Running title: extraction conditions of crude naringin from orange peel

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**Effect of extraction conditions on the yield of naringin in the orange (*Citrus sinensis*) peel byproducts**

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**Novelty statement:**

Soxhlet extraction produced better naringin yield than maceration extraction. The result showed that the addition of soaking step for 1 hour was found to better yield the naringin content. Other factors including ethanol concentration, material : solvent ratio, and extraction time caused significant impacts on the yield of naringin content. Crude naringin extract also exhibited antioxidant activity by scavenging DPPH radicals.

# Abstract

This study investigated extraction conditions to better yield the naringin content from the orange peel byproducts. The maceration and the Soxhlet extraction method were tested with different solvent concentrations (70, 80, 99.9%), soaking time (0.5; 1.0; 1.5 hours), the ratio of orange peel powder to solvent (1:8; 1:10; 1:12; 1:14; 1:18 w/v), extraction time (0.5 h to 7 h). As a result, the highest naringin content was achieved at 3.1014 ± 0,0245 mg/mL by applying the Soxhlet extraction method at 80oC for 6 h. The other extraction conditions were determined as followed: the 1:10 orange peel powder : solvent ratio, solvent concentration (ethanol) of 70%, soaking time of 1 h. The crude naringin extract exhibited an antioxidant activity by scavenging DPPH radicals with an IC50  value of 671.476 ± 2.54 µg/mL. The result showed that the naringin crude extract could be used as a potential nutraceutical and the knowledge gained from this study should be helpful for further studies in this potent resource.

**Keywords**: Antioxidant activity; Naringin; Orange peel; Soxhlet extraction

# Introduction

Citrus fruits, one of the vital crops, contribute to the annual worldwide production of more than 100 million tons and orange fruit consumption has been observed to continuously increase over the year (Allaf *et al*., 2013). Orange (*Citrus sinensis*), belonging to the Rutaceae family, is popularly consumed which triggered a large number of byproducts from the flavedo (10%) and albedo (25%) (Allaf *et al*., 2013; Escobedo-Avellaneda *et al*., 2014; Luengo *et al*., 2013). This byproduct is a valuable source of flavonoids such as hesperidin, naringin, and alkaloids which are mainly derived from the albedo (Luengo *et al*., 2013). Escobedo-Avellaneda *et al*. (2014) noted that the highest content of polyphenols (553.1 – 730 mg GA/100 g) was found in the albedo in comparison with the flavedo. Flavonoids from citrus have been demonstrated with many health-beneficial effects including antioxidant, antihyperglycemic, anti-inflammatory, antimicrobial, anticancer, etc. (Escobedo-Avellaneda *et al*., 2014; Tajaldini *et al*., 2020).

 Naringin has been found as a primary flavonoid in the orange peel (Kanaze *et al*., 2009; Peterson *et al*., 2006; Sawalha *et al*., 2009). Many studies have revealed that naringin served a role as a health-promoting nutraceutical. Naringin was found to successfully promote the antihyperglycemic effect in diabetic mice (Ahmed *et al*., 2017). Orange peel extract could be employed as an adjuvant therapy to alleviate side effects of chemotherapeutic agents as doxorubicin (Dox) in esophageal cancer stem cells (CSCs) treatment (Tajaldini *et al*., 2020). Naringin has been considered an anti-angiogenesis agent or having a capacity in repressing polymethyl methacrylate particles induced osteolysis (El Barky, 2020). Therefore, these health-promoting effects have increasingly drawn the attention of researchers for the utilization of these orange peel byproducts.

 The extraction process is considered an essential step for the use of bioactive compounds as naringin to further apply in food or pharmaceutical industries (Luengo *et al*., 2013). Considering bioactive compounds, solvent extraction is usually performed with different types of solvents depending on the phytochemical compounds (Allaf *et al*., 2013). Ethanolic extraction has been recently employed to extract the naringin compound from the orange peel which has been well discussed in many previously reported studies (El Barky, 2020; Sawalha *et al*., 2009). Vietnam is in a region of tropical and subtropical climate which facilitates the production of orange fruit, generating a vast number of orange peel byproducts but not perceiving the health-beneficial effects from their bioactive compounds. Besides, natural antioxidants alternative to synthetic one has been increasingly attracted health-conscious consumers (Ahn *et al*., 2008; Brewer, 2011; Nassar *et al*., 2008).

 Therefore, we, in this study, investigated the effects of extraction conditions on the yield of naringin content from orange peels such as material : solvent ratio, soaking time, ethanol concentration, extraction method, and time of extraction. The naringin content was determined and its antioxidant activity also was evaluated. The expected result will give guidance on the extraction process to better yield naringin content in the orange peel extract. Further application of this crude naringin extract could be employed to investigate their applicability in food or pharmaceutical industries.

# Materials and methodologies

# Sample preparation

The orange (*citrus sinensis*) peel byproduct was collected from the local market in Can Tho city. The flavedo of orange peel was manually removed by a sharp knife. The albedo was washed with water until completely clean and dried for 24 h at 55oC. The dried albedo was ground into fine powder and stored at room temperature for further analysis (Ahmed *et al*., 2017). Vitamin C, naringin, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Merck KGaA (Darmstadt, Germany). All other chemicals used in this study are analytical grade from standard commercial supplies.

# Determination of extraction method and ethanolic concentration

In this study, two extraction methods (maceration and Soxhlet) were employed to achieve the crude naringin extract with varied concentrations of ethanol (70%, 80%, and 99.9%). Briefly, each 15 g of albedo powder was included with 150 mL of ethanol solvent at different concentrations. For the maceration method, the mixture was kept at room temperature for 30 min. The Soxhlet extraction method was conducted by using a Soxhlet laboratory apparatus and operated at 80oC for 30 min. The extraction mixture was then filtrated through a filter paper (Whatman No. 1, Germany) for removing impurity solid particles. The filtrate was then evaporated to achieve the extract at 70oC using a rotary evaporator (IKA, RV 3 V-C, IKA Werke GmbH & Co. KG, Germany). The crude naringin extract from each method at different ethanol concentrations was determined its naringin content.

# Investigation of soaking time on the naringin content

Each 15 g of orange peel powder was included with 150 mL of ethanol at the selected concentration (70%) from section 2.2. The mixture was incubated at room temperature for different periods of time (0.5 – 1.5 h) and then extracted using the Soxhlet system (selected method from section 2.2) at 80oC for 30 min. The filtrate was analyzed its naringin content after the evaporation of the solvent in the extract.

# Investigation of orange peel powder : solvent ratio on the naringin content

Each 15 g of orange peel powder was mixed with ethanol solution at different ratios of orange peel powder to ethanol solution (1:8, 1:10, 1:12, 1:14, 1:18 g/mL). The mixture was incubated for 1 h (selected result from section 2.3) at room temperature and was extracted by the Soxhlet extractor at 80oC for 30 min followed by the solvent evaporation process of the filtrate. The sample was analyzed its naringin content.

# Investigation of extraction time on the naringin content

Each orange peel powder of 15 g was mixed with 70% ethanol solution at the ratio of 1:10 (selected result from section 2.4) and incubated for 1 h at room temperature before extracting by the Soxhlet system at 80oC for different periods of time (0.5 h - 7 h). The extract mixture was filtrated followed by the solvent evaporation process. The naringin content was determined to show the discrepancy in different extraction periods of time.

# Measurement of naringin content

The naringin determination was followed by the method of Kuntić *et al*. (2012) with slight modifications. Briefly, 1 ml of the crude naringin extract was mixed with 9 ml of 99.9% ethanol solution. The absorbance was recorded at the wavelength of 238 nm using a 722-Visible spectrophotometer (China Yangzhou Wandong Medical Co., Ltd, China). The naringin standard was prepared at different concentrations ranging from 0.02 mg/L to 0.004 mg/L.

# Antioxidant activity of crude naringin extract

The antioxidant activity was measured based on the DPPH radicals scavenging effect of crude naringin extract (Tabart *et al*., 2007). Briefly, 1 ml of crude naringin extract at serially diluted concentrations (2-12 µg mL) was mixed with 2 mL of DPPH (39.4 µg/mL) solution and was allowed to react for 30 min in the dark. The absorbance was read at 517 nm and vitamin C served as a positive control. The inhibitory concentration at 50% antioxidant activity of crude naringin extract and vitamin C standard was calculated by constructing the dose inhibition curve of crude naringin extract and vitamin C concentration versus the corresponding absorbance reduction at 517 nm.

# Statistical analysis

 Each experimental data was in three replicates and data was expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey’s HSD test were used to show statistically significant differences among mean values at the level of 5% (p < 0.05) by using a Statgraphics Centuration XVI software (Statpoint Technologies, Inc., USA).

# Results

# Effect of ethanol concentration and extraction method on the naringin content

Table 1 presents the variations in naringin content in the crude naringin extract when subjected to different extraction methods and ethanol concentration. There was a significance difference in the total naringin content among analyzed groups (p < 0.05). Specifically, the lowest naringin content was 0.41931 ± 0.0485 mg/mL when applying the Soxhlet method with 99.9% ethanol, whereas Soxhlet extraction with 70% ethanol was found to produce the highest naringin content of 0.6076 ± 0.0399 mg/mL. In the contrast, the naringin content when applying the maceration method with 70% ethanol was similar to that with 80% ethanol (p > 0.05), reaching approximately 0.0285 mg/mL. Similarly, 99.9% ethanol used for the maceration method was found to least produce the naringin content as compared to the others (0.0231 ± 0.0012 mg/mL).

 Table 1 also shows the discrepancy in the naringin content from the two extraction methods. Naringin extracted by the Soxhlet method was much higher than that of the maceration method. In summary, in this study, the highest content of naringin was obtained when subjected to the Soxhlet method with the ethanol concentration of 70%. Therefore, these parameters were selected to use for further experiments.

# Effect of soaking time on the naringin content

After selection of extraction method and ethanol concentration, the soaking time was investigated its influence on the naringin content. When the sample was incubated for a certain period, ranging from 0.5 h to 1 h, before the Soxhlet extraction process, there was a significant difference in naringin content at different periods of soaking time, shown in Fig. 1. The soaking time of 1 h in 70% ethanol solution before the Soxhlet extraction reached the highest naringin content (0.7654 ± 0.0471 mg/mL). The longer time of the soaking process was found to decrease the naringin content to 0.5360 ± 0.0690 mg/mL. Hence, the soaking time of 1 h was suitable to apply for subsequent experiments.

# Effect of orange peel powder : solvent ratio on the naringin content

The effect of orange peel powder : solvent ratio on the naringin content is listed in Table 2. Changing the ratio of orange peel powder to solvent induced a variation in the naringin content, ranging from 0.3840 ± 0.0506 mg/mL to 0.8007 ± 0.0122 mg/mL. The highest naringin content was found at 1:10 ratio, reaching a value of 0.8007 ± 0.0122 mg/mL. Later increase in the ratio of orange peel powder to solvent was observed with a decrease in naringin content. The 1:12 ratio produced the crude naringin extract with the reduced naringin content of 0.634 ± 0.0546 mg/mL, which continuously decreased to 0.384 ± 0.0506 mg/mL at the 1:18 ratio of orange peel powder to solvent. Therefore, the optimal orange peel powder : solvent ratio was selected at 1:10 ratio due to the highest naringin content obtained after the extraction process.

# Effect of extract time on the naringin content

Fig. 2 illustrates the naringin content at different periods of Soxhlet extraction. It can be seen that increasing time of extraction from 0.5 h to 6 h showed the increment in extraction efficacy but the further increase was found to show a decreasing trend. The naringin content at 0.5 h was 0.7958 ± 0.0273 mg/mL, increasing steadily to the peak of 3.1014 ± 0.0245 mg/mL at 6 h of extraction time. Over 6 h, the extraction yield of naringin was found to decline to 2.9092 ± 0.0245 mg/mL. In summary, the extraction time of 6 h was suitable for the extraction process to obtain the highest naringin content.

# Antioxidant activity

In this study, after selection of all variables for having the highest naringin content in the extraction process. IC50 of crude naringin extract was determined based on the DPPH radicals scavenging effect. The standard equations of correlation between the concentration of vitamin C standard and crude naringin extract and the corresponding absorbance reduction, shown in Table 3, showed a linear regression with R2 > 0.99. The IC50 values of crude naringin extract and vitamin C standard are presented in Table 3. The result showed that crude naringin had the IC50 value of 671.48 ± 2.54 µg/mL, whereas vitamin C standard only needed a low amount of 6.47 ± 0.16 µg/mL to exhibit 50% antioxidant activity. Therefore, further studies should be conducted to enhance the purity of naringin in the extract for better antioxidant performance.

# Discussion

In this study, the use of pure solvent was found to be unfavorable condition for the extraction process of naringin. Chew *et al*. (2011) reported that the binary-solvent system (water-ethanol) was found to achieve a higher yield of flavonoids than the mono-solvent system. This trend was in agreement with previously reported studies that the binary-solvent system of ethanol and water showed an improvement in the flavonoids extraction yield (Sheng *et al*., 2013; Turkmen *et al*., 2006; Xu *et al*., 2013). The increased efficacy in the extraction with the addition of water was ascribed to the increment in swelling of plant tissues by water, increasing the contact surface area between the plant matrix and the solvent (Xiao *et al*., 2008). In comparison of the two extraction methods, the Soxhlet extraction produced better yield of naringin content compared to the maceration method. This could be explained that the processed temperature at 80oC in the Soxhlet method accelerated the movement of naringin molecules in the orange peel, facilitating the release of this compound from plant tissues (Sheng *et al*., 2013). A similar finding was observed in the study of Giannuzzo *et al*. (2003) that the Soxhlet extraction promoted better yield of naringin content from the *Citrus paradisi* peel (15.2 ± 0.5 g/kg) compared to that from the maceration method (11.6 ± 0.6 g/kg).

 The addition of soaking time step showed an increment in the yield naringin during the extraction process. The soaking time of 1 h possibly improved the penetration of ethanol into the plant tissues, increasing the solubilization of naringin in the sample (Liu *et al*., 2018). Prolonged soaking time was found to release the flavonoid compounds due to leaching of these compounds into the soaking media, thereby it enhanced the extraction yield of naringin (Afify *et al*., 2012). However, it showed a limitation at longer time of soaking (1.5 h) which was compatible with a previous study done by Song *et al*. (2014). The ratio of orange peel powder to solvent is also an essential factor in the extraction process. The lower volume of solvent is inadequate to better yield the naringin content, whereas a large volume of ethanol can lead to unnecessary production cost and shows difficulty in the extraction process (Liu *et al*., 2018). In this study, the 1:10 ratio of orange peel powder to solvent was appropriate for the extraction process. The higher amount of solvent added to the mixture caused the adverse effect since the larger volume of the solvent led to excessive swelling of plant tissues of orange peel by water and absorbing the effective components (Guo *et al*., 2001). This finding was compatible with many previously reported studies (Sheng *et al*., 2013; Xiao *et al*., 2008; Xu *et al*., 2013).

 It is necessary to point out the suitable processing time to achieve the desired target. The Soxhlet extraction process in 6 h produced the crude naringin extract with the highest naringin content but a longer extraction time was found to reduce the extraction yield. This observed tendency was fairly similar to that reported in previous studies (Chew *et al*., 2011; Hao *et al*., 2002; Sheng *et al*., 2013). The prolonged extraction time was found to cause decay of naringin due to the oxidation process when exposed to environmental variables such as light, oxygen, temperature (Chan *et al*., 2009; Chirinos *et al*., 2007; Sheng *et al*., 2013). This also could be well explained upon Fick’s second law of diffusion that there will be a final equilibrium between the solute in the plant tissues and in the extraction solvent at a certain time (Silva *et al*., 2007). Therefore, the excessive extraction time cannot increase the extraction yield.

 The IC50 value of a compound is inversely correlated to its antioxidant activity, in other words, a lower IC50 value indicates a higher antioxidant capacity of the compound (Do *et al*., 2014). A higher IC50 value of the crude naringin extract showed lower efficacy in scavenging DPPH radicals compared to that of the vitamin C standard. This result was consistent with previously reported studies (Pereira *et al*., 2007; Pyrzynska & Pȩkal, 2013). In contrast, vitamin C is a pure antioxidant, with strong antioxidant capacity, with only a small amount of vitamin C also has the antioxidant capacity (Kalt *et al*., 1999). The lower antioxidant activity of naringin could be highly affected by heating treatment in prolonged time during Soxhlet extraction (Karabegović *et al*., 2014). The very high concentration of crude naringin needed for 50% antioxidant activity was mainly due to the purity of naringin in the crude naringin extract. Further steps for improving the purity of naringin should be conducted to enhance its antioxidant activity or to increase its applicability in food or pharmaceutical products.

# Conclusion

In this study, the effects of extraction variables on the extraction efficacy of naringin in the orange peel were successfully evaluated. All the parameters including extraction method, ethanol concentration, soaking time, orange peel powder : solvent ratio, and extraction time were found to significantly affect the extraction yield of naringin. The suitable extraction process to obtain the crude naringin was carried out at the 1:10 orange peel powder : solvent (70% ethanol) ratio, soaking time of 1 h, using the Soxhlet extraction method at 80oC for 6 h. The crude naringin extract showed the antioxidant activity upon DPPH radicals scavenging effect but requiring a high IC50 value compared to the vitamin C standard. Further purification processes should be studied to enhance the feasibility of crude naringin as a nutraceutical in the food or pharmaceutical industries.

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Author contributions

PXH: conceptualization, supervision, reviewing and editing. NHTT and CML: materials and methodologies, investigation. TNN and LDHB: data curation, data analysis. TDL: manuscript drafting, reviewing, and editing.

**Conflict of interest**

There is no conflict of interest to declare.

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**Fig. 1**: Naringin content at different soaking times. Superscripts (a, b) show statistical difference among analyzed groups.

**Fig. 2:** Naringin content at different extraction periods of time

**Table 1:** Naringin content at different extraction methods and ethanol concentrations

|  |  |
| --- | --- |
| Ethanol concentration (%) | Naringin content (mg/mL) |
| Maceration | Soxhlet |
| 70 | 0.0284 ± 0.0005a | 0.6076 ± 0.0399a |
| 80 | 0.0286 ± 0.0009a | 0.5232 ± 0.0179b |
| 99,9 | 0.0231 ± 0.0012b | 0.4193 ± 0.0485c |

Results are expressed as mean ± standard deviation. Superscripts (a, b, c) indicate the statistical significances within the same column.

**Table 2:** Naringin content at different ratios of orange peel powder to solvent

|  |  |
| --- | --- |
| Orange peel powder : solvent ratio | Naringin content (mg/mL) |
| 1:8 | 0.4409 ± 0.0106cd |
| 1:10 | 0.8007 ± 0.0123a |
| 1:12 | 0.6340 ± 0.0546b |
| 1:14 | 0.4968 ± 0.0029c |
| 1:18 | 0.3840 ± 0.0506d |

Data are presented as mean ± standard deviation. Superscripts (a, b, c, d) indicate the statistical significances in mean values.

**Table 3:** Inhibitory concentration at 50% antioxidant activity (IC50) of crude naringin extract and vitamin C standard

| Sample | IC50 (µg/mL) | Standard equation | R2 |
| --- | --- | --- | --- |
| Vitamin C | 6.47 ± 0.16 | y = 8.3163x - 3.7939 | 0.9937 |
| Crude naringin extract | 671.48 ± 2.54 | y = 0.0738x + 0.4451 | 0.9986 |

Data are presented as mean ± standard deviation. Small letters (a, b) indicate the statistical significance in IC50 values.