Published online: 6 August 2019

Comparative evaluation of different extraction method for biological compounds and antioxidant activity from young *Citrus maxima* Merr. for cosmetic utilization

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Abstract

In general, young fruits of pomelo are trimmed off and abandoned as agricultural waste during cultivation. To increase the value of those wastes, therefore, this research aimed to investigate the effects of extraction solvents on the biological activities of young fruits by the maceration technique. In this study, four different maceration solvent systems were used. The four systems were 95%, 75%, 50% ethanol, and propylene glycol, respectively. The age of young fruits used in this study was in three ranges: one-month-old, two-months-old, and three-months-old. The Folin-Ciocalteu reagent assay and aluminum chloride colorimetric method were used for measuring phytochemical constituents content. Antioxidant activity of the extract was measured by DPPH radical scavenging assay and hydrogen peroxide scavenging assay. The result in this study showed that one-month-old pomelo fruit extracted by 75% ethanol had the highest total phenolic content (31.64 ± 0.39 mg gallic acid equivalent/mL extract) and the highest total flavonoids content (5.41 ± 0.47 mg quercetin equivalent/mL extract). This specimen also had the strongest antioxidant activities. The extract had DPPH radical scavenging activity of 179.07 ± 8.48 mg ascorbic acid equivalent/mL extract and hydrogen peroxide scavenging activity of 124.63 ± 0.74 mg ascorbic acid equivalent/mL extract.

The summary showed that the phytochemical constituents and antioxidant activities decreased correspondingly to the maturing of the fruits. One-month-old fruit extracted by 75% ethanol was shown the highest phytochemical constituents and antioxidant activity. Further studies on phytochemical constituents, quantitative analysis, standardization, and formulation into cosmetic products should be conducted.

Keywords: *C. maxima* Merr./Tubtim-Siam/Total phenolic content/Total Flavonoid content/DPPH radical scavenging activity/Hydrogen peroxide scavenging activity

Introduction

Fruits and vegetables are the sources of polyphenolic compounds and flavonoids that have many essential bioactivities for health such as antioxidant, antithrombotic, anticancer, antidiabetic, and protection against cardiovascular disease (Khan, Zill-E-Huma, & Dangles, 2014). Citrus is one of the most famous plants in tropical climates on earth. Pomelo (*Citrus maxima* Merr., family Rutaceae) is one of the favorite citrus crops in Thailand. There are many types of pomelo cultivated, for example, *C. maxima* "Thong-dee," *C. maxima* "Kao-Nampueng," *C. maxima* "Ta-Koi," *C. maxima* "Tubtim- Siam," *C. maxima* "Kao-Tangkwa," and *C. maxima* "Kao-Yai" (Mäkynen et al., 2013).

C. maxima "Tubtim-Siam" is the Jewel of the Pakpanang river lagoon and an approved geographic indicator (GI) plant. Based on discussion with producers, who have Tubtim-Siam pomelo orchards, the author learned that they removed young fruits during the cultivation, which are an agronomical waste. It was reported that Tubtim-Siam pomelo is rich in citrus flavonoids having many bioactivities from the peel, pulp, and juice, for example, antioxidant properties, inhibitor of pancreatic lipase, antihyperlipidemic activity, and cholesterol esterase. These can be developed for functional food (Mäkynen et al., 2013). They also reduce of cellular oxidative stress, enhancement of GSH antioxidant capacity (Chularojmontri, Gerdprasert, & Wattanapitayakul, 2013), and enhancing cell migration and delaying cellular aging (Buachan, Chularojmontri, & Wattanapitayakul, 2014). Previous investigations have shown that the mature fruits of Tubtim-Siam pomelo provided benefits for health.

However, the biological activities of young pomelo fruits have not been reported. As mentioned earlier, this study aims to increase the value of waste and focuses on the investigation of phytochemical contents and antioxidant activities of the extract of young fruit for utilization in cosmetics as an active ingredient.

Study objectives

1. To investigate the optimum extraction conditions of *C. maxima* young fruits.
2. To evaluate total phenolic and total flavonoid contents of the obtained extract from different aged *C. maxima* young fruits.
3. To evaluate DPPH radical and hydrogen peroxide scavenging activity of the obtained extract from different aged *C. maxima* young fruits.

Scope of the study

1. Extraction of young *C. maxima* fruits which matured from 1 to 3 months.
2. Evaluation of total phenolic content and total flavonoid contents of the extract.
3. Evaluation of DPPH radical scavenging activity and hydrogen peroxide scavenging activity of the obtained extract.

Literature Review

Citrus fruits contain many flavonoids which are a sub-class of polyphenols, known as a phytochemical that cannot be synthesized by humans. The composition of flavonoids is not always the same in each part of citrus, but they vary according to the species and the parts of the citrus plant. For examples, lemon peel contains plenty of flavonoids glycoside which not found in the juice. Bitter citrus juice is rich in flavanone neohesperidoside such as naringin, neohesperidin, and neoeriocitrin. Flavanone rutinoside, narirutin, hesperidin, and didymin are found in mandarin, orange, and lemon juice (Tripoli et al., 2007).

Flavonoids from plants extract have long been used in cosmetics and dermatology. Ancient Egyptian used citrus fruits for the mummy making process. Ancient Roman people used citrus fruits for insecticides, antidotes, treatment for frostbite, and external wounds. It has been reported used for washing hair in ancient

India. Citrus fruits were used for herbal medicine as digestive medicines, cough remedies, and an expectorant in The Chinese herbal book (Matsuda et al.,2011).

There were many studies to increase the value of citrus waste from food industry which a good source of flavonoids. Peels and pulp from citrus by-products industry were reported to have antioxidant, antimicrobial, anticarcinogenic, antibacterial, and anti-inflammatory properties (Khan et al., 2014). Citrus pressed-cake extracts can down-regulate tyrosinase, TRP-1, TRP-2, and MITF expression in murine B16F10 melanoma cell (Kim et al.,2013). Citrus flavonoids are active compounds commonly used in anti-aging cosmetics due to their antiradical and metal chelation properties. Flavonoids are also able to form a complex with the proinflammatory factor, which produces anti-inflammatory activity (Arct & Pytkowska,2008). Some phenolic compounds from *C. sinensis* are responsible for anti-photoaging activity (Mukherjee et al., 2011). Flavanones, especially hesperidin, in which its structure is similar to hydroquinone can inhibit melanin synthesis, oxidative damage of collagen, and protect against UVA-induced damage of fibroblast (Zhu & Gao, 2008). Citrus flavonoids have been reported with antioxidant properties, excellent inhibition against α-amylase, α-acetylcholinesterase, α-glucosidase, β-glucuronidase, and tyrosinase in *vitro* (Abirami, Nagarani, & Siddhuraju, 2014).

Research methodology

1. Plant materials and their preparation

Young *C. maxima* fruits were gathered from Pakpanang district, Nakorn Si Thammarat province, Thailand in October 2018. They were classified into three groups according to the age of maturation (Figure 1): one-month-old, two-month-old, and three-month-old after having been fertilized. Fresh fruits were cut into small pieces, weighed (Two-digit digital weighing scale, Ohaus, USA) and dried in the oven (Hot air oven, Medcenter Einrichtungen GmbH, Germany) at 50 °C for seven days. The samples were stored in a cool and dry place before used in further experiments.

Figure 1 Young *C.maxima* fruits at different ages after fertilization

2. Extraction of fruits material

The dried sample was ground by electric grinder (electric grinder, Sharp EM-Smart4 Blend and Grinder 450W, Thailand). It was divided into four groups. Each group was extracted by maceration with 95% ethanol, 75% ethanol, 50% ethanol, or propylene glycol, for three days in a dark chamber at the ambient condition. The proportion of ground sample to solvent was a 1:5 ratio by weight. The extract was filtered (Filter paper, Whatman circle 90 mm Cat No 1001090, England) and kept in a refrigerator (refrigerator, Sharp, Japan) until use.

3. Total phenolic content by Folin-Ciocalteu reagent assay (Kumar & Chaiyasut, 2017)

Diluted plant extract (200 µL) was blended with 1.0 mL of 0.2 N Folin-Ciocalteu reagent. The mixed solution was left for 4 minutes at 25 °C before adding 7.5% sodium carbonate solution (800 µL). After mixing, the solution was incubated at room temperature in a dark chamber for 120 minutes. After that, the absorbance was measured at 725 nm by a spectrophotometer (UV-visible spectrophotometer, Bio Chroms, England). Gallic acid was applied as a standard. Total phenolic content was reported in terms of milligrams gallic acid equivalent per mL extract (GAE/mL extract).

4. Total flavonoid content by aluminum chloride colorimetric method (Kumar & Chaiyasut, 2017)

The diluted plant extract (1.0 mL) was mixed with 150 µL of 15% w/w sodium nitrite solution and then incubated at room temperature for 6 minutes. After incubation,

150 µL of 15% w/w aluminum chloride solution was combined and further incubated at room temperature for another 6 minutes. After that, 8% w/w sodium hydroxide solution (700 µL) was added to the mixture. The solution was incubated at room temperature in a dark chamber for 15 minutes. After that, the absorbance was measured at 510 nm by a spectrophotometer (UV-visible spectrophotometer, Bio Chroms, England). Quercetin was applied as a standard. Total flavonoid content of each extract was expressed in terms of milligrams quercetin equivalent per mL extract (mg QE/mL extract).

5. DPPH radical scavenging assay (Kumar & Chaiyasut, 2017)

The diluted plant extract was blended with 1.0 mL of 0.2 mM DPPH in 95% ethanol solution. The solution was stored at room temperature in a dark chamber for 30 minutes. After that, the absorbance was measured at 517 nm by a spectrophotometer (UV-visible spectrophotometer, Bio Chroms, England). DPPH radical scavenging activity was calculated as

% DPPH radical-scavenging activity = 100 ×(1- As÷Ac)

where Ac is the DPPH absorbance of the control at 517 nm and As is the absorbance of the sample at 517 nm. Ascorbic acid was used as a standard. DPPH radical scavenging activity of each extract was reported in terms of milligrams ascorbic acid equivalent per mL extract (mg VCE/mL extract).

6. Hydrogen peroxide scavenging activity (Kumar & Chaiyasut, 2017)

Diluted plant extract (1.0 mL) was added to 1.0 mL of 0.15% w/v hydrogen peroxide solution. Then absorbance was measured at 230 nm by the spectrophotometer (UV-visible spectrophotometer, Bio Chroms, England). Ascorbic acid was used as a standard. Hydrogen peroxide scavenging activity of each extract was reported in terms of milligrams ascorbic acid (vitamin C) equivalent per mL extract (mg VCE/mL extract).

7. Statistical analysis

All experiments were done in triplicate. The results were reported in terms of mean and standard deviation. The significance of differences was evaluated by using Student t-test and one-way ANOVA at the confidence level of 95%.

Results and discussion

From Table 1, the highest total phenolic content was detected in the one-month-old sample, which had been extracted by propylene glycol (32.76 ± 0.55 mg GAE/mL extract). The highest total flavonoid content has complied within the one-month-old sample, which obtained by 75% ethanol (5.41 ± 0.47 mg QE/mL extract). The highest DPPH radical scavenging activity (179.07 ± 8.48 mg VCE/mL extract) and the highest hydrogen peroxide scavenging activity (124.63 ± 0.74 mg VCE/mL extract) was discovered in the one-month-old sample which had been extracted by 75% ethanol. The lowest total phenolic content (6.46 ± 0.37 mg GAE/mL extract) and the lowest total flavonoid content (1.18 ± 0.36 mg QE/mL extract) was detected in the three-month-old sample, which had been extracted by 95% ethanol. The lowest DPPH radical scavenging activity observed in three-month-old samples obtained by propylene glycol (80.17 ± 5.42 mg VCE/mL extract), and the lowest hydrogen peroxide scavenging activity was observed in three-month-old samples which obtained by 95% ethanol (51.29 ± 0.51 mg VCE/mL extract).

Interestingly, results have shown an inverse correlation between age and the number of phytochemical constituents and antioxidant activities of the sample ($p < 0.05$). The comparison results in the same solvents system found that one-month-old pomelo fruit had the highest total phenolic content, total flavonoids content, and antioxidant activities. The best solvent system for highest phytochemical constituents and antioxidant activity was 75% ethanol.

Table 1 Total phytochemical constituents and antioxidant activities of different ages pomelo in four solvent systems

Biological activities	Solvent	Age of pomelo fruit (months)		
		1	2	3
Total phenolic contents (mg GAE/mL extract)	95% ethanol	17.34 ± 1.00^{c}	11.35 ± 0.37^{d}	6.46 ± 0.37^{d}
	75% ethanol	31.64 ± 0.39^{a}	28.47 ± 0.54^{a}	21.60 ± 0.48^{a}
	50% ethanol	20.70 ± 0.36^{b}	20.47 ± 0.06^{c}	15.80 ± 0.72^{b}
	propylene glycol	32.76 ± 0.55^{a}	22.22 ± 0.32^{b}	14.75 ± 0.27^{c}
Total Flavonoids contents (mg QE/mL extract)	95% ethanol	2.53 ± 0.29^{d}	1.27 ± 0.30^{c}	1.17 ± 0.36^{b}
	75% ethanol	5.41 ± 0.47^{a}	3.78 ± 0.43^{a}	2.37 ± 0.24^{a}
	50% ethanol	4.27 ± 0.23^{b}	3.31 ± 0.29^{a}	1.32 ± 0.16^{b}
	propylene glycol	3.62 ± 0.26^{c}	2.35 ± 0.04^{b}	1.08 ± 0.08^{b}
DPPH radical scavenging (mg VCE/mL extract)	95% ethanol	137.37 ± 12.13^{b}	130.87 ± 0.61^{a}	111.55 ± 12.56^{b}
	75% ethanol	179.07 ± 8.48^{a}	134.90 ± 1.70^{a}	138.79 ± 15.04^{a}
	50% ethanol	133.16 ± 9.26^{b}	123.24 ± 4.70^{b}	123.19 ± 10.27^{ab}
	propylene glycol	91.60 ± 3.13^{c}	90.66 ± 5.12^{c}	80.17 ± 5.42^{c}
Hydrogen peroxide Scavenging (mg VCE/ mL extract)	95% ethanol	63.75 ± 0.54^{d}	58.49 ± 0.16^{d}	51.29 ± 0.51^{d}
	75% ethanol	124.63 ± 0.74^{a}	95.29 ± 0.95^{a}	93.51 ± 2.70^{a}
	50% ethanol	95.69 ± 2.92^{c}	91.92 ± 1.98^{b}	82.97 ± 1.65^{b}
	propylene glycol	110.16 ± 0.90^{b}	82.91 ± 1.24^{c}	68.02 ± 0.86^{c}

Note: The mean of three replicates ± SD in a column with different letters indicates statistical differences at $p < 0.05$

Commonly, Ascorbic acid as a significant antioxidant compound and its content is the indicator for measuring antioxidant of the citrus plant. There are many influences which affect to the ascorbic acid level in fruit, for example; species, preharvest, cultivar rootstock, light, climate, fertilization, hormone and, ripening (Magwaza et al., 2017).

The main phytochemical constituents in citrus fruits are phenolic and flavonoid compounds that directly affect antioxidant activities. Considering their chemical structures allow reducing properties as hydrogen or electron-donating, ability to steady the unpair electron and metal-chelating potential. (Zou et al., 2016). There are many investigate shown that phytochemicals of citrus are varied with its origin, species, different tissue, and method of processing. Antioxidant activity and flavonoid content of peel and pulp at the mature stage of 28 local Chinese pomelos and four grapefruits were different. The pomelo peels of Rio Red and Cocktail had the highest naringin content (9,871.69 mg/kg fresh weight) and neohesperidin content (7,011.15 mg/kg fresh weight) which also shown highest antioxidant capacity ((Xi et al., 2014). The higher of total flavonoid content and total phenolic content of five different oranges peel was correlated with antioxidant activity and anti-inflammatory activity (Chen, Tait, & Kitts, 2017). There were some reports which shown phenolic content and flavonoid content from different part of Tubtim-Siam pomelo and extraction method. Total phenolic content (107.23 ± 0.62 GAE/g dry weight of extract), individual flavonoid constituents and DPPH scavenging activity (8.64 ±0.79 mg VCE/g dried extract) of mature Tubtim-Siam pulp extracted by methanol was reported (Mäkynen et al.,2013). Freeze-dried Tubtim-Siam juice was measured for total phenolic content (690 mg GAE/L fruit juice), and specific citrus flavonoids by HPLC (Buachan et al., 2014).

Accordingly, our results showed that solvent systems and phytochemical constituents content were effected to antioxidant activity, which were similar to the previous studies. However, in the previous studies, researchers expressed the results in different units from our experiment, and our tests used primary screening method that can not report exactly quantity of phytochemicals, which complicated comparison with our results.

From another analysis point, results were explained a significant reverse correlation between age and the amount of total phenolic content, total flavonoids content, DPPH radical scavenging activity, and hydrogen peroxide scavenging activity. The explanation of the reduction of flavonoids in mature fruits is the dilution of them by cell growth (Castillo, Benavente, & Rio, 1992).

Conclusion

During the cultivation of C. maxima "Tubtim-Siam pomelo," there are many young fruits which are cut down and left as waste. This research proposed to investigate the extraction process and biological activities in different ages of young C. maxima fruits to increase the value from wastes material. It used the traditional maceration technique with four different solvent systems, which were 95%, 75%, 50% ethanol, or propylene glycol. The Folin-Ciocalteu reagent assay and aluminum chloride colorimetric method were used for measuring phytochemical constituents content. Antioxidant activity of the extract was measured by DPPH radical scavenging assay and hydrogen peroxide scavenging assay. The result revealed a high inverse relationship between age and the level of total phenolic content, total flavonoids content, DPPH radical scavenging activity, and hydrogen peroxide scavenging activity. The best sample that showed the highest phytochemical constituents and antioxidant activities was one-month-old fruit extracted by 75% ethanol.

The stability of young pomelo fruit extracts should be considered in further experiments. The one-month-old fruit extracted by 75% ethanol should be chosen to develop into cosmetics, and clinical efficacies of formulations should be considered.

The spectrophotometric technique for judgment of phenolic compounds is basic and quick, which expresses the estimation of phytochemical constituents but loses precision for individual compounds. The method that works for the separation and qualification of polyphenolic compounds is High-Performance Liquid Chromatography (Ignat, Volf, & Popa, 2011), which should be designated for further analyses.

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