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RESEARCH ARTICLE

Characterisation and phytochemical screening of ethanolic extract *Citrus reticulata* peel and its anti-inflammatory activity using protein denaturation method

Fitri Wulandari, Endang Sri Sunarsih, Khumairotul Musnaini
Pharmacy Study Programme, Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia

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Correspondence
Fitri Wulandari
Pharmacy Study Program
Faculty of Medicine
Universitas Diponegoro
Semarang
Indonesia
fitriwulandari@lecturer.undip.ac.id

Abstract

Background: Inflammation is an initial systemic response that occurs in the body and necessitates prompt management to prevent complications and death. Natural therapies, including *Citrus reticulata*, rich in hesperidin, a potent anti-inflammatory flavonoid, are gaining attention due to accessibility and safety. **Objective:** The aims of this study were to characterising Citrus reticulata peel simplicia and extract to ensure their quality phytochemical screening and determining its anti-inflammatory activity. **Method:** Simplicia characterisation involves testing water content, total ash content, and acid-insoluble ash content while the extract is tested for water and total ash content. Phytochemical screening is done using the tube method, and the anti-inflammatory activity test is conducted using the protein denaturation method. **Result:** The extract had water content and total ash content of 14.91% and 1.89%, respectively. The ethanol extract of *Citrus reticulata* peel contains flavonoids, alkaloids, saponins, tannins, quinones, steroids, and terpenoids. It shows anti-inflammatory activity with an IC50 value of 132.13 µg/mL, with a difference of approximately 55% to achieve the same level of protein denaturation inhibition as positive control dexamethasone, with an IC50 value of 73.14 µg/mL. **Conclusion:** *Citrus reticulata* peel simplicia and ethanol extract met quality standards, contain flavonoids with anti-inflammatory properties, and inhibit inflammation by up to 50% at 132.13 µg/mL.

Introduction

Each citrus tree in Indonesia produces 1000 to 2000 fruits per year (Endarto & Martini, 2016). *Citrus reticulata* Blanco, commonly known as Jeruk Siam, is the Latin name for this citrus variety (USDA, n.d.). The peel of *Citrus reticulata* is typically discarded as waste by people after consumption. However, it contains active compounds beneficial to health, including flavonoids, alkaloids, saponins, tannins, quinones, steroids, and terpenoids. The predominant compound in *Citrus reticulata* peel is flavonoid (70.82 mg/g), specifically hesperidin. Hesperidin belongs to the subclass of flavonone glycosides and is known for its anti-inflammatory, anti-cancer, antioxidant, anti-atherogenic, and antibacterial activities. As an anti-inflammatory agent, hesperidin can significantly reduce inflammatory mediators such as cytokines, enzymes, and adhesion molecules (Tejada *et al*., 2017).

Inflammation is a normal early response of the immune system in blood vessels' tissues to stimuli such as pathogens, damaged cells, and irritants (Bouhlali *et al*., 2016). The purpose of inflammation is to contain tissue damage, often resulting in oedema. However, oxidative stress during the inflammatory response can lead to the progression of dangerous pathological conditions if not promptly addressed (Nawrin*et al*., 2021). Inflammation can be managed using anti-inflammatory drugs, including both steroids and non-steroidal medications

(Kedi *et al*., 2018). Nevertheless, natural remedies have become necessary in society due to their perceived safety and easy accessibility.

The human body primarily comprises proteins found in muscles, bones, skin, hair, and nearly every other part or tissue in the body. Damage or denaturation of proteins caused by various external stimuli, such as heat, chemicals, etc., can lead to structural damage to the tertiary and secondary proteins, triggering an inflammatory response and causing various inflammatory diseases (Osman *et al*., 2016). Therefore, substances capable of inhibiting protein denaturation indicate anti-inflammatory activity.

In vitro testing can be conducted to measure the activity of natural substances before animal testing, considering the limitations of animal testing due to interspecies differences, making extrapolation very difficult due to the lack of analogue-isomorphism with human species, especially at the cellular and molecular levels where diseases occur. In vitro, anti-inflammatory testing can be performed using various methods, one is the protein denaturation inhibition method using Bovine Serum Albumin (BSA), which is stable and non-reactive. The principle of this method involves measuring the turbidity absorbance caused by protein denaturation at its maximum wavelength using a UV-VIS spectrophotometer. Anti-inflammatory activity is expressed as Inhibitory Concentration 50% (IC50) (Williams *et al*., 2008).

Therefore, based on the explanation above, the researchers conducted characterisation and phytochemical screening to assess the quality as an effort to optimise the use of natural materials as candidates for therapy and performed in vitro anti-inflammatory testing using the protein denaturation inhibition method on 96% ethanol extract of *Citrus reticulata* peel.

Methods

Material

The materials used include *Citrus reticulata* peel (orange plantation in Mungkid, Magelang, Central Java, Indonesia), 96% ethanol (Kimia Indrasari), HCl, Wagner reagent (Nitra Kimia), 10% FeCl, Lieberman reagent, 1M NaOH, concentrated sulfuric acid (Kimia Jaya Labora), silica gel (Pharmacy Health), tannic acid (Nitra Kimia), dexamethasone tablets, BSA (Himedia), tris base.

Tools

The equipment used includes an oven (Memmert), glassware (Pyrex), blender (FCT-Z200), analytical balance (Mettler Toledo), filter paper (Nitra Kimia), Bunsen burner, tripod stand, water bath (Memmert), porcelain crucible, UV-VIS spectrophotometer (Shimadzu UV-1200), hot plate, dehydrator, muffle furnace, and desiccator.

Preparation of simplicia and 96% ethanol extract of Citrus reticulata peel

Fresh eight kg of Citrus reticulata fruit was collected. The peel was separated from the fruit flesh, then underwent wet sorting, washed with running water, drained, cut, and dried using a dehydrator at 40℃ for 48 hours. After drying, it underwent dry sorting. The simplicia form was pulverised using a blender. The powder was weighed and placed in a sealed container, and silica gel was added to prevent moisture.

This study used the maceration technique for the extraction method. 200 grams of simplicia powder were placed in a glass jar, then added with 96% ethanol in a ratio of 1:10, stirred, and left to stand overnight. Subsequently, the soaked powder was filtered using filter paper. Remaceration was performed four times. The obtained filtrate was evaporated at 50°C. The resulting extract was stored in dark bottles, and the yield was calculated.

Characterisation of simplicia and 96% ethanol extract of Citrus reticulata peel

The moisture content test was conducted by weighing five grams of simplicia and placing it in the sample holder of a moisture analyser, which was then sealed. The instrument automatically measured the moisture content of the simplicia powder. Meanwhile, the moisture content test for the extract was performed by placing one gram of extract in a crucible, drying it in an oven at 105°C for five hours, cooling it, and weighing it until a constant weight was obtained.

The total ash content test for both simplicia and the extract was conducted by calibrating and weighing a porcelain crucible and then weighing two grams of simplicia. The powder was incinerated in a furnace at 800°C until it turned into ash, cooled, and weighed until a constant weight was achieved.

The acid-insoluble ash test was conducted by taking the remaining ash from the total ash content and adding 25 mL of diluted hydrochloric acid (HCl). The mixture was boiled for five minutes. The solution was then filtered using ash-free filter paper. The filter paper was incinerated in a furnace, cooled, and weighed until a constant weight was achieved.

Phytochemical screening of 96% ethanol extract of Citrus reticulata peel

Phytochemical screening of extracts uses a tube test method to see the content of alkaloid compounds, saponins, flavonoids, tannins, quinones, steroids and terpenoids. Identifying the presence or absence of phytochemical compounds contained in extracts is based on specific colour changes or foam formation in extracts containing saponins.

Anti-inflammatory activity test using protein denaturation method

This study conducted the anti-inflammatory test using the protein denaturation method. First, the Tris Buffer Saline (TBS) solution of 500 ml dissolved 4.35 grams of sodium chloride (NaCl) into aqua PA 200 ml. Then 0.65 gr of tris base was added, and aqua PA was added to 400 ml. Glacial acetic acid is added to adjust the pH in the range of 6.2–6.5 and added with aqua PA to 500 ml. Next, a Bovine Serum Albumin (BSA) solution dissolves 0.2 grams of BSA in 100 ml of TBS. The positive control solution was prepared using dexamethasone dissolved in aqua PA with 2000, 1000, and 500 ppm concentrations. The negative control was prepared using 500 µl aqua PA dissolved in the 5 ml BSA solution. Sample preparation of siamese orange peel extract was carried out by weighing 200 mg of extract and dissolved in aqua PA into concentrations of 8000, 2000, 1000, and 500 ppm. The sample solutions were re-diluted using BSA solution at concentrations of 800, 400, 200, and 100 ppm, then incubated at 37℃ for 30 minutes and heated at 72℃ for five minutes. Then, it was cooled at room temperature, and absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 660 nm. The inhibition of protein denaturation that reflected anti-inflammatory activity was calculated using the following equation:

$$\text{Inhibition (\%)} = (A-B)/A \times 100\%$$

A = negative control absorbance; B = absorbance of sample solution. IC50 (concentration at 50% inhibition) is calculated by finding the linear regression equation between concentration (X) and %inhibition (Y).

Results

In this study, the 96% ethanol extract of *Citrus reticulata* peel yielded 19.56%. Table I shows the characterisation of simplicia and extract has generally met the standards according to the Indonesian Herbal Pharmacopoeia (Ministry of Health Republic of Indonesia, 2000), while the Voigh reference was used to identify that the concentrated extract contains 5-30% moisture content.

Table I: Characterisation of *Citrus reticulata* peel simplicia and extract

Simplicia characterisation parameters	Result (%) ± SD	FHI* (%)
Moisture content	7.90 ± 0.396	≤10
Total ash content	3.78 ± 0.265	≤ 4
Acid insoluble ash content	0.09 ± 0.057	≤ 2
Extract characterisation parameters	**Result (%) ± SD**	**Voight and FHI* (%)**
Moisture content	14.91 ± 1.99	5-30
Total ash content	1.89 ± 0.14	≤ 4

*Farmakope Herbal Indonesia (FHI)

Phytochemical screening was conducted to identify the active compounds present in the extract. The results of the phytochemical screening showed that the *Citrus* reticulata peel extract tested positive for flavonoids, alkaloids, saponins, tannins, quinones, steroids, and terpenoids, as seen in Table II.

Table II: Qualitative phytochemical screening of ethanol extract of *Citrus reticulata* peel using tube test

Phytochemical compounds	Result
Flavonoid	Positive
Alkaloid	Positive
Saponin	Positive
Tanin	Positive
Kuinon	Positive
Steroid	Positive
Terpenoid	Positive

The results of the anti-inflammatory test using the protein denaturation inhibition method are shown in Table III. The results indicate that the *Citrus reticulata* peel extract has anti-inflammatory activity with an IC50 value of 132.12 ppm, meaning that the extract can inhibit protein denaturation by 50% at this concentration. In comparison, the positive control dexamethasone has an IC50 value of 73.14 ppm. Therefore, as a reflection, the natural extract shows a difference of up to 55% to achieve the same activity level as the positive control regarding its anti-inflammatory properties.

Table III: The result of anti-inflammatory test of *Citrus reticulata* peel extract and dexamethasone as positive control

Concentration of *Citrus reticulata* peel extract (ppm)	Absorbance ± SD	% Inhibition	IC50 (ppm)
100	0.634 ± 0.012	52.13	132.12
200	0.464 ± 0.073	64.89	
400	0.425 ± 0.072	67.95	
800	0.268 ± 0.024	79.80	
Concentration of dexamethasone* (ppm)	**Absorbance ± SD**	**% Inhibition**	**IC50 (ppm)**
50	0.821 ± 0.066	32.42	73.14
100	0.383 ± 0.004	68.46	
200	0.267 ± 0.020	78.00	
400	0.193 ± 0.004	84.09	

*Positive control

Discussion

In characterising Citrus reticulata Peel Simplicia and Extract, this study conducts the water content test because water is a preferred medium for microorganisms. When the water content of a substance is too high, microorganisms can easily grow and damage the material, impacting its safety and quality. Therefore, it is essential to determine the water content in simplicia and extracts. Total ash content indicates the inorganic compounds in the extract, which is important for assessing material safety. Examples of inorganic compounds include heavy metals, transition metals, and light metals. Heavy metals are crucial to monitor because they can pose health risks if they enter the body in large quantities. Therefore, the acid-insoluble ash test can be performed to assess heavy and transition metals' content. Both metals form compounds that are insoluble in water when dissolved in acid. In this study, the characterisation results of simplicia and the extract met the quality requirements, with water content, total ash content, and acid-insoluble ash content complying with the standards (Ministry of Health Republic of Indonesia, 2000). It indicated that the method used in this research is the right method to produce the herb with good quality and safety standards so it can be implemented sustainably.

Phytochemical screening was conducted to identify the active compounds present in the extract. The results of the phytochemical screening showed that the Citrus reticulata peel extract tested positive for flavonoids, alkaloids, saponins, tannins, quinones, steroids, and terpenoids, as seen in Table II. This is consistent with the research conducted by Santoso *et al*. and Khotimah *et al*., which also showed the presence of these compounds after the qualitative phytochemical screening of 96% ethanol extract of Citrus reticulata peel (Khotimah *et al*., 2017; Santoso *et al*., 2020)

The anti-inflammatory test using the protein denaturation inhibition method is presented in Table III. In these tables, it can be observed that as the sample concentration decreases, the obtained absorbance values also decrease. This indicates low turbidity, suggesting inhibition by compounds with suspected anti-inflammatory activity in the Citrus reticulata peel extract. A study on the anti-protein denaturation effect of Citrus nobilis peel methanol extract (Malik *et al*., 2021) obtained an inhibition percentage of approximately 85% at the highest concentration (200 mg/mL). In this study, a % inhibition percentage of 79.8% was obtained at the highest concentration of 800 µg/mL. The ethanol polarity index (5.2) is higher than that of methanol (5.1), indicating that ethanol is a more polar solvent than methanol. In this research, 96% ethanol, a mixture of ethanol and water, was used to enhance its polarity as a solvent and enable it to extract more polar compounds that play a role in anti-inflammatory activity. This might explain why the concentration used in Malik's study was higher to obtain similar percentage inhibition results to this study.

The 50% inhibition concentration (IC50) of the Citrus reticulata peel extract was 132.12 ppm, showing a difference of up to 55% compared to the positive control with an IC50 value of 73.14 ppm. This difference might be due to dexamethasone, a synthetic chemical compound in the steroid group, exhibiting strong anti-inflammatory activity (Del Grossi Moura *et al*., 2018). Therefore, dexamethasone cannot be proportionally compared to the ethanol extract of Citrus reticulata peel, a natural herbal remedy. However, as a reflection, dexamethasone has a smaller IC50 than the extract, indicating that dexamethasone has higher activity in inhibiting protein denaturation or has higher anti-inflammatory activity than the ethanol extract of Citrus reticulata peel.

The mechanism of hesperidin as an anti-inflammatory agent is known to inhibit MAPK (mitogen-activated protein kinase), one of the intracellular pathways involved in pro-inflammatory responses that can lead to the release of inflammatory mediators. Excessive release of inflammatory mediators can cause inflammation. Hesperidin's inhibition of MAPK can reduce inflammation (Denaro *et al*., 2021).

The predicted binding sites for the anti-denaturation action on BSA are believed to be in the regions of lysine residues and lysine-rich aromatic and aliphatic tyrosine residues of BSA. It is hypothesised that therapeutic molecules (extracts) can activate tyrosine-rich motif receptors along with threonine, regulating the signal transduction biological pathways for its overall biological actions (Denaro *et al*., 2021).

Conclusion

The study's simplicia and extract of Citrus reticulata peel met quality standards. They contained several phytochemical compounds, including flavonoids, alkaloids, saponins, tannins, quinones, steroids, and terpenoids, with flavonoids being the major contributor to the anti-inflammatory effect. The *Citrus reticulat*a peel extract exhibited anti-inflammatory activity with an IC50 value of 132.12 ppm, tested using the protein denaturation inhibition method.

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References

Apraj, V. D., & Pandita, N.S. (2014). Pharmacognostic and phytochemical evaluation of citrus reticulata blanco peel. *International Journal of Pharmacognosy and Phytochemichal Research*, **6**(2), 328–331. https://impactfactor.org/PDF/IJPPR/6/IJPPR,Vol6,Issue2,Article33.pdf

Bouhlali, E. dine T., Sellam, K., Bammou, M., Alem, C., & Filali-Zehzouti, Y. (2016). In vitro antioxidant and anti-inflammatory properties of selected Moroccan medicinal plants. *Journal of Applied Pharmaceutical Science*, **6**(5), 156–162. https://japsonline.com/admin/php/uploads/1876_pdf.pdf

Del Grossi Moura, M., Cruz Lopes, L., Silva, M. T., Barberato-Filho, S., Motta, R. H. L., & De Cássia Bergamaschi C. (2018). Use of steroid and nonsteroidal anti-inflammatories in the treatment of rheumatoid arthritis: Systematic review protocol. *Medicine*, **97**(41), e12658. https://doi.org/10.1097/MD.0000000000012658

Denaro, M., Smeriglio, A., Trombetta, D., (2021). Antioxidant and anti-inflammatory activity of citrus flavanones mix and its stability after in vitro simulated digestion. *Antioxidants*, **10**(2), 140. https://doi.org/10.3390/antiox10020140

Ministry of Health Republic of Indonesia. (2000). *General standard parameters of medicinal plant extracts. vol. 1*. Departemen Kesehatan RI. https://akfarstfransiskusxaverius.ac.id/wp-content/uploads/2023/08/51_Parameter-standar-umum-ekstrak-tumbuhan-obat-1.pdf

Endarto, O., & Martini, E. (2016). *Guidelines for cultivating healthy* oranges. World Agroforestry Centre (ICRAF) Southeast Asia Regional Program. https://apps.worldagroforestry.org/downloads/Publications/PDFS/BL16186.pdf

Kedi, P. B. E., Meva, F. E., Kotsedi, L., Nguemfo, E. L., Zangueu, C. B., Ntoumba A. A., Mohamed, H. E. A., Dongmo, A. B., & Maaza, M. (2018). Eco-friendly synthesis, characterization, in vitro and in vivo anti-inflammatory activity of silver nanoparticle-mediated Selaginella myosurus aqueous extract. *International Journal of nanomedicine*, **13**, 8537–48. https://doi.org/10.2147/IJN.S174530

Khotimah, K., Rahmawati, & Mukarlina. (2017). Antifungal activity of ethanol extract of Siam orange peel against Phytophthora sp. Im5 from the base of the stem of the Siamese orange plant (Citrus nobilis var. microcarpa). *Jurnal Untan Acld*, **6**(3), 188–193. https://dx.doi.org/10.26418/protobiont.v6i3.22474

Malik, A., Najda, A., Bains, A., Nurzyńska-Wierdak, R. & Chawla, P. (2021). Characterization of citrus nobilis peel methanolic extract for antioxidant, antimicrobial, and anti-inflammatory activity. *Molecules*, **26**(14), 4310. https://doi.org/10.3390/molecules26144310

Nawrin, K., Billah, M. M., Ahmed, F., Hossin, A., Rahman Tushar, R. R., & Islam, M. N. (2021). Protective potential of C. sinensis fruit peel aqueous extract on in vitro inflammation. *Pharmacotherapy and Pharmascience* Discovery, **1**(10), 10–18. https://www.ptherpscid.com/wp-content/uploads/2021/08/C.-sinensis-Anti-inflammatory-1.pdf

Osman, N. I., Sidik, N. J., Awal, A., Adam, N. A., & Rezali, N. I. (2016). In vitro xanthine oxidase and albumin denaturation inhibition assay of barringtonia racemosa L. and total phenolic content analysis for potential anti-infl ammatory use in gouty arthritis. *Journal of intercultural ethnopharmacology*, **5**(4), 343–349. https://doi.org/10.5455/jice.20160731025522

Santoso, J. D., Prasetyaningsih, A., & Madyaningrana, K. (2020). Potency of Citrus reticulata Ppeel extract as active compound of nonAlcohol based gel hand sanitizer. *Sciscitatio*, **1**(2), 79–86. https://doi.org/10.21460/sciscitatio.2020.12.32

Tejada, S., Pinya, S., Martorell, M., Capó, X., Tur, J. A., Pons, A., & Sureda, A. (2017). Potential anti-inflammatory effects of hesperidin from the genus Citrus. *Current medicinal chemistry*, **25**(37), 4929–4945. https://doi.org/10.2174/0929867324666170718104412

USDA. (n.d.). *Citrus reticulata Blanco*. United States Department of Agriculture (USDA). Retrieved January 17, 2024, from https://plants.usda.gov/home/plantProfile?symbol=CIRE3

Williams, L. A. D., O'Connar, A., Latore, L., Dennis, O., Ringer, S., Whittaker, J. A., Conrad, J., Vogler, B., Rosner, H., & Kraus, W. (2008). The in vitro anti-denaturation effects induced by natural products and non-steroidal compounds in heat treated (Immunogenic) bovine serum albumin is proposed as a screening assay for the detection of anti-inflammatory compounds, without the use of animals, in the early stages of the drug discovery process. *The West Indian Medical Journal*, **57**(4), 327–331. https://pubmed.ncbi.nlm.nih.gov/19566010/