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Naringin Extracted from Vietnamese *Citrus Maxima* Peels: Extraction and Optimization

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ABSTRACT

Background: This study investigated the extraction procedure, identification and purification level of naringin from *Citrus maxima* peels, a readily available food waste resource.

Methods: The extraction employed efficient multi-stage extraction, including hot extraction, alkaline treatment, liquid-liquid extraction and crystallization. The high-purity Naringin validation steps confirmed naringin purity *via* melting point determination, high-performance liquid chromatography and mass spectrometry. Thirdly, the optimization of extraction conditions was performed in varying parameters as optimal solvent concentration, temperature and time for maximum naringin yield from *Citrus maxima*.

Result: Naringin extraction conditions were optimized in varying parameters, including solvent concentrations, extraction times and temperatures. Optimal extraction conditions were determined to be 80% ethanol, an extraction time of 27 minutes and an extraction temperature of 60°C. The extracted naringin exhibited high purity (99.50%) in high-performance liquid chromatography measurement, a melting point of 166.5°C and a molecular weight of 580.1763 g/mol in mass spectrometry measurement. These findings demonstrate the feasibility of efficiently extracting high-purity naringin from *Citrus maxima* peels, offering a sustainable approach to valorize this abundant by-product.

Key words: *Citrus maxima*, Extraction, Naringin, Optimal condition, Peels.

INTRODUCTION

Citrus maxima, a pomelo species within the Rutaceae family, is a tropical fruit native to Southeast Asia. While pomelo species exhibit geographical variations in size, aroma and flavor, they are consistently recognized for their rich phytochemical composition (Hamid *et al.,* 2024). *C. maxima* fruits are characterized by a diverse array of secondary metabolites, including carbohydrates (fructose, glucose and sucrose), non-starch polysaccharides (pectin, cellulose and hemicellulose), vitamins (C, folate and β-carotene) (Sharma *et al.,* 2024), (Ben Hsouna *et al.,* 2023) and various phytochemical classes such as flavonoids, limonoids, coumarins, terpenoids and carotenoids, which are present in both extracts and essential oils (Sapkota *et al.,* 2022).

The diverse phytochemical composition of *C. maxima*, as previously discussed, includes a significant presence of flavonoids. These compounds, one of the largest groups of plant secondary metabolites, are synthesized within plant cells and are abundant across a wide range of flora (Roy *et al.,* 2022). In plant systems, flavonoids contribute to the vibrant coloration of fruits, flowers and vegetables, as well as play crucial roles in maturation and defense against bacterial, fungal and viral pathogens and insect predators (Kumar *et al.,* 2021), (Gupta *et al.,* 2021). Notably, *C. maxima* peels (Fig 1A) have been proven to be particularly rich in specific flavones, including naringin, neohesperidin, eriocitrin, hesperidin and neoeriocitrin. Among these, naringin constitutes (Fig 1B) the predominant flavanone in *C. maxima* (4′, 5, 7-trihydroxyflavanone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside) (Ding *et al.,* 2022), primarily concentrated in the albedo, the white spongy inner portion of the peel, contributes to the fruit's characteristic bitter taste.

As a major flavanone component of *C. maxima* peel, naringin has garnered significant attention for its diverse therapeutic potential. Reported effects include anti-inflammatory, antibacterial, anti-allergic, anti-cancer and antiviral properties (Wang *et al.,* 2021), (Tutunchi *et al.,* 2020). Notably, its capacity to reduce blood lipid levels has been a primary focus of pharmacological research (Yang *et al.,* 2022). Due to the situation that commercially available naringin extracts are very expensive, Vietnam provides a huge opportunity in supplying pomelo peel for naringin

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production. By efficiently extracting naringin from this readily available resource, demand could be met and costs drastically reduced for naringin-based pharmaceuticals and food products. However, the substantial volume of citrus waste generated annually by the food industry, estimated at approximately 15×10^6 tons globally (Food and Agriculture Organization of the United Nations (FAO), 2016), (Yalim *et al.,* 2020), poses a significant environmental challenge due to its high chemical and biological oxygen demand. Utilizing citrus peels for naringin extraction offers a sustainable approach to mitigate this environmental impact while simultaneously creating value from a byproduct. In tropical countries like Vietnam, where pomelo is widely cultivated and consumed, only the juice and flesh are typically utilized, leaving the peels as waste destined for burning or animal feed (Phat *et al.,* 2020). Therefore, exploring the efficient extraction of naringin from these peels is crucial to reducing the environmental burden and capitalizing on the inherent potential of this resource.

This research was designed to determine the optimal extraction conditions, specifically solvent concentration and temperature, for naringin from *C. maxima* peels. Furthermore, the purity of the extracted naringin was evaluated.

MATERIALS AND METHODS

Selection and preparation of materials

C. maxima peels were collected from fruits harvested in Can Tho, Vietnam, in early September 2024. Upon collecting, the peels were washed, thinly sliced and dried in a forced-air oven at 60°C. The dried peels were subsequently ground into a fine powder prior to being stored in sealed plastic bags at 4°C with controlled low humidity until further analysis at the Pharmaceutical Laboratory of Ho Chi Minh City International University (Vietnam National University).

Naringin extraction

100 g of *C. maxima* peel powder was extracted with 80% ethanol in a water bath maintained at 70°C for one hour. The resulting extract was filtered through filter paper to remove residual particulate matter. Subsequently, the volume of the filtrate was reduced to one-fifth using an IKA rotary evaporator. To remove pectin, the extract underwent alkaline treatment (Victor *et al.,* 2018). Calcium hydroxide (0.1 M) was used to adjust the pH of the solution to the range of 11-11.5, causing pectin to precipitate as calcium pectate. The solution was then filtered again to eliminate the pectin precipitate. Afterward, non-polar and low-polarity compounds were removed *via* liquid-liquid extraction using n-hexane. The extract and n-hexane were combined in a separatory funnel, vigorously shaken and allowed to separate into two distinct layers. The lower layer was collected and the pH to 4-4.5, reported as optimal for naringin crystallization by using 0.1 M hydrochloric acid. The solution was subsequently stored at 4°C for 24 hours to facilitate crystallization (Nguyen *et al.,* 2024), (Ly *et al.,* 2021). The resulting crude crystals were washed with cold water and dried in a Büchi vacuum dryer.

Column chromatography purification

Silica gel (0.04-0.06 mm) was used as the stationary phase for column chromatography (Simas *et al.,* 2013), (Vila-Real *et al.,* 2011), with a 9:1 ethyl acetate/methanol mixture as the mobile phase. A sample of 0.6 g crude naringin crystals was mixed with 2.0 g silica gel, ground to a fine powder and loaded onto the column. Elution fractions were collected: 15 mL for the initial two fractions, followed by 8 mL for the subsequent nine. Thin-layer chromatography (TLC), using the same 9:1 ethyl acetate/methanol mobile phase, was performed to analyze all fractions, with visualization under UV light. Fractions containing naringin were then recrystallized in a desiccator (Varun *et al.,* 2017).

Characterization by melting point

Melting points were measured using a melting point apparatus for both the naringin standard (Sigma) and the extracted naringin, using 0.5 cm capillary columns.

High-performance liquid chromatography

The purification level of naringin extracted from *C. maxima* peel was determined using HPLC on a Thermo Scientific Surveyor MSQ Plus Mass Spectrometer System. Samples were dissolved in acetonitrile/water/acetic acid 20/80/2.5

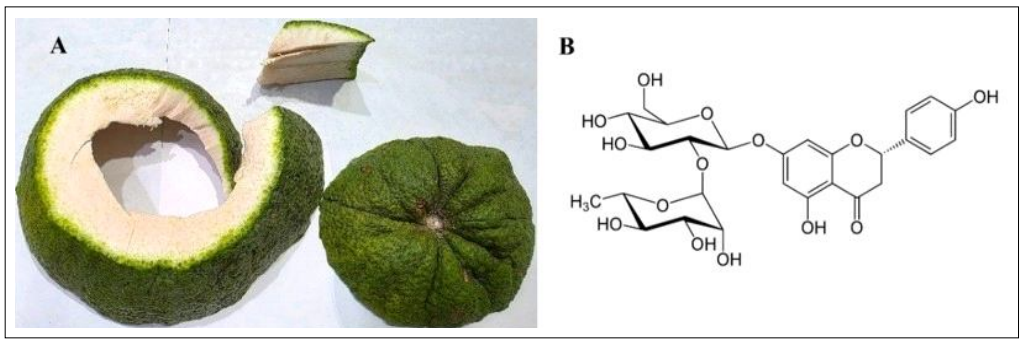


Fig 1: A: *Citrus maxima* peels. B: Naringin (4', 5, 7-trihydroxyflavanone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside) structure.

(v/v/v) and 20 µL injections were applied to a C18 HD (250 × 4 mm i.d.) column. Naringin was eluted isocratically with the same solvent system (acetonitrile/water/acetic acid at 20/80/2.5 (v/v/v) at a flow rate of 1.0 mL/min. Detection was performed using a UV detector at 280 nm. Purity was determined by comparing the peak area of naringin to the total peak area of all detected compounds and by comparing retention time and peak area to a calibrated naringin standard.

Molecular mass spectrum

0.10 g samples were introduced to the Mass Spectrometer Systems, high-performance benchtop instrument (X500-QTOF) (Moulard *et al.,* 2011) and mass spectra were acquired in TOF MS mode within a range of 70-1500 m/z, beginning from 0.25 minutes post-injection. To enhance spectral clarity, noise was filtered using a multiplier of 1.5 and Gaussian smoothing was applied with a 0.5-point window. This processing facilitated the accurate determination of naringin ion and characteristic fragment ions, allowing for unambiguous identification.

Pressurized solvent extraction (PSE)

Naringin was extracted from *C. maxima* peels using a Büchi Speed Extractor E-914/E-916 (Stabrauskiene *et al.,* 2022), (BÜCHI Labortechnik, 2019), a technique combining high temperature and pressure to enhance analyte solubility and diffusion. The sample cells were loaded according to Büchi instructions. A base layer of quartz sand was followed by a 1:1 mixture of 5 g *C. maxima* peels and 5 g quartz sand, topped with quartz sand to within 1 cm of the cell's upper edge. Dust and sand were removed and cellulose filter paper was tightly pressed onto the sample using a plunger prior to the extraction. Each extraction run began with a tightness test using nitrogen. Cells were heated to the desired temperature, maintaining a pressure of 200±20 bar. Three extraction cycles were performed, each consisting of a 2-minute heating phase, a 5-minute holding phase and a 2-minute discharge phase. Extracted solutions were collected in 250 mL vials. After the final cycle, cells were flushed with solvent and gas. Absolute ethanol and distilled water were used as solvents, selectable *via* four solvent valves.

Optimization of extracting conditions

Büchi Speed Extractor was utilized to investigate the effects of sample-to-solvent ratio, temperature and extraction time (Lý *et al.,* 2021). Solvent concentration (40%, 50%, 60%, 70% and 80% v/v) was varied while maintaining a constant temperature of 70°C and three extraction cycles; temperature (60°C, 70°C, 80°C, 90°C and 100°C) was varied using 80% ethanol and three cycles; and extraction time was manipulated by altering the number of cycles (one, two and three) with 80% ethanol at 70°C. All experiments were performed in triplicate and naringin content was quantified colorimetrically, with absorbance values compared against a standard curve.

Statistical analysis

All tests were done in triplicate and SPSS version 22.0 software was used for statistical assessment. One-way analysis (ANOVA) was applied to determine the significant differences between means at $p<0.05$.

RESULTS AND DISCUSSION

Crystal yield

After storage at 4°C for two days, the crude extract was washed with cold water and dried, yielding 1.7142 g of green, clustered powder. The yield of crude crystals was influenced by three key factors: Alkaline treatment, liquid-liquid extraction and acidification.

C. maxima peels contain significant pectin, which interferes with naringin crystallization. Pectin was removed by adjusting the pH to 11-11.5 using 0.1 M $Ca(OH)_2$, inducing pectin precipitation as calcium pectate. $Ca(OH)_2$ also isomerizes flavonoids and solubilizes chalcones, which are later reversed by acidification (Lo Curto *et al.,* 1992). Due to the high viscosity of calcium pectate, repeated filtration (2-3 times) was necessary for complete removal. Incubation for 10-15 minutes after $Ca(OH)_2$ addition was crucial to ensure complete reaction and prevent post-filtration precipitation.

Non-polar compounds were removed using n-hexane. The progression of extraction was monitored by the fading color of the hexane layer, with a colorless, transparent layer indicating completion. While no specific hexane-to-extract ratio was defined, a gradual fading of the yellow color with each 50 mL hexane addition was observed. To reverse the flavonoid isomerization and chalcone solubilization from the alkaline treatment, 0.1 M HCl was used to adjust the pH to 4-4.5, which is optimal for naringin crystallization (Chakraborty *et al.,* 2024). From 100 g of *C. maxima* peels, 1.7142 g of crude naringin crystals were obtained. The pale green color and clustered powder form of the crude crystals were attributed to residual chlorophyll from the flavedo, as whole peels were used in the extraction.

Column chromatography and naringin crystal yield

Eleven fractions (Fig 2) obtained from column chromatography were analyzed using thin-layer chromatography (TLC). A single compound exhibiting an Rf value near 1 was observed in fraction one, indicative of a highly non-polar substance. Two distinct spots, with Rf values of 0.44 and 0.63, were shown in fraction two. A single spot, with an Rf of 0.44, was displayed in fractions three through eleven, with intensity observed to decrease progressively from fraction three to eleven.

The presence of chlorophyll (Rf = 0.97) was suggested by the green color and an Rf value of approximately 1 in the first fraction. A low concentration of naringin (Rf = 0.44) and an unidentified compound (Rf = 0.63) were indicated in fraction two. Naringin was identified in fractions three to eleven, with a decreasing concentration gradient observed from fraction three. The presence of minor contaminants,

which could not be completely removed, was suggested by a faint band at the solvent front. It is likely that these contaminants form a coating or core within the naringin crystals. Following column chromatography, the final nine fractions were combined and dried in a desiccator for 24 hours, resulting in the recrystallization of naringin. Large, needle-shaped crystals were collected, yielding 0.5789 g.

Analysis of melting point

The melting points of naringin standard (Sigma, Switzerland) and the extracted crystal were 165.8°C and 166.5°C, respectively. The melting point of naringin was reported to be 166°C. This value was then re-evaluated by measuring the melting point of standard naringin (at 166.5°C). As for the naringin collected from the extraction, this data was recorded at 165.8°C. It can be observed that the differences were insignificant, indicating the high purity of the crystals.

This significantly high purity level was reconfirmed by HPLC chromatogram as presented in Fig 3, peak 5 exhibited an absorbance of 2094.116 mAU, indicating a naringin of 99.50% from *C. maxima* peel. This evidence supports the conclusion that the naringin obtained is highly pure, containing a mere 0.5% impurities, thus validating the suitability of this extraction and purification process for industrial-scale naringin production.

The remaining part contained eight contaminants in small amounts. By running MS, the molecular weight of naringin was recorded at 580.1763 g/mol as Fig 4, which was close to the value of standard naringin and theoretical naringin, at 580.2385g/mol and 580g/mol respectively (Shilpa *et al.,* 2023). The differences were due to the contaminants present in the crystals.

Optimum conditions for extraction using the PSE system
Naringin standard curve

A series of naringin standards was prepared at the following concentrations: 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, 0.3125 mg/mL, 0.15625 mg/mL and 0.078125 mg/mL. A standard curve was constructed by plotting absorbance at 420 nm against concentration. The standard curve's equation was determined to be y= 1.7571 × (R^2 = 0.9999). All values are presented as means ± standard deviation.

Naringin content was investigated using a colorimetric assay, based on the development of a yellow color upon alkaline treatment (Alam *et al.,* 2014). An increase in yellow color intensity was observed with increasing naringin concentration. The high accuracy and reliability of the standard curve were demonstrated by its strong correlation, as evidenced by an R^2 value of 0.9999.

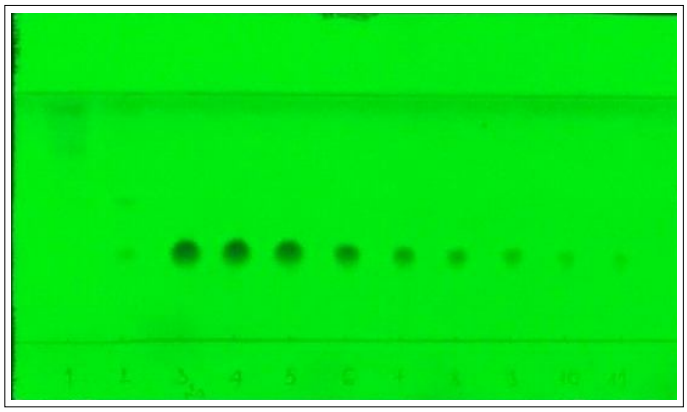


Fig 2: Fractions of crude crystal obtained through thin-layer chromatography.

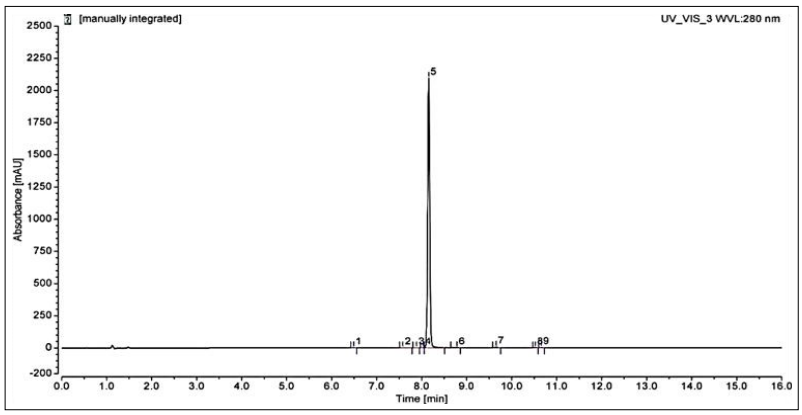


Fig 3: HPLC chromatogram of extracted naringin.

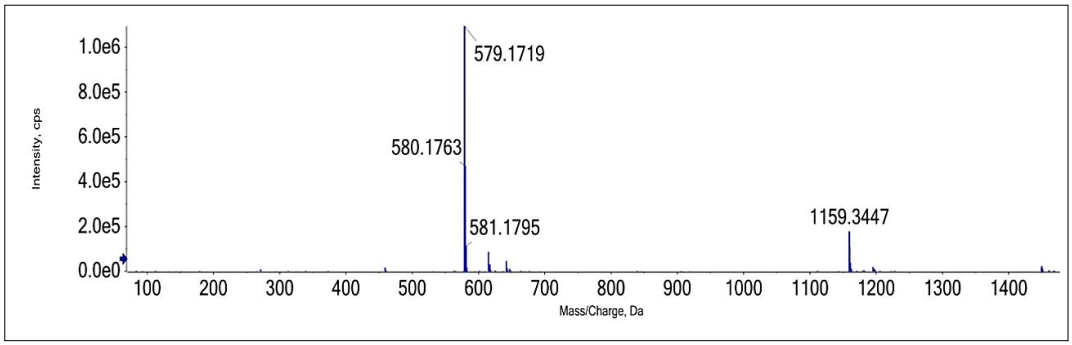


Fig 4: Mass spectrophotometer spectrum.

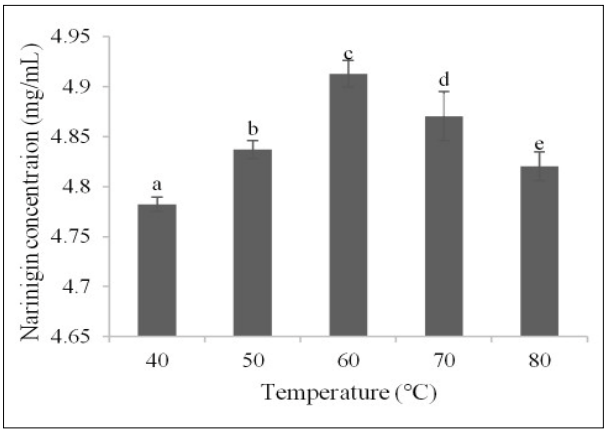


Fig 5: Effects of temperature on naringin extraction.

The temperature for maximum yield

To determine the optimal extraction temperature for naringin (Ly *et al.,* 2021), experiments were conducted using a fixed solvent concentration of 80% ethanol and three extraction cycles while varying the temperature across a range of 40°C, 50°C, 60°C, 70°C and 80°C. The results demonstrated that naringin content was significantly influenced by temperature. Specifically, the highest naringin concentration (4.91±0.013 mg/mL) was achieved at 60°C. While 70°C yielded a slightly higher mean value (4.97±0.025 mg/mL), the difference was not statistically significant. Lower naringin concentrations were observed at 50°C (4.84±0.0091 mg/mL), 80°C (4.82±0.014 mg/mL) and 40°C (4.78±0.0071 mg/mL). These findings suggest that 60°C represents the optimal temperature for naringin extraction under the given conditions. The observed decrease in yield at higher temperatures (70°C and 80°C) may be attributed to the potential thermal degradation of naringin. The relationship between extraction temperature and naringin concentration is visually represented in Fig 5.

Several studies have indicated that temperature is one of the most important parameters that could affect the extraction efficiency of flavonoids from plant materials (Vuong *et al.,* 2011), (Wissam *et al.,* 2012). The naringin yield increased with the increase in temperature; however, from 70°C to 80°C, the naringin yield started decreasing. A previous study by Krishnaiah *et al.* (2012) reported that bioactive compounds, including flavonoids, could be volatized and depleted at high temperatures. Since increasing temperature provides kinetic energy for the reaction, the extraction can be developed by increasing temperature. However, with high temperatures, the energy also causes the motion of molecules to become unstable and hence, hampers the extraction process.

The results showed that temperature had a significant impact on the naringin concentration. At 60°C, the highest naringin content was yielded and therefore, this temperature was chosen for the naringin extraction process.

The solvent concentration for maximum yield

Fig 6 shows the extraction yields of naringin by using different solvent concentrations from 60% to absolute ethanol when the temperature was kept constant at 70°C and the number of extracting cycles was three. The highest naringin content was yielded (4.91±0.013 mg/mL) by ethanol 80%, followed by ethanol 90% (4.82±0.0044 mg/mL), ethanol 70% (4.80±0.016 mg/mL), absolute ethanol (4.77±0.017 mg/mL) and ethanol 60% (4.75±0.0088 mg/mL).

Naringin, like other flavonoids, exhibits solubility characteristics determined by its chemical structure and the polarity of the extraction solvent (Dong *et al.,* 2023). Ethanol, a safe, economical and effective solvent for pomelo peel extraction, is particularly well-suited for naringin due to its capacity to dissolve both polar and non-polar components of the molecule (Andrade *et al.,* 2022). This property, shared with water, makes polar solvents ideal for extracting naringin. Naringin yield increased with ethanol concentration from 60% to 80%, reaching a maximum of 4.91±0.0013 mg/mL. This suggests increased polarity efficiency with higher ethanol concentrations. However, a decrease in yield was observed at 99% ethanol, possibly due to a polarity mismatch with naringin in *C. maxima* peels. The 80% ethanol extraction yielded significantly higher naringin concentrations ($\rho<0.05$) compared to other concentrations. These findings align with Ioannou *et al.* (2018), which also reported optimal flavonoid yields at 80% ethanol, although their study utilized orange peel and supercritical CO_2 extraction (Ioannou *et al.,* 2018).

The time for maximum yield

Fig 7 shows the extraction yield of naringin in three-time intervals from 9 to 27 minutes when other extraction conditions were as follows: 80% ethanol and a temperature of 70°C. As each extraction cycle done by the Speed Extractor machine included three steps - heat up, hold and discharge, which were set for 2, 5 and 2 minutes, respectively, each cycle lasted 9 minutes and the machine could only run up to three cycles maximum. 27 minutes of extraction was the optimum extracting time (4.91±0.013 mg/mL), followed by 18 minutes (4.80±0.0090 mg/mL) and 9 minutes (4.71±0.014 mg/mL).

It was hypothesized that varying contact times between *C. maxima* peels and solvent would impact naringin yield. Sufficient extraction time is necessary for solvent saturation before replacement. Using a Büchi Speed Extractor, each extraction cycle comprised 2 minutes heating, 5 minutes holding and 2 minutes discharging, totaling 9 minutes per cycle, with a maximum of three cycles. An extraction time of 27 minutes (three cycles) yielded the highest naringin concentration (4.91±0.013 mg/mL). Due to the machine's limitations, this was determined as the optimal extraction time for this PSE method. Therefore, 27 minutes was concluded to be the optimal extraction time for maximizing flavonoid yield within the constraints of the PSE method.

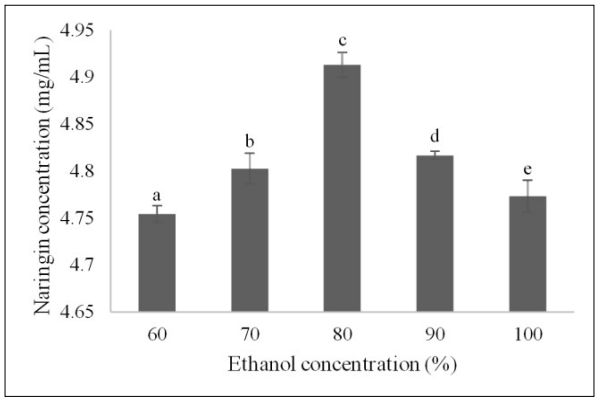


Fig 6: Effects of solvent concentration on naringin extraction.

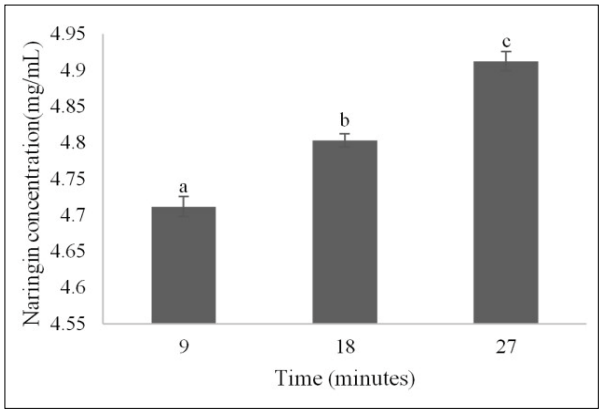


Fig 7: Effects of time on naringin extraction.

CONCLUSION

This study successfully demonstrated the extraction of naringin from *C. maxima* peels using a sequential process involving hot ethanol extraction, alkaline treatment for pectin removal, liquid-liquid extraction to eliminate non-polar compounds and acidification to facilitate crystallization. Optimal extraction conditions were determined to be 80% ethanol, 27 minutes of extraction time and 60°C extraction temperature. The extracted naringin was characterized by high purity (99.50%), a melting point of 166.5°C and a molecular weight of 580.1763 g/mol.

Furthermore, this research identified *C. maxima* peels as a novel and readily available source for naringin extraction. This finding offers a valuable avenue for utilizing a previously underutilized resource, potentially contributing to sustainable practices within the citrus processing industry. While this study established effective extraction parameters, further investigation into the impact of extraction time, pH and sample-to-solvent ratios on naringin yield is warranted. Such studies will provide a more comprehensive understanding of the extraction process and contribute to the optimization of naringin recovery from *C. maxima* peels.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect loss resulting from the use of this content.

Informed consent

There was no animal use applied in this research.

Conflict of interest

The authors declare to have no conflict of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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