the polyphenols and flavonoids yields of the pomelo flavedo extract as well as its influence on the antioxidant activity of the extract. The qualitative and quantitative analysis of phytochemicals in the extract were also evaluated.

2 Materials and methodologies

2.1 Materials

Da Xanh pomelo variety harvested in July-August was purchased from the local market, Ben Tre province. The fruits were washed to remove dust and impurity particles. The flavedo was manually peeled off by a sharp knife. The measured thickness of the flavedo was about 5 ± 2 mm. The flavedo was subjected to a convection dryer at 60 °C until reaching the moisture content of less than 5%. The dried flavedo was ground into fine powder by using a grinder and then sieved by a 0.25 mm mesh. The flavedo powder was stored in the dark at room temperature for further analysis.

2,6-dichlorophenolindophenol (DCPIP), L-ascorbic acid, Folin-Ciocalteu reagent (F-C), gallic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid), naringin, and hesperidin were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. All other analytical chemicals were from standard commercial supplies.

2.2 Pomelo flavedo extract by different methods

In this study, the prepared flavedo powder was introduced to different extraction methods. In brief, the procedure for each method was described as followed:

Soxhlet extraction: Five grams of flavedo powder was included in 150 mL of absolute ethanol solution. The mixture was subjected to the Soxhlet extractor at the processed temperature of 80 ± 2 °C with different repeated reflux cycles (2-3-4-5 cycles, 35 ± 2 min per cycle).

Ultrasound assisted extraction (UAE): Five grams of flavedo powder was included in 150 mL of absolute ethanol solution. The mixture was extracted in association with an ultrasound system (40 kHz, 120 W) at varied temperatures (45-50-55-60 °C) for 20 min. To determine extraction time, the mixture was extracted at different time periods (15-20-25-30 min) at selected temperature (60 °C).

Microwave assisted extraction (MWE): Five grams of flavedo powder was included in 150 mL of absolute ethanol solution. The mixture was introduced to a microwave system (ME71A/SV, Samsung Electronics Co., Ltd., Korea) at different applied power (150-300-450-600 W). After selecting the suitable applied power, the extraction time was evaluated at varied time periods (15-20-25-30 min).

2.3 Polyphenols yield

Polyphenols content was determined following a Folin-Ciocalteu colorimetric approach as previously described by Chandra et al. (2014). The pomelo flavedo extract (PFE) was serially diluted at different concentrations. Aliquot (0.5 mL) of PFE was included with 10% Folin-Ciocalteu solution (2.5 mL) and allowed to react for 5 min in the dark. Then, a volume of 2 mL of 7.5% Na_2CO_3 was added to the mixture and incuabted for 1 h at room temperature before reading absorbance at 765 nm by using a UV-Vis spectrophotometer (model GENESYS™ 10S, Thermo Fisher Scientific Inc., USA). Gallic acid was used as a standard solution. The polyphenols content was expressed as milligram galic acid equivalent per gram of dry weight (mg GAE/g DW). The polyphenols yield (%) was calculated by the polyphenols content of the PFE per the polyphenols content of the control sample (flavedo powder).

2.4 Flavonoids yield

The flavonoids yield was measured according to the method of Mahboubi et al. (2013) with slight modifications. Briefly, 0.5 mL of PFE at different diluted concentrations was mixed with 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M CH_3COOK, and 4.3 mL of distilled water. The mixture was kept for 30 min at room temperature and the absorbance was recorded at 415 nm. Quercetin served as a standard solution. The flavonoids content was expressed as milligram quercetin equivalent per gram dry weight (mg QE/g DW). The flavonoids yield (%) was calculated by flavonoids content of the PFE per flavonoids content of the flavedo powder.

2.5 Antioxidant activity

The antioxidant activity of PFE was determined by using a DPPH assay and ABTS assay. For DPPH assay, 0.5 mL of PFE was mixed with 1.5 mL of DPPH solution and incubated in the dark for 30 min. The absorbance was recorded at 517 nm. Vitamin C served as a positive control. The DPPH radicals scavenging

effect was expressed as vitamin C equivalent (mg AA/g DW) using a standard equation: y = -0.1239x + 0.991 (R^2 = 0.99999).

In terms of ABTS assay, ABTS solution was prepared by mixing 10 mL of 7.4 M ABTS with 2.6 mM $K_2S_2O_8$ and incubated in the dark for 24 h. The mixture was diluted by adding absolute ethanol to adjust the absorbance to approximately 1.1 ± 0.02 at 734 nm. Aliquot (0.5 mL) of PFE was inlcuded with ABTS solution and allowed to keep for 30 min. The absorbance was read at 734 nm and vitamin C served as a positive control. The ABTS scavenging effect was expressed as vitamin C equivalent (mg AA/g DW) by using a standard equation: y = -0.1266914x + 0.6672952 (R^2 =0.99936).

2.6 Qualitative and quantitative analysis of phytochemical constituents in the PFE

The qualitative analysis was carried as followed by a previous study done by Ciulei (1982) to characterize the presence of Alkaloids, Cyanidin, Steroid, Terpenoid, Tannin and reducing sugar in the PFE. High-performance liquid chromatography (HPLC) was employed for the quantitative analysis of PFE. The PFE was diluted with methanol (1:10 w/v), centrifuged at 12000 rpm and then filtrated by using a 0.22 membrane (Merck GKA, Germany). Aliquot (3 µL) of sample was subjected to HPLC system (1260 Infinity II LC, Agilent Technologies, Inc., USA) with the symmetry column (C18, 5 µm 250 x 4.6 mm). Methanol and 0.01% orthophosphoric acid mixture at the 5:95 ratio was employed as a mobile phase with a flow rate of 1 mL/min. Analytes (naringin and hesperidin) were detected and monitored by a UV detector at 280 nm (Saeidi et al., 2011).

2.7 Statistical analysis

Experiments were performed in triplicate and data were expressed as mean ± standard deviation. Statistical analysis was determined by using a Statgraphics Centurion XV.I software, Statgraphics Technologies, Inc., Virginia. Analysis of variance (ANOVA) and Tukey's HSD test were applied to compare experimental mean values at the level of 5% ($p < 0.05$).

3 Results and discussion

3.1 Effect of Soxhlet extraction condition on the polyphenols yield, flavonoids yield, and antioxidant activity of PFE

Figure 1 presents the effect of reflux cycles from the Soxhlet extraction on the polyphenols yield, flavonoids yield, DPPH and ABTS radical scavenging capacities of the obtained PFE.

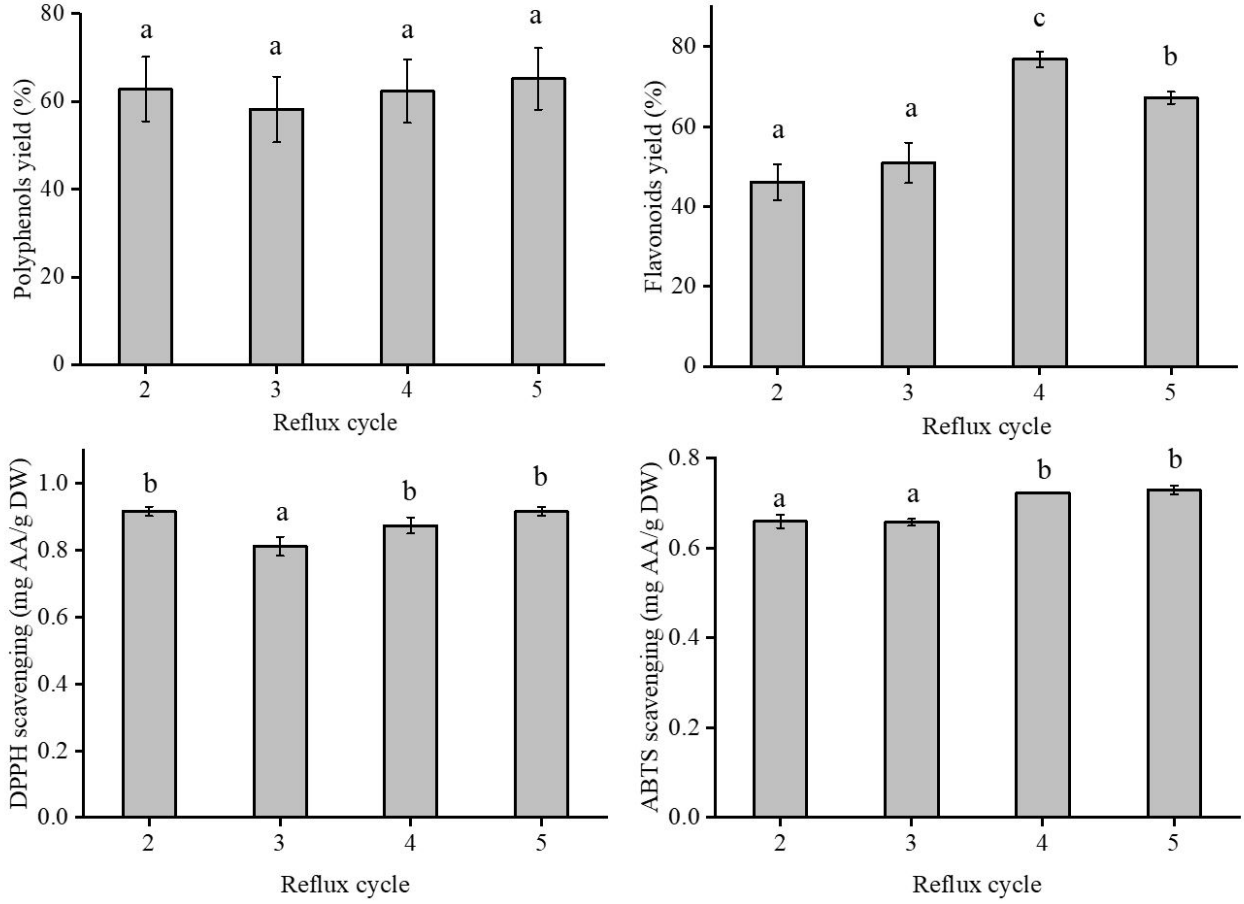

Figure 1. Effect of reflux cycles in the Soxhlet extraction on the polyphenols yield, flavonoids yield, and antioxidant activity based on DPPH and ABTS scavenging capacities of resulting PFE.

The highest flavonoids yield, 76.84 ± 1.48%, was obtained after 4 reflux cycles. The increased extraction efficiency was due to the solvent circulation which increased the ability to entrain phytochemical compounds. However, the prolonged exposure to high heating temperature from the Soxhlet apparatus easily led to the decomposition of flavonoids (Mojzer et al., 2016). On the contrary, the increased flux cycle could not promote better polyphenols yield, around 62%. This result followed the Fick's second law of diffusion, the extraction rate was slowly reduced until reaching the final equilibrium between the extraction solvent and plant tissues. Therefore, further reflux cycles could not increase the polyphenols yield (Alara et al., 2018). The PFE extracted with 4 reflux cycles also revealed the highest DPPH and ABTS scavenging effects with respect to 0.87 ± 0.02 mg AA/g DW and 0.72 ± 0.00 mg AA/g DW. The observed trend was in agreement with a previous report done by Chew et al. (2011). The highest antioxidant activity of *Orthosiphon stamineus* extract accompanied with the highest phenolic compounds after 120 min of Soxhlet extraction. The antioxidant activity, in some cases, is highly dependent on not only high phytochemical content but also the characteristic structures or interrelationships between the phenolic compounds in the extract (Huang et al., 2005). Therefore, the identification of the predominant phenolic compounds in accordance with antioxidant mechanisms and their synergistic effects should be further investigated to provide great insight into the correlation between the phytochemicals content and antioxidant activity. In this studied condition, the Soxhlet extraction with 4 reflux cycles was considerably suitable to obtain the highest flavonoids yield and antioxidant activity.

3.2 Effect of ultrasound extraction conditions on the polyphenols yield, flavonoids yield, and antioxidant activity of PFE

The effects of extraction temperature and extraction time of the UAE process on the polyphenols yield, flavonoids yield, and antioxidant activity by scavenging DPPH and ABTS radicals are depicted in Figure 2. The elevated temperature could not enhance the polyphenols and flavonoids extraction yield, remaining the extraction yield around 70% and 80%, respectively. However, the antioxidant activity by scavenging DPPH and ABTS radicals achieved the highest value of 1.91 ± 0.02 mg AA/g DW and 0.69 ± 0.01 mg AA/g DW at 60 °C, respectively. The higher temperature assisted with the ultrasound wave might accelerate the molecular movement of phenolic compounds in plant tissues, facilitating better release of high antioxidant activity-exhibiting phenolic groups into the extracting liquid (Ma et al., 2009).

Similarly, the extraction time was not found to induce a significant variation in the polyphenols and flavonoids yield. Normally, the polyphenols extraction process was observed to experience the first stage of fast release of polyphenols in the mixture followed by a slower release of these compounds (Jovanović et al., 2017). The exposure of polyphenols to high temperature, prolonged time, and enzymatic hydrolysis might

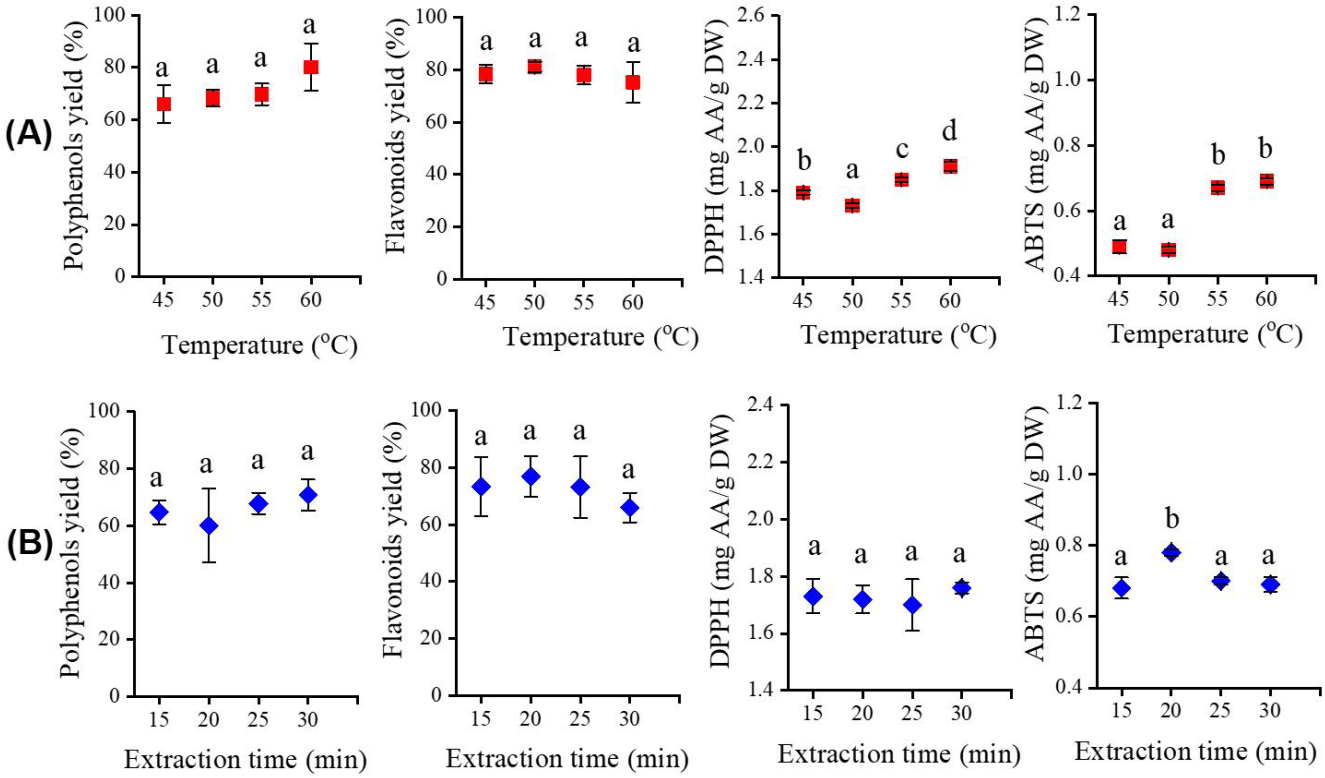

Figure 2. Effect of A) temperature and B) extraction time in the UAE on the polyphenols yield, flavonoids yield, and DPPH and ABTS scavenging effect of resulting PFE.

induce the oxidation process of polyphenols, leading to the decay of polyphenols (Vergara-Salinas et al., 2012). Besides, the prolonged time of ultrasonication was noted to partially cause the destruction of antioxidants due to the formation of free radicals during the sonication process (Bamba et al., 2018; Horžić et al., 2012). In this study, the extraction time for 15 min promoted a 70% release of polyphenols and flavonoids compounds, whereas later increase in the extraction time could not induce higher extraction efficiency. d'Alessandro et al. (2012) also reported that polyphenols were rapidly released in the first 15 min, but slower secretion occurred in the next 4 h. Besides, the DPPH scavenging effect was insignificantly different among the tested group ($p > 0.05$), reaching a value of 1.7 mg AA/g DW. Meanwhile, the PFE extracted by the UAE method for 20 min significantly showed the highest antioxidant capacity with a respective value of 0.78 ± 0.01 mg AA/g DW. The result showed that the extraction conditions of the UAE method at the power of 120 W, the temperature of 60 °C for 20 min were suitable in this studied condition to obtain the highest polyphenols yield, flavonoids yield, and antioxidant activity.

3.3 Effect of microwave assisted extraction conditions on the polyphenols yield, flavonoids yield, and antioxidant activity of PFE

In the microwave assisted extraction process, the applied power and extraction time play a critical role in the extraction efficiency of polyphenols and flavonoids compounds, shown in Figure 3. The applied power at 150 W revealed the highest polyphenols yield and flavonoids yield with respect to 84.12 ± 2.42% and 92.83 ± 3.78%. The higher applied power did not significantly increase the polyphenols yield and flavonoids yield. The microwave heating induced the alteration in the structural characteristics of plant tissue by increasing the capillary-porous properties and the water absorption capacity of plant cells (Kratchanova et al., 2004). This improved the extraction efficacy of phytochemical components in the flavedo. It was also reported that the microwave heating promoted a thermal gradient between the extracting medium and plant cells, facilitating the liberation of phenolic compounds (Hayat et al., 2010). A similar finding was observed in the study of Ghanem et al. (2012), the phenolics content in the citrus peel was unchangeable at the applied power of 100 W and 180 W but it was found to increase by 33.6% at the power level of 450 W. Phenolic compounds in green asparagus was reported to decay when applying an increased microwave power (Nguyen et al., 2019). On the other hand, the flavonoids yield was noticeably reduced by the microwave treatment when increasing the microwave power. The flavonoids yield decreased to 73.71 ± 4.62% at 600 W. The high power of microwave treatment caused the increment in electric field strength and fast dielectric heating within the sample, contributing to the degradation of flavonoids compounds (Hayat et al., 2010). The PFE obtained from the MWE at an applied power of 150 W exhibited the highest antioxidant capacity based on DPPH and

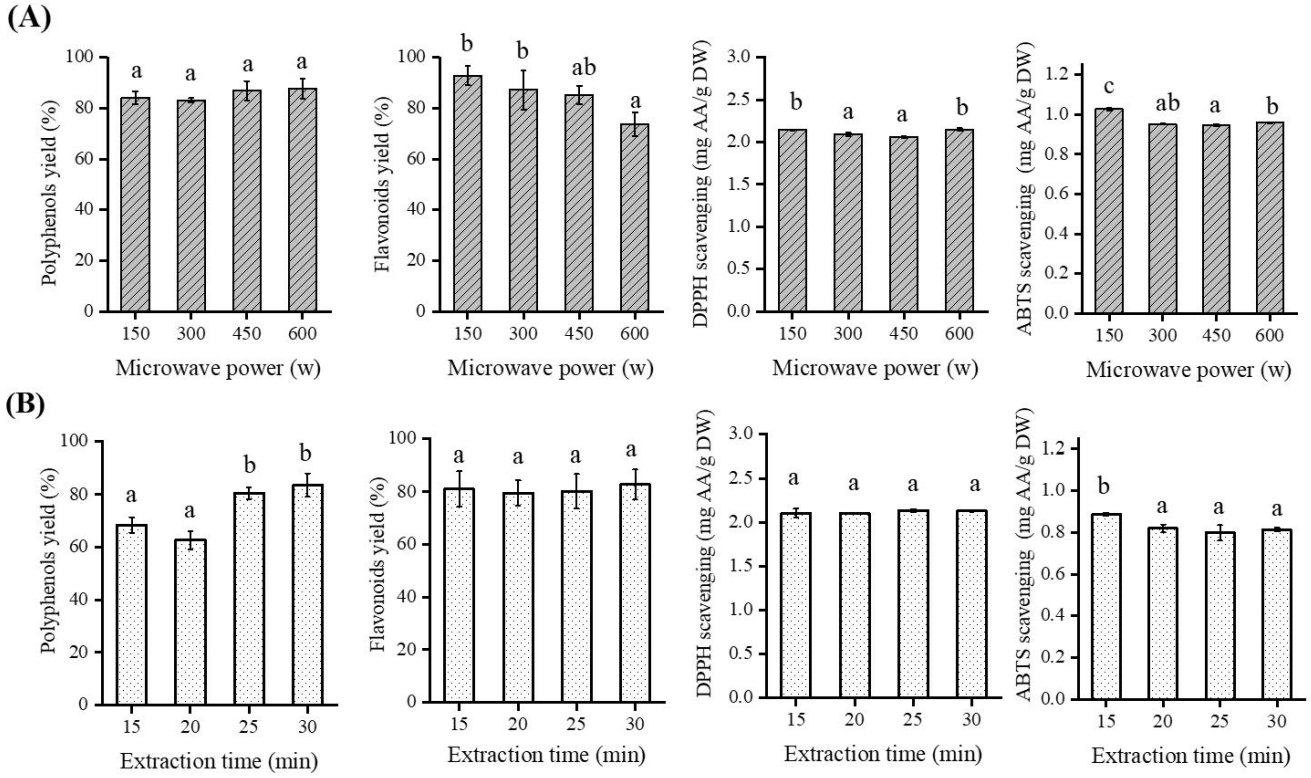

Figure 3. Effect of A) microwave power and B) extraction time in the MWE on the polyphenols yield, flavonoids yield, and DPPH and ABTS scavenging effect of resulting PFE.

ABTS scavenging effects, reaching the values of 2.11 ± 0.05 mg AA/g DW and 0.89 ± 0.01 mg AA/g DW, respectively.

The extraction time in the MWE process also showed the noticeable influence on the polyphenols yield. The PFE achieved the highest polyphenols yield (80.56 ± 6.56%) after 25 min of extraction and further extraction time could not help release more phenolic compounds. Hayat et al. (2010) also showed that the microwave treatment showed the improvement in antioxidant activity as a result of increased phenolic compounds in the citrus mandarin peel extract, but longer irradiation time might cause the adverse effect due to the degradation of these compounds. However, in this study, the flavonoids yield and antioxidant activity by scavenging DPPH and ABTS radicals were less affected by the prolonged irradiation of microwave treatment. The DPPH and ABTS scavenging capacities were mainly dependent on the heat-stable flavonoids (flavones and flavanones) in the pomelo flavedo (Tian et al., 2018), thereby the insignificant difference in antioxidant activity of PFE at different microwave power was possibly due to the heat-stability of flavonoids under this studied condition. An extended treatment time should be conducted to observe the significant difference in the TFC and antioxidant activity of PFE. Therefore, according to the studied conditions, the microwave power of 150 W and the extraction time of 25 min were appropriate to promote the highest polyphenols yield, flavonoids yield, and antioxidant activity of PFE by the MWE method.

3.4 Comparing the impacts of extraction methods on the polyphenols yields, flavonoids yield, and antioxidant activity of resulting PFE

After selecting the suitable process parameters for each extraction method, the comparison of each extraction method according to the polyphenols yield, flavonoids yield, and antioxidant activity of resulting PFE was evaluated, shown in Figure 4.

In general, the Soxhlet extraction showed the lowest efficiency, achieving 63.27 ± 1.92% of polyphenols yield, 67.22 ± 1.92% of flavonoids yield, and DPPH and ABTS scavenging effects with respect to 0.87 ± 0.02 mg AA/g DW and 0.72 ± 0.00 mg AA/g DW. This could be ascribed to the prolonged exposure to high processed temperature of 80 °C in the Soxhlet extraction process, leading to the faster degradation of phytochemical compounds (M'hiri et al., 2017). The MWE showed the highest yield of polyphenols (80.56 ± 2.21%) and flavonoids (84.54 ± 3.53%), as well as the highest DPPH and ABTS scavenging effects, 2.13 ± 0.02 mg AA/g DW and 0.80 ± 0.04 mg AA/g DW. It was reported that the phenolic compounds were easily susceptible to the ultrasound treatment than microwave treatment. To be more specific, p-coumaric and p-hydroxybenzoic acids were rapidly degraded by the ultrasound treatment at 40°C for 20 min, whereas none alteration of these compounds was observed when subjected to the microwave treatment at the high temperature of 175 °C (Ma et al., 2008). The lower efficacy in the UAE could be possibly due to the local high pressure and high temperature by cavitation collapse, leading to easier degradation of phenolic compounds (Raso et al., 1999). A similar finding was observed in the study of Bagherian et al. (2011), the MWE was found to better yield pectin content than the UAE. Many previous studies also highlighted that the MWE showed the most improvement in the content of polyphenols and flavonoids compounds from citrus peels compared to other methods (M'hiri et al., 2015; Ma et al., 2008; Nayak et al., 2015; Wandee et al., 2019). Therefore, it could be concluded that the MWE showed the most extraction efficiency in these studied conditions.

3.5 Qualitative and quantitative analysis of PFE

Table 1 lists the presence of phytochemical compounds in the PFE. As a result, all the targeted compounds showed positive results including alkaloids, coumarin, cyanidin, reducing sugar, steroid, tannin, and terpenoids. Different varieties of pomelo fruit or extraction methods may reveal the discrepancy in the presence of phytochemical compounds in the extract. Balamurugan et al. (2014) showed the presence of cardiac glycosides except saponin, flavonoids, terpenoid, and protein in

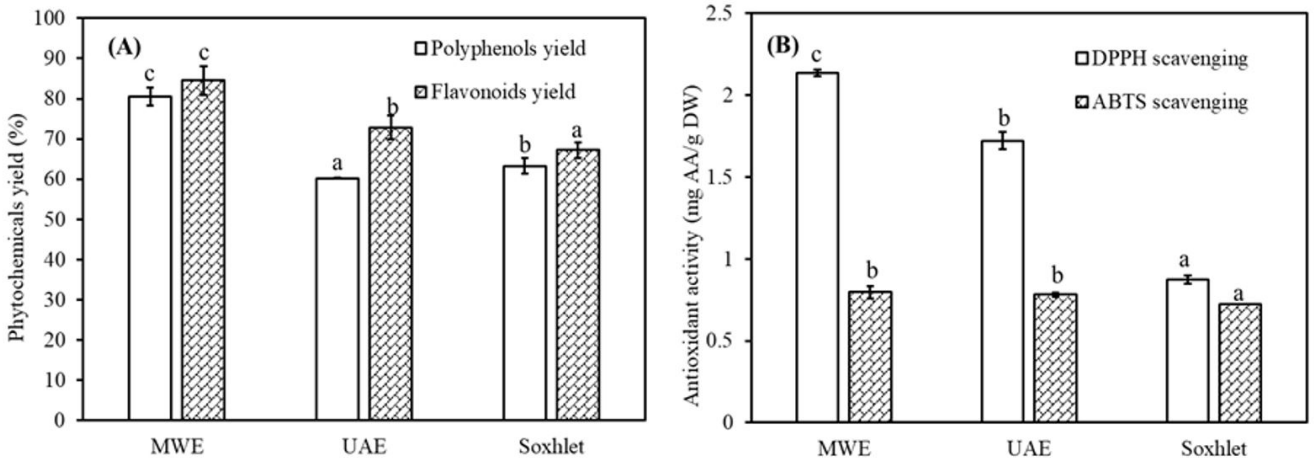

Figure 4. Comparison of extraction methods according to the yield of polyphenols and flavonoids content, and DPPH and ABTS scavenging effects of resulting PFE.

the methanolic pomelo peel extract. The typical flavonoids such as quercetin, naringin, and hesperidin were characterized as the predominant compounds in the pomelo flavedo (Grzegorzewski, 2010; Methacanon et al., 2013). The presence of alkaloids, reducing sugar, and carbohydrates were obviously identified in the pomelo peel extract by the Soxhlet extraction method, whereas the maceration extraction only presented positive results on alkaloids and terpenoids (Khan, 2018).

The HPLC chromatogram for the quantitative analysis of naringin and hesperidin in the PFE is presented in Figure 5 and Table 2.

The naringin and hesperidin content in the PFE were 1582.53 mg/mL and 23.56 mg/mL corresponding to 64.42 ± 2.90 mg/g DW and 0.97 ± 0.02 mg/g DW, respectively. The variation in the naringin and hesperidin content is highly dependent on the pomelo varieties and extraction methods, or types of extracting solvent. The naringin content in this study was considerably higher than that extracted by the microwave treatment at 331 W for 15 min in a study of Liu et al. (2017) (8.38 ± 0.2 mg/g). The naringin content was also higher than those, which ranged from 23 mg/g to 32 mg/g, from previously reported studies (Sudto et al., 2009; Xu et al., 2007). Meanwhile, the hesperidin content, in some previous reports, was not found in the pomelo varieties from China (Nogata et al., 2006; Phuong et al., 2021). The high content of naringin in the PFE suggested a promising potential in further application of these compounds as nutraceuticals for food or pharmaceutical industries.

Table 1. Qualitative analysis of phytochemical compounds in the PFE.

Test method		Water	Ethanol	PFE
Anthraquinones		+	+	+
Alkaloids	Bouchadat	+	+	+
	Mayer	+	+	+
	Dragendoff	+	+	+
Coumarin		+	+	+
Cyanidin		+	-	+
Reducing sugars		+	+	+
Steroid		+	+	+
Tannin		+	+	+
Terpenoid		+	+	+

+ = shows the presence; - = the absence of the compound in the sample.

Table 2. Naringin and hesperidin content in the PFE.

Composition	Naringin	Hesperidin
Content (mg/g DW)	64.42 ± 2.90	0.97 ± 0.02

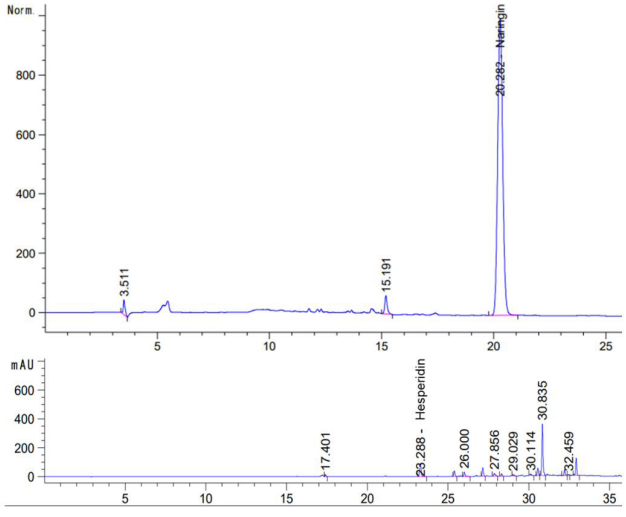

Figure 5. HPLC chromatogram of PFE.

4 Conclusion

In this study, the effects of extraction conditions from different extraction methods including Soxhlet extraction, UAE, MWE were successfully evaluated. The result showed that the highest yield of polyphenols and flavonoids in the PFE, and antioxidant activity were obtained by the MWE method. Naringin and hesperidin were the predominant compounds in the PFE, suggesting the potent application of the PFE in food or pharmaceutical industries due to the high amount of these bioactive compounds. Further analysis should be conducted to identify the predominant compounds in the extract that are mainly responsible for the antioxidant capacity. Besides, the optimization of process parameters could be conducted to better yield bioactive compounds in the PFE.

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