TOTAL LIMONOID CONCENTRATION AND ANTIOXIDANT CAPACITIES OF EXTRACTS FROM SEEDS OF DIFFERENT CITRUS VARIETIES USING DIFFERENT SOLVENTS

Pham Hai Son Tung[1], Pham Van Hung[1], Chau Tran Diem Ai[2], Nguyen Thi Nguyen[2], Nguyen Thi Lan Phi[2*]

[1]*International University, VNU-HCM*

[2]*University of Technology, VNU-HCM*

*Email: *lanphi@hcmut.edu.vn*

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ABSTRACT

Citrus seeds contain high amounts of limonoids which prove their role in health promoting properties. In this study, total limonoid aglycones, limonin content and antioxidant capacities of the extracts of three kinds of citrus seeds including Dao lime, Vinh orange and Thanh Tra pomelo were investigated. The limonoids were extracted with ethyl acetate, acetone, methanol and dichloromethane using the conventional extraction method. The total limonoid aglycones was quantified by a colorimetric method using DMAB indicator reagent, while the antioxidant activies were determined by both DPPH and ABTS radical scavenging assays. The results indicated that pomelo seeds contained the highest amount of total limonoid aglycones (44.04 μg/g) when extracting with ethyl acetate, followed by those of orange seeds (27.96 μg/g) and lime seeds (27.66 μg/g). The limonoid contents of the methanol-based extracts from lime, orange and pomelo seeds were higher than other solvent-based extracts (19.06, 12.44 and 13.72 μg/g, respectively). The results also indicated that the extracts of pomelo seeds had higher DPPH and ABTS radical scavenging activities than those of lime and orange seeds, whereas the extracts of the orange and lime seeds exhibited no significant difference in antioxidant capacities. The methanol showed the best extraction yield, whereas the dichloromethane was the least effective solvent among all solvents.

Keywords: Citrus seeds, limonoids, limonin, antioxidant capacity.

1. INTRODUCTION

Citrus fruits have been recognized as one of the most healthful sources in human diet. While citrus health-promoting properties are conventionally associated with their vitamin C content, recent studies on bioactive compounds, specifically limonoids, have proved their major role in preventing chronic diseases. Citrus limonoids are a group of highly oxygenated triterpenoid compounds found in plant species of Rutaceae and Meliaceae family. They occur in high concentrations as aglycones and glucosides in fruit tissues and seeds [1, 2]. Limonoid glucosides are water-soluble and more abundant in juices and pulps, whereas limonoid aglycones are mainly responsible for a bitter taste of the citrus fruits because they are fat-soluble and present in seeds and peels [1, 3]. Significant progress has demonstrated the number of biological activities of these compounds including anti-inflamatory, antiviral, anti-proliferative and anticarcinogenic activities *in vivo* system. Structural features that make an impact on cholesterol reduction in blood have been identified [4]. Limonoids are reported to be nontoxic to animals and noncancerous cells of mammals [5, 6].

Limonoids of citrus seeds have been also considered as natural antioxidants, which interfere in free radical reactions associated with tumorgenesis in mammalian systems [7-10]. Among 56 limonoids have been identified in citrus, limonin is the first characterized compound of these phytochemicals knowing as a constituent of citrus since 1841 [11]. The studies *in vivo* and *in vitro* indicated that limonin is a potential bioactive compound and it exhibits a number of significant biological activities. Limonin has been involved in the inhibition of development of oral tumors in hamster cheek pouch model and has been found to influence GST enzyme activity in liver and small intestine mucosa of rodent models. Treatment with limonin has showed a considerable effect in inhibition of DMBA-induced neoplasia in experimental mice [12]. Besides anticancer activity, limonin has antimicrobial activities, cholesterol lowering, antiviral activities and acts as protective agent against low-density lipoprotein (LDL) oxidation [13, 14].

There are a variety of methods used to extract limonoids in citrus seeds including supercritical fluid [15], microwave-assisted process [16], ultrasound [17], and solvent extraction [18, 19]. However, solvent extraction is more popular than another one based on its advantages. Firstly, several solvent extractions can be performed in parallel. Secondly, it required a little in training and can extract more sample mass than other methods. For those reasons, the solvent extraction method was preferred to extract phenolic and antioxidant compounds [20, 21]. Regarding extraction of citrus limonoids, little information in effectiveness of different solvents used for extraction has been reported. Moreover, little information in the limonoid and limonin contents of seeds of different citrus species grown in Vietnam have been found [22]. Therefore, the objective of this study is to investigate the total limonoid aglycones, limonin content and antioxidant capacities of extracts from different kinds of citrus varieties (Dao lime, Vinh orange, Thanh Tra pomelo) using the solvents having different polarity including ethyl acetate, acetone, methanol and dichloromethane.

2. MATERIALS AND METHODS

2.1. Materials

Fresh seeds of three kinds of popular citrus fruits in Vietnam (Dao lime, Vinh orange, Thanh Tra pomelo) were collected and dried under the sunlight for 3 days to achieve moisture content of below 10%. The dried seeds were ground and stored in air-tight plastic bag, and then placed in a desiccator for further analysis.

The seed powder was deffated with n-hexane using a Soxhlet system for 8 h at 80 ^{o}C. The deffated residue was dried and used for limonoid extraction.

2.2. Extraction methods

The extraction method was carried out based on the report of Soong and Barlow [23]. One gram of each seed powder was refluxed with 20 mL of each solvent (ethyl acetate, acetone, methanol or dichloromethane) using a water bath at 70 °C for 20 min. The mixture of sample and solvent was then centrifuged at 4 °C with a speed of 5,000×g for 15 min. The supernatant was filtered and kept while the residue was repeatedly extracted for two more times. All supernatants were combined and made up properly to get exactly 60 mL of solutions.

2.3. Measurement of total limonoid equivalents using DMAB reagent

The colorimetric methods using a DMAB indicator reagent for quantification of total limonoid was employed by method of Andrew and Phil [24].

The DMAB indicator reagent was prepared as follows: 24 mL of 70% perchloric acid was combined with 30 mL of acetic acid (glacial) to prepare stock acid solution. The DMAB reagent was freshly prepared by dissolving 0.56 g of DMAB in 15 mL of stock acid solution.

Measurement of total limonoids: 330 μL of DMAB indicator and stock acid solution were added into 220 μL of sample and then incubate at room temperature for 30 min. Next, the absorbance was measured at 503 nm by a UV-Vis spectrophotometer. Limonin standard was prepared in acetonitrile (0.1 mg/mL). The results were expressed as μg/g.

2.4. HPLC analysis of limonin

The reconstituted sample (20 μL) in acetonitrile was injected to C18 reversed phase column and eluted isocratically at 1 mL/min flow rate using a mobile phase composed of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). The gradient elution was conducted, starting at 85% of solvent A, reduction to 77% in 5 min, to 74% after 25 min, further reduction to 60% at 30 min and completing the gradient at 54% at the end of 45 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before next run [25]. The elution was monitored at UV wavelength 210 nm and carried out at room temperature.

2.5. Antioxidant capacity by DPPH radical scavenging assay

The DPPH radical scavenging activity was measured using a method reported by Liyana-Pathirana and Shahidi [26]. Briefly, 3.9 mL of 0.075 mM DPPH solution prepared in methanol was mixed with 0.1 mL of sample. The mixture was vigorously vortexed and kept under subdued light at room temperature. The absorbance was recorded at 515 nm after exactly 30 min of reaction. The reduction was recognized by the color of solution faded overtime. Blank solution was prepared from 0.1 mL of methanol and the absorbance was read at t = 0. Each sample was measured in triplicate. The scavenging of DPPH was calculated according to the following equation:

$$\%\ \text{DPPH scavenging} = \{(Abs_{t=0} - Abs_{t=30}) / Abs_{t=0}\} \times 100$$

where: $Abs_{t=0}$ = absorbance of DPPH radical + methanol at t = 0 min

$Abs_{t=30}$ = absorbance of DPPH radical + phenolic extracts at t = 30 min.

2.6. Antioxidant capacity by ABTS cation radical-scavenging assay

The ABTS radical-scavenging activity was carried out according to the previous report by Robert *et al*. [27]. ABTS cation chromophore was generated by reacting 7 mM ABTS with 2.45 mM potassium and allowing the mixture to stand in the dark for at least 16 h at room temperature. The mixture was then diluted with absolute ethanol and measured at 734 nm to give an absorbance of 0.70 ± 0.02. After addition of 1 mL of ABTS ethanolic solution to 10 μL of sample, the absorbance was taken exactly 1 min after initial mixing and up to 6 min. All the assays were carried out in triplicate. Ascorbic acid was used as positive standard under the same condition. The antioxidant activity of the extracts was calculated as concentration of ascorbic acid equivalents (mM) at 1 and 6 min. The percentage inhibition of absorbance was plotted and calculated by the following formula:

$$\%\text{ABTS scavenging} = (1 - A_f / A_o) \times 100$$

Where: A_o = absorbance of solution without the presence of sample/standard
A_f = absorbance of solution after addition of sample/standard.

2.7. Data analysis

All values shown were the means of triplicate measurements. All statistical analyses were performed by the SPSS (Statistical Package for Social Sciences) version 22.0 to determine difference in means at 5% significance level. The difference among samples were evaluated by One-way ANOVA and Post-hoc's multiple tests. All data were reported as the means ± standard deviations.

3. RESULTS AND DISCUSSION

3.1. Total limonoid aglycone of citrus seeds

Total limonoid aglycone of citrus seeds is indicated in Table 1. The limonoid aglycones extracted from the lime and orange seeds using methanol were higher than those of other solvents (27.66 µg/g and 27.96 µg/g, respectively), whereas the limonoid aglycones extracted from the pomelo seeds using ethyl acetate (EtOAc) were higher than those using other solvents (44.01 µg/g). In contrast, dichloromethane (DCM) was the least effective one applied for limonoids extraction. The total limonoid aglycone extracted with DCM was the lowest as compared to those using EtOAc, acetone (AcOH) or methanol (MeOH). In addition, the total limonoid aglycones extracted with MeOH was higher than those extracted with AcOH. Ethyl acetate was considered to be the best solvent to extract some of the medium polar compounds, whereas the acetone and methanol were widely used for the extraction of medium polar and polar compounds like aglycons and glucosides [28, 29]. Therefore, the ethyl acetate and methanol could extract more compounds than other solvents resulting in higher total limonoid contents of the extracts using these solvents. Among three kinds of citrus varieties, pomelo seeds contained the highest amounts of limonoid aglycones (44.04 µg/g), followed by the orange seeds (27.19 µg/g) and lime seeds (24.01 µg/g) when extracting with EtOAc. Hasegawa *et al*. [1] reported that the limonoid aglycones concentrations in citrus seeds were higher than the limonoid glucosides, which is likely to significantly contribute to antioxidant capacity of limonoids. According to Miyake *et al*. [29], the predominant aglycones in citrus seeds was limonin followed by nomilin. Besides, some predominant acidic limonoids like nomilinic acid and diacetylnomilinic acid occupy approximately 20% of total aglycones in citrus seeds [1].

Table 1. Total limonoid contents of citrus seeds (µg/g limonin equivalent, LE)[1,2]

Sample	Total limonoids (µg/g)			
	EtOAc	AcOH	MeOH	DCM
Lime	24.01 ± 1.63^{cB}	24.79 ± 1.63^{bB}	27.66 ± 2.39^{bA}	17.49 ± 1.20^{bC}
Orange	27.19 ± 1.55^{bA}	25.37 ± 1.62^{bB}	27.96 ± 0.78^{bA}	20.45 ± 1.19^{aC}
Pomelo	44.04 ± 2.79^{aA}	39.92 ± 2.48^{aB}	41.73 ± 1.55^{aB}	19.58 ± 1.61^{aC}

[1]EtOAc, ethyl acetate; AcOH, acetone; MeOH, methanol; DCM, dichloromethane.
[2]Values (Means ± SD, n = 3) followed by different superscript letters in the same column or by capital letters in the same row are significantly different ($p < 0.05$).

3.2. HPLC analysis of limonin in pomelo's seeds

Amounts of limonin found in the seeds of three kinds of citrus are given in Table 2. Overall, methanolic extracts were accounted for the highest limonin concentration as compared to other solvent-based extracts. Although pomelo sample demonstrated the highest total limonoid content in ethyl acetate extracts, its limonin content (8.32 μg/g) appeared to be the lowest among all citrus seed extracts. The extraction method was important to obtain the limonin content of citrus seeds. In this study, the solvent-based immersion method was used, which resulted in lower total limonoids and limonin contents obtained from Thanh Tra pomelo as compared to the results reported by Phuong *et al*. [22], who used the Soxhlet system to extract. The limonin contents of the DCM-based extracts were also the lowest as compared to other solvent-based extracts and there was not significantly different between limonin contents of the EtOAc-based and AcOH-based extracts. The lime seeds had the highest amount of limonin (19.06 μg/g), whereas there was not significantly different between the limonin contents of the orange seeds (12.44 μg/g) and pomelo seeds (13.72 μg/g). The different amounts of total limonoids and limonin might affect antioxidant capacity and bioactivities of the extracts.

Table 2. Limonin contents of citrus seeds analyzed by HPLC system[1,2]

Sample	Limonin content (μg/g)			
	EtOAc	AcOH	MeOH	DCM
Lime	10.71 ± 0.20^{aB}	10.47 ± 0.50^{aB}	19.06 ± 0.30^{aA}	3.66 ± 0.20^{bC}
Orange	9.04 ± 0.40^{bB}	8.72 ± 0.20^{bB}	12.44 ± 0.22^{cA}	3.08 ± 0.10^{bC}
Pomelo	8.32 ± 0.25^{cB}	8.03 ± 0.10^{bB}	13.72 ± 0.15^{bA}	4.16 ± 0.30^{aC}

[1]EtOAc, ethyl acetate; AcOH, acetone; MeOH, methanol; DCM, dichloromethane.

[2]Values (Means ± SD, n = 3) followed by different superscript letters in the same column or by capital letters in the same row are significantly different ($p < 0.05$).

3.3. DPPH radical scavenging capacity of limonoids extracting from citrus's seeds

DPPH radical scavenging activity was approached to investigate the reaction between a nitrogen-center radical and sample antioxidants. The discoloration rate indicated the scavenging potential of the extracts. The results given in Table 3 showed the different antioxidant activities among the solvent-based extracts and among the citrus seeds. The MeOH-based extracts exhibited the highest antioxidant capacities than those of other solvent-based extracts. In contrast, the DCM-based extracts had the lowest antioxidant capacity. The antioxidant capacities of the EtOAc-based extracts and AcOH-based extracts were not significantly different. Haroen *et al*. [28] reported that the different biological activities of citrus extracts were due to the difference in the phytochemical composition of various crops and due to the extraction method. Considering that limonoid aglycones concentrations in citrus seeds are higher than limonoid glucosides [1] and limonin is a predominant aglycone followed by nomilin [30], which is likely to significantly contribute to antioxidant capacity of limonoids. In this study, the antioxidant capacities of the extracts were also consistent with the limonin contents rather than the limonoid aglycone concentration. The extracts from pomelo seeds had the highest antioxidant activity (38.53%), whereas, the antioxidant activities of the orange seeds (37.21%) and lime seeds (36.75%) were not significantly different. Previous study demonstrated that limonin had relative stronger antioxidant activity than others, especially in LDL oxidation assay system [1]. Therefore, with higher limonoid aglycones and limonin contents, the pomelo seeds had the higher antioxidant activity compared to other citrus seeds.

Table 3. Antioxidant capacity (% inhibition) of citrus seed extracts using DPPH assay[1,2]

Sample	DPPH scavenging (%)			
	EtOAc	AcOH	MeOH	DCM
Lime	20.46 ± 0.71^{bB}	20.55 ± 0.70^{cB}	36.75 ± 0.71^{bA}	18.58 ± 0.52^{aC}
Orange	21.23 ± 0.34^{bC}	22.98 ± 0.52^{bB}	37.21 ± 0.26^{bA}	18.76 ± 0.48^{aD}
Pomelo	23.10 ± 0.07^{aC}	25.65 ± 0.58^{aB}	38.53 ± 0.45^{aA}	18.84 ± 0.32^{aD}

[1]EtOAc, ethyl acetate; AcOH, acetone; MeOH, methanol; DCM, dichloromethane.

[2]Values (Means ± SD, n = 3) followed by different superscript small letters in the same column or by capital letters in the same row are significantly different ($p < 0.05$).

3.4. ABTS cation radical scavenging assay

ABTS decolorization assay was applied to evaluate the relative antioxidant ability in aqueous phase. The antioxidant components having lower redox potential than that of ABTS cation were able to scavenge the color of the radical proportionate to their amount. The measurement was compared with ascorbic acid used as positive standard compound. Table 4 illustrates effects of the citrus seed extracts on the suppression of ABTS radical cation upon the duration of 6 min. Similar to the results of the DPPH radical scavenging, the MeOH-based extracts exhibited the highest antioxidant capacity, whereas the DCM-based extracts had the lowest determined by the ABTS scavenging assay. The methanol-based extracts of the pomelo seeds had the significant ABTS radical scavenging activity (50.77%), whereas the antioxidant capacity of the lime seed and orange seed extracts were 32.26% and 33.75%, respectively.

Table 5 shows the amounts of antioxidants of the citrus seed extracts expressed in mM ascorbic acid equivalent corresponding to their inhibition at 1 min and 6 min. The trend of these concentrations increased over time due to the rise of the ABTS scavenging ability. While the methanol-based extracts of lime and orange seeds indicated the similarity in the inhibition of absorbance, their concentrations were significantly different ($p < 0.05$) after 6 min. Apart from dichloromethane extracts, pomelo seeds performed its domination in all fraction results in comparison with other citrus seeds. The ethyl acetate-based and acetone-based extracts of orange seeds were observed with similar concentration at 1 min ($p < 0.05$) and maintained their similarities after 6 min (4.94 mM and 5.26 mM, respectively). As discussed above, the different antioxidant capacities of the extracts were due to the different phytochemical compounds in the extracts, in which the limonin played an important role with higher concentration and biological activities than other limonoids of citrus seeds [30].

Table 4. Antioxidant capacity (% inhibition) of citrus seed extracts after 6 min of reaction using ABTS assay[1,2]

Sample	% Inhibition			
	EtOAc	AcOH	MeOH	DCM
Lime	25.03 ± 0.14^{cC}	26.48 ± 0.43^{cB}	32.26 ± 0.40^{cA}	16.36 ± 0.50^{aD}
Orange	26.29 ± 0.56^{bC}	27.83 ± 0.50^{bB}	33.75 ± 0.43^{bA}	16.41 ± 0.57^{aD}
Pomelo	30.91 ± 0.37^{aC}	33.61 ± 0.32^{aB}	50.77 ± 0.61^{aA}	16.13 ± 0.21^{aD}

[1]EtOAc, ethyl acetate; AcOH, acetone; MeOH, methanol; DCM, dichloromethane.

[2]Values (Means ± SD, n = 3) followed by different superscript small letters in the same column or by capital letters in the same row are significantly different ($p < 0.05$).

Table 5. Antioxidant activity as ascorbic acid equivalents (mM) at specific time points[1,2]

Sample	Conc (mM) at 1 min			
	EtOAc	AcOH	MeOH	DCM
Lime	4.61 ± 0.06^{cC}	4.90 ± 0.08^{cB}	6.15 ± 0.14^{dA}	2.95 ± 0.12^{aD}
Orange	4.89 ± 0.12^{bC}	5.21 ± 0.06^{bB}	6.33 ± 0.12^{cdA}	2.80 ± 0.12^{cD}
Pomelo	5.77 ± 0.10^{aC}	6.31 ± 0.12^{aB}	9.50 ± 0.12^{bA}	2.78 ± 0.04^{cD}

Sample	Conc (mM) at 6 min			
	EtOAc	AcOH	MeOH	DCM
Lime	4.68 ± 0.03^{cC}	4.98 ± 0.09^{cB}	6.19 ± 0.08^{dA}	2.86 ± 0.11^{bD}
Orange	4.94 ± 0.12^{bC}	5.26 ± 0.11^{bB}	6.50 ± 0.09^{cA}	2.87 ± 0.12^{bD}
Pomelo	5.91 ± 0.08^{aC}	6.47 ± 0.07^{aB}	10.06 ± 0.13^{aA}	2.82 ± 0.04^{cD}

[1]EtOAc, ethyl acetate; AcOH, acetone; MeOH, methanol; DCM, dichloromethane.
[2]Values (Means ± SD, n = 3) followed by different superscript small letters in the same column or by capital letters in the same row are significantly different ($p < 0.05$).

4. CONCLUSIONS

The total limonoid aglycones, limonin contents and antioxidant capacity of the extracts from different citrus seeds using different organic solvents were investigated in the present study. The results indicated that the extract from pomelo seeds had higher limonoid content and antioxidant capacity, whereas the extracts from the lime and orange seeds showed no significant difference. The methanol was found to be the best solvent for extracting the limonoids from the citrus seeds, whereas the dichloromethane appears to be the least effective among other solvents due to their low yield of limonoids. These information are useful for futher developing an effective extraction method and investigating the biofunctional properties of citrus seeds.

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There is no conflict of this study.

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TÓM TẮT

HÀM LƯỢNG LIMONOID TỔNG VÀ KHẢ NĂNG KHÁNG OXY HÓA TỪ DỊCH CHIẾT CỦA CÁC LOẠI HẠT CHI CITRUS SỬ DỤNG CÁC DUNG MÔI KHÁC NHAU

Phạm Hải Sơn Tùng[1], Phạm Văn Hùng[1], Châu Trần Diễm Ái[2],
Nguyễn Thị Nguyên[2], Nguyễn Thị Lan Phi[2*]
[1]*Trường Đại học Quốc tế - ĐHQG TP.HCM*
[2]*Trường Đại học Bách khoa - ĐHQG TP.HCM*
*E-mail: *lanphi@hcmut.edu.vn*

Hạt cam quýt có chứa một hàm lượng lớn các limonoid có hoạt tính sinh học cao có lợi ích sức khoẻ con người. Trong nghiên cứu này, hàm lượng limonoid aglycones, hàm lượng limonin và khả năng kháng oxy hoá của dịch chiết từ ba loại hạt họ cam quýt bao gồm chanh Đào, cam Vinh và bưởi Thanh Trà được tiến hành nghiên cứu. Limonoids được chiết xuất bằng cách sử dụng các dung môi hữu cơ khác nhau bao gồm ethyl acetate, aceton, methanol và dichloromethane. Hàm lượng limonioid tổng được xác định bằng các phương pháp đo màu bằng cách sử dụng chất phản ứng chỉ thị DMAB, trong khi đó khả năng kháng oxy hoá được xác định bởi cả hai phương pháp sử dụng DPPH và ABTS. Kết quả cho thấy rằng dịch chiết từ hạt bưởi có chứa hàm lượng limonoid aglycones cao nhất (44,04 µg/g) khi chiết bằng ethyl acetate, sau đó đến dịch chiết hạt cam (27,96 µg/g) và dịch chiết hạt chanh (27.66 µg/g). Hàm lượng limonin của dịch chiết từ hạt chanh, hạt cam và hạt bưởi bằng methanol cao hơn so với khi sử dụng các dung môi khác, tương ứng 19,06; 12,44 và 13,72 µg/g. Kết quả cũng chỉ ra rằng dịch chiết hạt bưởi có khả năng kháng oxy hoá cao hơn so với dịch chiết hạt chanh và hạt cam, trong khi đó dịch chiết của hai loại này có khả năng kháng oxy hóa không khác nhau. Dung môi methanol được coi là phù hợp nhất để tách dịch chiết hạt quả citrus, trong khi đó dung môi dichloromethane có khả năng tách được limonids là thấp nhất trong số các dung môi đã sử dụng.

Từ khóa: Hạt quả họ cam quýt, limonoids, limonin, khả năng kháng oxy hóa.