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Determination of Phenolic and Flavonoid Total Levels and Antioxidant Activity of Ethanol, Ethyl Acetate, and n-Hexane Extracts of *Citrus reticulata* Blanco Fruit Peel by DPPH and ABTS Methods

(*Penetapan Kadar Total Fenol dan Flavonoid serta Aktivitas Antioksidan Ekstrak Etanol, Etil Asetat, dan n-Heksan Kulit Buah Citrus reticulata Blanco dengan Metode DPPH dan ABTS*)

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ABSTRACT

Background: The peel of *Citrus reticulata* Blanco has been traditionally used in medicine due to its diverse qualities. This practice is linked to the bioactive substances in the fruit peel, including phenolic compounds and flavonoids, which protect against oxidative stress induced by free radicals. **Objectives:** This study aimed to evaluate the total concentrations of phenolics and flavonoids, as well as the antioxidant properties, in the ethanol, ethyl acetate, and n-hexane extracts of *Citrus reticulata* Blanco peel. The evaluation utilized the DPPH (2,2-diphenyl-1-picrylhydrazil) and ABTS (2,2'-azinobis-(3-ethylbenzothiazolin-6-sulphonate)) methods. **Material and Methods:** Extraction was conducted using the maceration technique with ethanol, ethyl acetate, and n-hexane solvents at ambient temperature for 24 hours. Total phenolic and flavonoid content was quantified using UV-Vis spectrophotometry. Antioxidant activity was assessed by the extract's ability to counteract free radicals from DPPH and ABTS. **Results:** The ethanol extract of *Citrus reticulata* Blanco peel exhibited higher total phenolic and flavonoid content compared to ethyl acetate and n-hexane extracts. Phenolic concentrations were 142.02, 74.60, and 57.17 mg GAE/g for ethanol, ethyl acetate, and n-hexane extracts, respectively. Flavonoid concentrations were 45.96, 40.22, and 38.54 mg QE/g for ethanol, ethyl acetate, and n-hexane extracts, respectively. The ethanol extract demonstrated the highest antioxidant activity, with IC50 values of 23.49 µg/mL for DPPH and 31.97 µg/mL for ABTS. **Conclusions:** This study reveals that the peel of *Citrus reticulata* Blanco contains phenolic compounds and flavonoids with significant antioxidant potential. Among the extracts, the ethanol extract shows the highest total phenolic and flavonoid content and antioxidant activity. These findings provide a basis for developing pharmaceutical and health supplements aimed at enhancing antioxidant levels and overall health.

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INTRODUCTION

Indonesia is one of the tropical regions in the world that is famous for its diversity of flora. The diversity of flora is one of the pillars of wealth in Indonesia. Cultivation and utilization of various types of plants continue to be carried out by various types of research and development processes, especially medicinal preparations, and supplements. Various types of medicinal plants are believed to ward off diseases triggered by increasing free radicals in the body(Agbo, 2015; Chen et al., 2019; Tristantini et al., 2021).

Biochemists and health workers say that reactive oxygen compounds or in other words a form of free radicals which are compounds with unpaired electrons. This compound is formed in the body and triggered by various factors. A compound that can eliminate, withstand, and clean up the effects of free radicals is called an antioxidant. Antioxidants are compounds that can eliminate, cleanse, and withstand the effects of radicals. Antioxidants stabilize free radicals by supplementing free radicals' lack of electrons (Cerón-Carrasco, 2014; Erkekoglou, 2017; Sério, 2014).

Citrus reticulata Blanco, or "jeruk siam" in Indonesia, has a distinctive, refreshing aroma, tastes good and sweet, and has a sour, fresh taste. The hue of the fruit's outer layer exhibits a yellowish tinge, while the inside flesh of the fruit can be readily detached from its outer peel. *Citrus reticulata* Blanco contains less vitamin C than *Citrus sinensis* but higher vitamin A. Where one of the health benefits of vitamin A is maintaining healthy eye function (Ikawati et al., 2019; Wu et al., 2021; W. Zhang et al., 2022). Based on the aforementioned description, it is important to undertake a comprehensive research endeavor on the total phenolic and flavonoid levels and antioxidant activity of *Citrus reticulata* Blanco ethanol extract with the DPPH and ABTS methods so that it can be used as traditional medicine and health supplements that have efficacy, safety, and quality.

The objective of this research endeavor is to conduct a comprehensive analysis of the levels of total phenolic and flavonoids, as well as the antioxidant activity, utilizing the IC_{50} determination method. The focus of this investigation is on the ethanol (polar), ethyl acetate (semipolar), and n-hexane (non-polar) extracts. The DPPH and ABTS methods will be employed to assess the antioxidant activity, and the correlation between IC_{50} values obtained from the DPPH and ABTS methods will be determined. This study is expected to provide antioxidant information from the three types of extracts from the skin of the *Citrus reticulata* Blanco fruit to academics, researchers, and the public in general.

MATERIAL AND METHODS

Materials

The tools used in this study were blenders, macerators, glass funnels, measuring cups, beakers, test tubes, drip pipettes, test tube racks, water baths, spatulas, analytical scales, a set of distillation tools, desiccators, crucible dishes, evaporation cups, electric stoves, crucible pliers, furnaces, ovens, *vacuum rotary evaporators*, measuring flasks, Erlenmeyer, micropipettes, vials, stirring tubes, mortars, stampers, filter paper, ash-free filter paper, aluminum foil, UV 254 lamp, UV 365 lamp, silica gel 60 F254 cuvette, and UV-Vis spectrophotometry.

The materials used in this study were *Citrus reticulata* Blanco (jeruk siam) fruit peel simplisia powder, 96% ethanol solvent, ethyl acetate, n-hexane, vitamin C, toluene, aquadest, ammonia, chloroform, Dragendorff reagent, Mayer reagent, Steasny reagent, diluted HCl, $FeCl_3$ 1 %, amyl alcohol, gelatin, Mg powder, Liebermann-Burchard reagent, NaOH, ether, gallic acid, quercetin, DPPH (2,2-diphenyl-1-picrylhydrazyl), and ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

Methods

Test Sample Preparation

Preparing test materials includes material collection, determination of test plants, and processing raw materials into simplisia. The plant material used was the *Citrus reticulata* Blanco (jeruk siam) fruit peel obtained from the Wanareja area, Garut Regency, West Java. The test plant parts that have been collected were then carried out by a plant determination process at the Bogoriense Herbarium, Botanical Field of the Biological Research Center – LIPI (*Indonesian Institute of Sciences*), Bogor. The raw materials that have been collected were then carried out a wet sorting, washing, knitting, and drying process without exposure to direct sunlight, then a dry sorting process is carried out, and a grinding process was carried out using a blender and stored for use (Daulay et al., 2019; Husni et al., 2020; Sinaga et al., 2021).

Determination of Simplisia Characteristics

Examination of simplisia characteristics, which include determination of moisture content (% v/w), drying shrinkage (% w/w), total ash content (% w/w), acid insoluble ash content (% w/w), water insoluble ash content (% w/w), water-soluble essence (% w/w), and ethanol soluble juice content (% w/w) were carried out by methods from WHO, Ministry of Health, and other scientific journals according to standards (Depkes RI, 2000; Kemenkes, 2014; Purkon et al., 2021; Purkon, Kusmiyati, et al., 2022; WHO, 1998, 2011).

Phytochemical Screening in Simplisia and Test Extracts

Characteristics of simplisia were examined, and test extracts were screened for alkaloids, phenols, flavonoids, saponins, tannins, quinones, and steroids using standardised techniques from various scientific sources (Akbaribazm et al., 2020; Altemimi et al., 2017; Depkes RI, 2000; WHO, 1998, 2011; Y. J. Zhang, 2015).

Extraction of Fruit Skin of *Citrus reticulata* Blanco

Extraction from fruit skin of *Citrus reticulata* Blanco. was extracted using a 96% ethanol, which is a polar solvent, ethyl acetate (semipolar), and n-hexane (non-polar) solvents. The macerator was loaded with 530 grams of simplisia derived from the fruit peel of *Citrus reticulata* Blanco, followed by the addition of a solvent consisting of 96% ethanol. This simplisia was also done on different macerators for ethyl acetate and n-hexane solvents until all simplisia powder was submerged and allowed to stand for 3 x 24 hours with a periodic stirring process. The liquid extract was afterwards concentrated through the utilization of a rotating vacuum evaporator. The extract was afterwards placed in a steamer dish that had been secured, and an evaporation procedure was conducted in a waterbath until the extract's weight reaches a consistent value. The outcomes were then weighed (Altemimi et al., 2017; Kanlayavattanakul et al., 2019; Mohammadi, 2016).

Determination of Total Phenolic Levels

Preparation of standard solutions of gallic acid

To create a standard solution with a concentration of 1000 ppm gallic acid, 100 mg of gallic acid was accurately weighed and subsequently dissolved in a methanol solvent. The solvent was added incrementally until the final volume of the solution reaches 100 mL. The solution was then made dilution series of 30, 40, 50, 60, and 70 ppm. The pipetting process was then carried out as much as 0.3, 0.4, 0.5, 0.6, and 0.7 mL and then sufficient with methanol solvents up to 10 mL (Granato et al., 2018; Mahindrakar, 2020; Rahmanisa & Oktaria, 2016).

Measurement of standard solutions of gallic acid

A volume of 0.4 mL of Folin-Ciocalteu reagent was added to solutions with concentrations of 30, 40, 50, 60, and 70 ppm. Next, the mixture was agitated till achieving homogeneity. It was then left undisturbed for a duration of 4 to 8 minutes. Subsequently, 4 mL of a 7.5% Na_2CO_3 solution was introduced, and the resulting mixture was vigorously shaken until homogeneity was attained. Subsequently, a 10 mL volume of methanol solvent was introduced and left undisturbed for a duration of 30 minutes at ambient temperature. Subsequently, measurements were conducted utilizing a UV-Vis spectrophotometer within the wavelength range of 700-800 nm in order to ascertain the maximum

wavelength. The calibration curve was established a relationship between the content of gallic acid (expressed in µg/mL) and its corresponding absorbance (Farasat, 2014; Rababah, 2015; Vongsak, 2013).

Determination of total phenolic content by the Folin-Ciocalteu method

Stock solutions of ethanol, ethyl acetate, and n-hexane extracts of *Citrus reticulata* Blanco fruit peel were made as much as 100 ppm, and a dilution process of 700 ppm concentration was carried out on the three test extracts that were pipetted as much as 0.5 mL and added with 0.4 mL of Folin-Ciocalteu reagent and then shaken. The silencing process was carried out for 4-8 minutes, and 4.0 mL of 7.5% Na_2CO_3 solution is added to the mixture. A solution containing 10 mL of methanol was added, and the resulting mixture was left undisturbed at room temperature for a period of 30 minutes. The determination of the sample's absorbance was conducted with a UV-Vis spectrophotometer, specifically at a designated wavelength of 678 nm. This measurement was performed three times to ensure accuracy. The phenol content of the sample, expressed in terms of gallic acid per gram of extract, was produced based on the acquired data (Abramovič et al., 2017; Sembiring, 2018; Zohra, 2019).

Determination of Total Flavonoid Levels

Preparation of Standard Solution of Quercetin

A quantity of 10 mg of quercetin standard raw material was measured and subsequently dissolved in a methanol solvent, resulting in the attainment of quercetin levels of 1000 ppm. All concentrations are made from a standard solution of quercetin 1000 ppm, namely: 40, 50, 60, 70, and 80 ppm. By way of pipetting process consecutively, as much as 0.4, 0.5, 0.6, 0.7, and 0.8 mL. At each concentration of the standard quercetin solution, 1 mL of 3.2% $AlCl_3$ and 8 mL of 5% acetic acid were added and sufficient with methanol solvent up to 10 mL. Subsequently, an incubation procedure was conducted for a duration of 30 minutes at ambient temperature, followed by the quantification of absorbance using a UV-Vis spectrophotometer set at a wavelength of 411 nm (Jebitta, 2016; Rafi, 2018; Sulastri, 2018).

Determination of Total Flavonoid Levels

The weighing process was carried out with 100 mg of ethanol, ethyl acetate, and n-hexane extracts. Then dissolved in 100 mL of methanol and a dilution process with a concentration of 700 ppm for the three extracts and then taken 1 mL and added 1 mL of $AlCl_3$ 2% and 8 mL of acetic acid 5%. Subsequently, the incubation procedure was conducted for a duration of 30 minutes under ambient conditions, and the measurement of absorbance was performed using a UV-Vis spectrophotometer set at a wavelength of 411 nm. The experimental procedure involved doing three replications of the sample solution in order to determine the flavonoid levels, specifically in terms of quercetin equivalents (Ahmed, 2017; Fidrianny, 2015; Zengin, 2016).

The Assessment of Antioxidant Activity Using the DPPH Method

The evaluation of antioxidant activity using the DPPH method involves several steps, including sample preparation, determination of the maximum wavelength, preparation of the DPPH solution, preparation of the comparison solution (vitamin C), and measurement of the IC_{50} value for each test sample.

Sample preparation

Stock solutions weighing up to 10 mg of ethanol, ethyl acetate, and n-hexane extracts were prepared. These solutions were then diluted in 100 mL of ethanol p.a. in a measuring flask, resulting in a concentration of 100 ppm. From the stock solution, then a dilution process with concentrations of 10, 20, 30, 40, and 50 ppm was carried out for ethanol extract. As for ethyl acetate extract, dilutions of 30, 40, 50, and 60 ppm were carried out, and for n-hexane extracts, dilutions of 50, 60, 70, 80, and 90 were carried out.

Determination of maximum wavelength

A volume of 1 mL of a 25 parts per million (ppm) solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was carefully transferred using a pipette. Subsequently, 3 mL of ethanol of analytical grade (p.a.) was added to the solution. Subsequently, the solution was allowed to rest in a light-deprived environment for a duration of 30 minutes, following which its measurements were obtained utilizing a UV-Vis spectrophotometer set at a wavelength range of 400-800 nm. The result of the determination of the maximum wavelength shows the greatest absorbance at λ 517 nm.

Preparation of DPPH solution

To achieve a concentration of 100 parts per million (ppm), a quantity of up to 10 mg of the substance was dissolved in 100 mL of ethanol solvent p.a. The resulting solution was prepared in a measuring flask. Subsequently, the dilution procedure was executed to achieve a concentration of 25 parts per million (ppm) (Abramovič et al., 2017; Baliyan et al., 2022; Fidrianny, 2013a).

Preparation of comparison solution (vitamin C)

A quantity of Vitamin C weighing 10 mg was dissolved in 100 mL of ethanol within a measuring flask, resulting in a concentration of 100 parts per million (ppm). Subsequently, the dilution procedure was conducted using a combination series of 2, 4, 6, 8, and 10 parts per million (ppm) derived from the initial stock solution.

Examination of IC_{50}

A volume of 1 mL was pipetted for each sample concentration of the test and comparison extract (vitamin C), which was subsequently transferred into a test tube. Subsequently, a volume of 1 mL of a 25 ppm DPPH solution was added to each individual combination. The homogenized mixture was placed in a light-restricted setting and left undisturbed for a period of 30 minutes. Following this, the quantification of the absorption of the combination was performed using a UV-Vis spectrophotometer, with a specific focus on the wavelength of 517 nm. Once the absorbance value has been acquired, the percentage of inhibition (%) of the solution was determined by employing the following formula:

$$\text{Percentage of Inhibition (\%)} = \frac{(Control\ Absorbance - Sample\ Absorbance)}{Control\ Absorbance}\ x\ 100\%$$

The percentage of inhibition (%) obtained then determined the value of IC_{50}, a concentration capable of inhibiting 50% of free radicals. The IC_{50} value can be determined by doing a linear regression analysis on the concentration series and the corresponding percentage of inhibition.

Evaluation of Antioxidant Activity by using ABTS Method

Testing of antioxidant activity by the ABTS method includes sample preparation, determination of maximum wavelength, preparation of ABTS solution, preparation of comparison solution (vitamin C), and determination of IC_{50} of each test sample.

Sample preparation

Stock solutions were prepared by dissolving 10 mg of ethanol, ethyl acetate, and n-hexane extracts in 100 mL of ethanol solvent p.a. in a measuring flask, resulting in a concentration of 100 ppm. From the stock solution, dilutions were carried out with concentrations of 10, 20, 30, 40, and 50 ppm for ethanol and ethyl acetate extracts and 30, 40, 50, 60, and 70 ppm for n-hexane extract extracts (Abramovič et al., 2017; Sridhar & Charles, 2019; Sultana, 2012).

Determination of maximum wavelength

Transfer 1 mL of ABTS solution into a vessel using a pipette, followed by the addition of 5 mL of ethanol p.a. in a light-restricted environment. The measurement of absorbance for the solution was conducted utilizing a UV-Vis spectrophotometer, specifically at a wavelength range of 400-800 nm. The findings on the determination of the maximum wavelength indicate that the highest level of absorbance occurs at a wavelength of 742 nm.

Preparation of ABTS solution:

(i) Solution a: 20 mg of ABTS was weighed and dissolved with 5 mL of aqua-dest. Then the incubation process was carried out for 12 hours.

(ii) Solution b: 7 mg of $K_2S_2O_8$ was weighed and dissolved with 5 mL of aqua-dest. Then the incubation process was carried out for 12 hours.

(iii) Solutions a and b were mixed in a dark room and sufficient in volume with ethanol solvent p.a. up to 25 mL (Abramovič et al., 2017; Fidrianny, 2013a; Sridhar & Charles, 2019).

Preparation of comparison solution (Vitamin C)

Vitamin C was weighed as much as 10 mg and dissolved with 100 mL ethanol solvent into a measuring flask to obtain a concentration of 100 ppm. Then, from the stock solution, a dilution process was carried out with concentration series: 3, 4, 5, 6, 7, and 8 ppm (Abramovič et al., 2017; Bhandari, 2016; Roshanak, 2016).

IC_{50} Examination

Each concentration of test and comparison samples (vitamin C) was pipetted as much as 1 mL and inserted into a test tube. Then add 1 mL of ABTS solution. The process of homogenizing the mixture was carried out in a controlled environment with restricted light conditions. The absorbance of the mixture was then measured using a UV-Vis spectrophotometer, specifically at a wavelength of 742 nm. Once the absorbance value had been acquired, the calculation of the solution's percentage of inhibition (%) was performed utilizing the subsequent formula:

$$\text{Percentage of Inhibition (\%)} = \frac{(Control\ Absorbance - Sample\ Absorbance)}{Control\ Absorbance}\ x\ 100\%$$

The percentage of inhibition (%) was obtained then determined the value of IC_{50}, a concentration that could inhibit 50% of free radicals. The IC_{50} value could be calculated from a linear regression curve between the percentage of inhibition (%) and the concentration series (Abramovič et al., 2017; Agbo, 2015; Ajiboye et al., 2017; de Moura et al., 2022; Fidrianny, 2013a, 2013b; Masisi et al., 2021).

RESULTS AND DISCUSSION

Collection of *Citrus reticulata* Blanco fruit peel material obtained from Cibolerang Village, Karangsari Village, Karangpawitan District, Garut Regency, West Java, Indonesia. This plant was then carried out a determination process at Herbarium Bogoriense, Botanical Field of the Biological Research Center – LIPI (*Indonesian Institute of Sciences*) Bogor to ensure the identity of the plant used was the species *Citrus reticulata* Blanco with No. 2657/IPH.1.01/H.07/XII/2018. The finished fruit skin is then carried through a wet sorting process to select the part of the fruit skin that is still good, fresh, and separated from the impurities by washing process with running water. The drying process was accelerated by displays, which were done in drying cabinets. Then a dry sorting process is carried out to ensure there are no more impurities in the simplisia, and continued the process of reducing the particle size of the simplisia with a blender so that the penetration of the solvent into the simplisia cell membrane is more

accessible and allows the compounds contained in more ingredients to be attracted when the extraction process is carried out (Daulay et al., 2019; Handayani et al., 2017).

The simplisia obtained is further subjected to a characterization to determine the quality/quality of the simplisia of the skin of the fruit *Citrus reticulata* Blanco as depicted in Table 1. The characterization process involves evaluating different attributes, such as moisture content (expressed as a percentage by volume/weight), drying shrinkage (expressed as a percentage by weight), total ash content (expressed as a percentage by weight), acid-insoluble ash content (expressed as a percentage by weight), water-soluble ash content (expressed as a percentage by weight), water-soluble essence (expressed as a percentage by weight), and ethanol-soluble essence (expressed as a percentage by weight). The purpose of this study is to conduct a comprehensive analysis of the characteristics of the herbal substances utilized, with the aim of ascertaining their identity, validity, and overall quality. The study of *Citrus reticulata* fruit peel simplisia is of utmost importance in the field of drug and supplement development due to its direct correlation with both efficacy and safety (Gurning & Sinaga, 2020).

Table 1. Examination Results of *Citrus reticulata* Blanco Fruit Peel Characteristics

No.	Characterization	Examination Results (%)
1.	Water Content	4
2.	Shrinkage Drying	5.4
3.	Total Ash Content	10.7
4.	Insoluble Acid Ash Content	2
5.	Water-Soluble Ash Content	8
6.	Water Soluble Essence	7.6
7.	Ethanol Soluble Essence	2.3

According to the study's findings, the azeotrope distillation process, which combines two challenging-to-separate chemicals, produced water content of 4% v/w. This moisture content check is carried out to determine the range of moisture content that is less than <10% and can maintain the quality of simplisia, quality control (prevent the growth of microorganisms), determination of dry weight, and establish the stability of the active ingredient of a simplisia (Gurning & Sinaga, 2020; Husni et al., 2020; Purkon, Fadhlillah, et al., 2022). The drying shrinkage value of simplisia is 5.4% v/w. The distinction between moisture content and drying shrinkage lies in the quantity of water present in the simplisia, with moisture content referring to the overall water content within the simplisia, and drying shrinkage encompassing the whole of the simplisia content that is capable of evaporating subsequent to the drying process (Sinaga et al., 2021).

Furthermore, the process of determining ash content is conducted at a temperature of 600°C in order to optimize the ashing procedure. The overall ash content, acid-insoluble ash content, and water-soluble ash content were determined as 10.7%, 2%, and 8% w/w, respectively. The determination of ash content

aims to determine the mineral levels contained in the plant place and control the contaminants of other inorganic objects, such as soil and sand bound to plants. Acid-insoluble ash content is intended to determine the value of metal oxides that come from outside the plant, while water-soluble ash content is to determine metal oxides derived from minerals in the plant (Jang et al., 2021; Kalaskar et al., 2021; Purkon et al., 2021). The water-soluble essence content is determined to be 7.6% w/w, whereas the ethanol-soluble essence content is found to be 2.3% w/w. Determination of water-soluble essence content and ethanol-soluble essence content aims to show the amount of compound content that can be extracted by water or ethanol solvent so that it can be used to determine the chemical profile and validate the correct extraction technique effectively and efficiently (Gómez-Mejía et al., 2019).

The maceration method was chosen due to its simple working process and its classification as a cold extraction technique, which minimizes the risk of compound degradation. The extraction process of *Citrus reticulata* Blanco fruit peel uses three types of solvents, namely: polar solvents (ethanol), semipolar (ethyl acetate), and non-polar (n-hexane). The use of these three types of solvents aims to determine the yield level of each type of solvent, attract compounds according to the level of polarity, and use different solvents whose polarity levels allow to extract of different polyphenol components so that the antioxidant properties obtained from the extraction of each type of solvent can obtain each different compound (Andriyanto et al., 2022; Fikranus Shofa et al., 2022; Isnawati &; Retnaningsih, 2018). The percentage yield value of test extracts on ethanol, ethyl acetate, and n-hexane extracts was 5.35%, 3.05%, and 0.60% w/w, respectively.

Table 2. Results of Phytochemical Screening on Simplisia and Ethanol, Ethyl Acetate, and n-Hexane Extracts from the *Citrus reticulata* Blanco Fruit Peel

No.	Secondary Metabolite Group	Simplisia	Extract		
			Ethanol	Ethyl Acetate	n-Hexane
1.	Flavonoid	+	+	+	+
2.	Saponin	+	+	+	+
3.	Tannin	-	-	-	-
4.	Alkaloid	-	-	-	-
5.	Steroid	+	+	+	+
6.	Quinon	+	+	+	+
7.	Phenol	+	+	+	+

Information: (+): Detected
(-): Undetected

A phytochemical screening analysis was conducted on simplisia as well as ethanol, ethyl acetate, and n-hexane extracts derived from the peel of *Citrus reticulata* Blanco fruit. The objective of this analysis was to identify the specific class of secondary metabolite chemicals present in simplisia and the three aforementioned extracts. The results of phytochemical screening on simplisia and the three types of test

extracts on *Citrus reticulata* Blanco are flavonoids, saponins, steroids, quinones, and phenols, as seen in Table 2. Flavonoid and phenolic compounds are both compounds known to have strong antioxidant activity because of their chemical structure, which will be hydroxyl groups (OH^-) and aromatic rings. The hydroxyl group in the compound can neutralize harmful free radicals within the body to protect cells from oxidative stress damage (Alvarez-Arellano et al., 2020; Ge et al., 2020a; Pasupuleti et al., 2020).

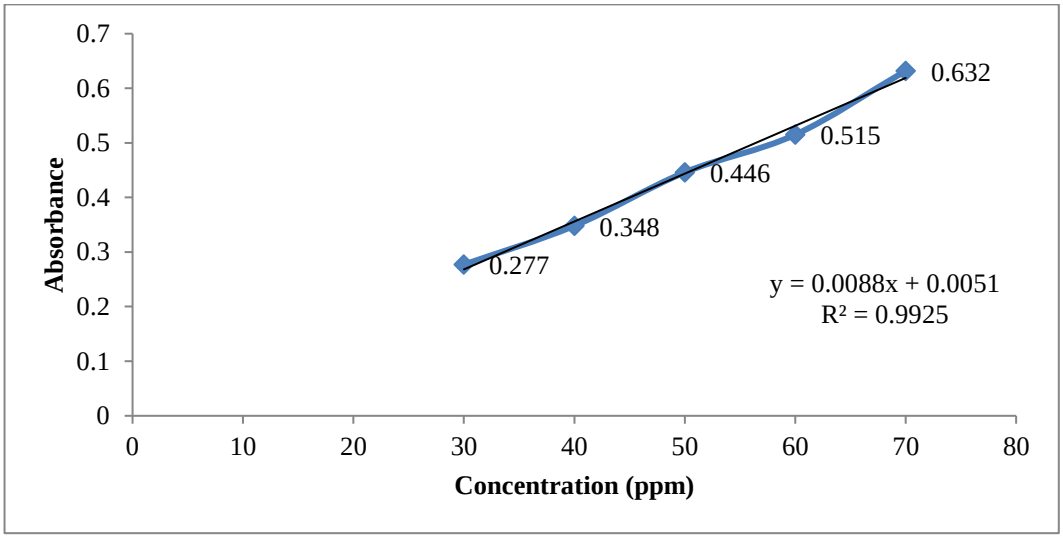

Figure 1. Result of the gallic acid calibration curve (total phenolic)

The standard curve value of gallic acid has a linear regression equation of y = 0.0088x + 0.0051 with an R^2 value of 0.9925, as seen in Figure 1. Determination of total phenolic levels using the Folin-Ciocalteu reagent is characterized by the formation of blue complex compounds based on the reducing power of the phenol hydroxy group. The selection of gallic acid (GAE) as a standard solution for quantifying the total phenolic content is based on its status as a derivative of hydroxybenzoic acid, which belongs to the simple phenol acid group and possesses stable qualities. Subsequently, the introduction of a sodium carbonate (Na_2CO_3) solution is implemented to establish an alkaline environment, facilitating the dissociation of protons in phenolic compounds into phenolic ions. This reaction can only take place in an alkaline setting, enabling the interaction between phenolic compounds and Folin-Ciocalteu reagents (Badri et al., 2013; Granato et al., 2018; Hasnaeni &; Aminah, 2019; Kustiawan et al., 2021; Nur et al., 2019; Pandey, 2018).

The data presented in Table 3 displays the total phenolic levels of ethanol extract (EECR), ethyl acetate (EAECR), and n-hexane (NECR) in the peel of *Citrus reticulata* Blanco fruit, which respectively amounted to 142.02, 74.60, and 57.17 mgGAE/g. The values for the total flavonoid content obtained from the ethanol extract (EECR), ethyl acetate (EAECR), and n-hexane (NECR) were 45.96, 40.22, and 38.54 mgQE/g, respectively, as presented in Table 4. The total phenolic content exceeds the total

flavonoid content due to the presence of considerable phenolic chemicals in the extract that are not exclusively classified as flavonoids. Phenolic compounds are known to protect against UV-B exposure and prevent cell death and protect DNA from dimerization and damage (Hanin & Pratiwi, 2017; Oktavia & Sutoyo, 2021; Tungmunnithum et al., 2018).

Table 3. Average total phenolic levels obtained from the ethanol extract (EECR), ethyl acetate (EAECR), and n-hexane (NECR) extracts derived from *Citrus reticulata* Blanco fruit peel

No.	Test Extract Group	Absorbance Average ($\bar{x}$ ± SD)	Average of Gallic Acid Equivalent Levels (µL/mL; $\bar{x}$ ± SD)	Volume (L)	Dilution Factor	Test Sample Weight (mg)	Average of Total Phenolic Levels (mg GAE/g Sample; $\bar{x}$ ± SD)
1.	EECR	0.630 ± 0.0113	71.01 ± 1.2807	0.01	20x	0.1	142.02 ± 2.5614
2.	EAECR	0.333 ± 0.0061	37.30 ± 0.6945	0.01	20x	0.1	74.60 ± 1.3884
3.	NECR	0.257 ± 0.0354	28.66 ± 4.0276	0.01	20x	0.1	57.17 ± 7.7949

Note: EECR = Ethanol Extract of *Citrus reticulata* Blanco fruit peel
EAECR = Ethyl Acetate Extract of *Citrus reticulata* Blanco fruit peel
NECR = n-Hexane Extract of *Citrus reticulata* Blanco fruit peel

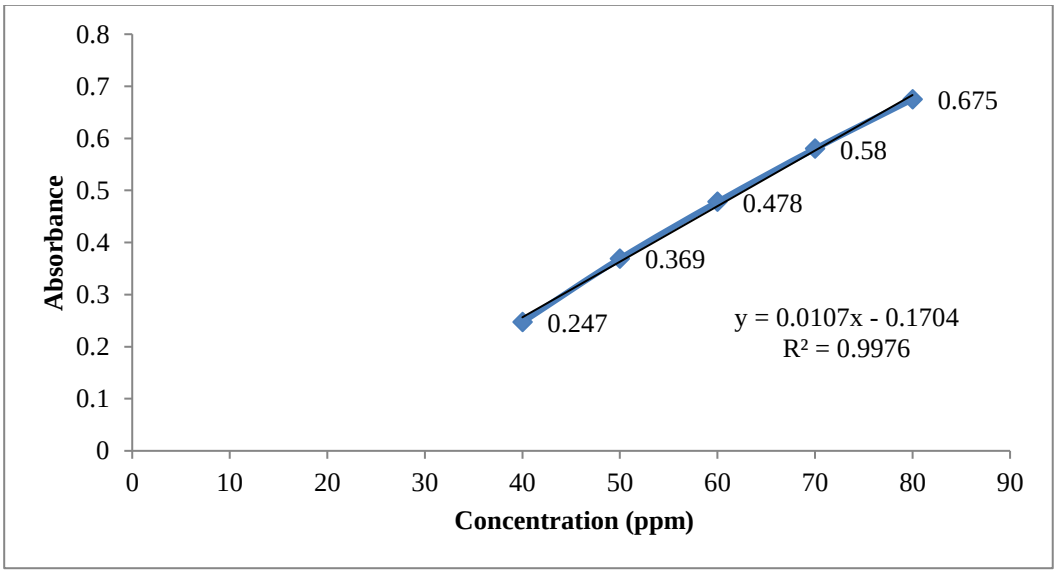

Figure 2. Results of the quercetin calibration curve (total flavonoids)

The calibration curve data for quercetin compounds, which is commonly used to quantify total flavonoid levels, has a linear regression equation of y = 0.0107x – 0.1704. This equation demonstrates a strong correlation, as indicated by the high R2 value of 0.9976, as depicted in Figure 2. The quantification of overall flavonoid content by a colorimetric technique employing a standard solution of quercetin (QE). Subsequently, the addition of $AlCl_3$ compounds is performed, facilitating the formation of complexes

with quercetin molecules. The interaction between the components causes a change in the wavelength of light, causing it to move into the visible spectrum. As a result, the solution exhibits a yellow hue (Jebitta, 2016; Rafi, 2018; Sulastri, 2018).

Table 4. Average Levels of Total Flavonoids from Ethanol Extract (EECR), Ethyl Acetate (EAECR), and n-Hexane (NECR) derived from *Citrus reticulata* Blanco Fruit Peels

No.	Test Extract Group	Absorbance Average ($\bar{x}$ ± SD)	Average of Quercetin Equivalent Levels (µL/mL; $\bar{x}$ ± SD)	Volume (L)	Dilution Factor	Test Sample Weight (mg)	Average Total Flavonoid Levels (mg QE/g Sample; $\bar{x}$ ± SD)
1.	EECR	0.321 ± 0.0021	14.11 ± 0.1946	0.01	10x	0.1	45.96 ± 0.1946
2.	EAECR	0.260 ± 0.0020	8.37 ± 0.1870	0.01	10x	0.1	40.22 ± 0.1870
3.	NECR	0.242 ± 0.0010	6.69 ± 0.0935	0.01	10x	0.1	38.54 ± 0.0935

Information: EECR = Ethanol Extract of *Citrus reticulata* Blanco fruit peel
EAECR = Ethyl Acetate Extract of *Citrus reticulata* Blanco fruit peel
NECR = n-Hexane Extract of *Citrus reticulata* Blanco fruit peel

In the assessment of antioxidant activity, two methods were employed, specifically the DPPH method (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). The data obtained from the experiment assessing the antioxidant activity of vitamin C was utilized for comparative analysis. The experiment conducted the DPPH method at a maximum wavelength of 517 nm. The obtained IC_{50} value was 8.522 µg/mL (ppm), determined using a linear regression curve equation of $y = 5.015x + 7.2614$ and an R^2 value of 0.9769, as depicted in Figure 3 and Table 5. In Table 5, the result of antioxidant testing with the DPPH method for ethanol extract of *Citrus reticulata* Blanco fruit peel (23,490 µg/mL) is categorized as a strong antioxidant because it is in the range of 10-50 ppm. These results follow the exposure to research by Jadid, et al. (2017) that the antioxidant activity of a compound using levels of 10-50 ppm is a strong category. Meanwhile, ethyl acetate and n-hexane extracts of 52,812 and 92,506 µg/mL, respectively, are categorized as strong antioxidants because they are in the range of 50-100 ppm. The DPPH method is utilized to assess antioxidant activity by measuring the capacity of antioxidants, phenolic compounds, and flavonoids to decrease DPPH radicals by hydrogen capture processes. This method is also a method of measuring antioxidant capacity using synthetic radicals in polar organic solutions. This purple DPPH solution (containing DPPH radicals) will be bound by H^+ ions derived from antioxidant compounds, causing the intensity of the purple color to decrease (Abramovič et al., 2017; Baliyan et al., 2022; Ge et al., 2020b; Sulastri & Ikram, 2017).

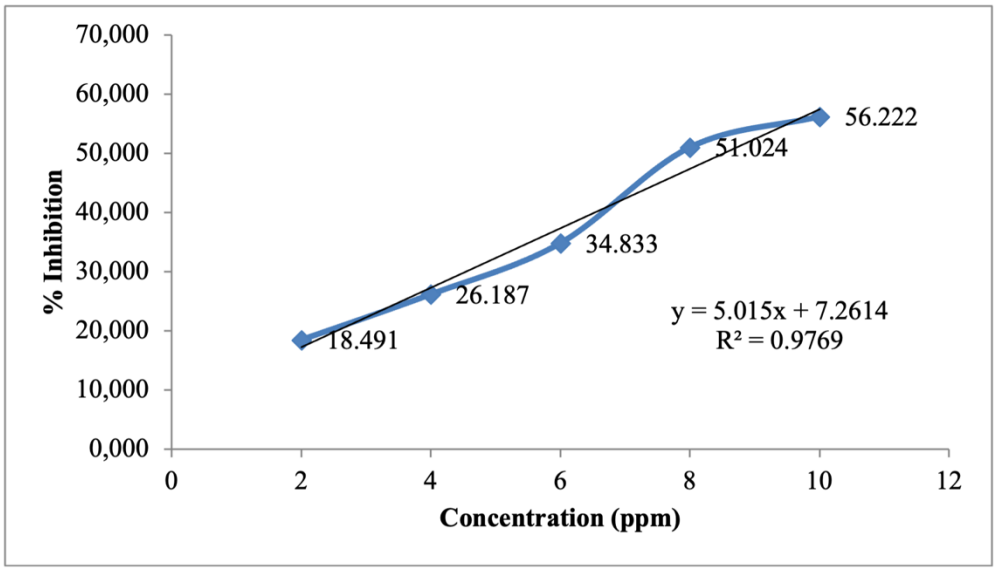

Figure 3. The antioxidant activity curve of vitamin C using the DPPH method

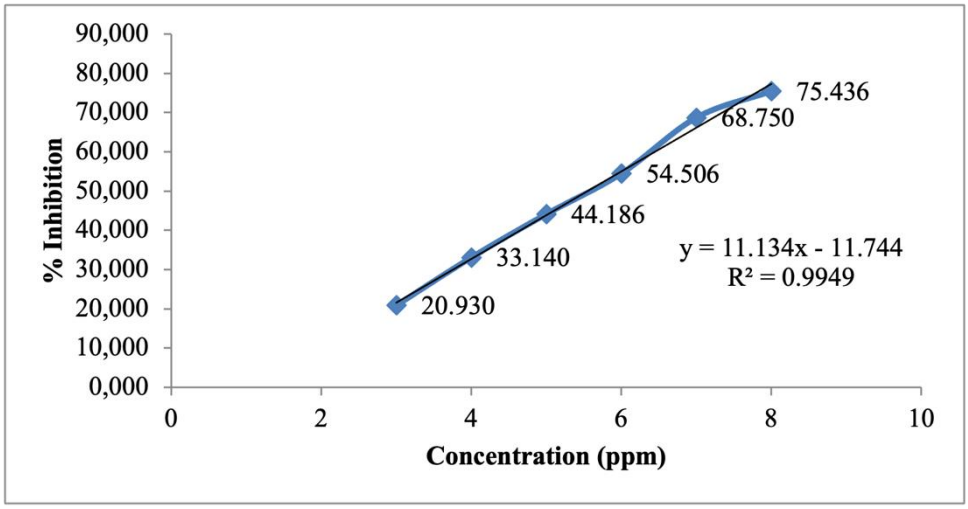

Figure 4. The content of vitamin C and its corresponding inhibition percentage (%) as determined by the ABTS technique, which measures antioxidant activity

Table 5. Antioxidant Activity Data of Ethanol Extract (EECR), Ethyl Acetate (EAECR), and n-Hexane (NECR) from *Citrus reticulata* Blanco Fruit Peel and Vitamin C with DPPH Method and ABTS Method

No.	Test Group	IC_{50} Value with DPPH Method (µg/mL or ppm; $\bar{x} \pm SD$)	IC_{50} Value with ABTS Method (µg/mL or ppm; $\bar{x} \pm SD$)
1.	EECR	23.490 ± 0.062	31.971 ± 0.133
2.	EAECR	52.812 ± 0.091	41.617 ± 0.146
3.	NECR	92.506 ± 0.082	66.078 ± 0.113
4.	Vitamin C	8.522 ± 0.107	5.546 ± 0.144

Information: EECR = Ethanol Extract of *Citrus reticulata* Blanco fruit peel
EAECR = Ethyl Acetate Extract of *Citrus reticulata* Blanco fruit peel
NECR = n-Hexane Extract of *Citrus reticulata* Blanco fruit peel

The results obtained from the ABTS method, which is commonly employed to evaluate antioxidant activity, demonstrated that vitamin C, employed as a standard solution, displayed an IC_{50} value of 5.546 µg/mL (ppm). This was determined based on the highest ABTS wavelength of 742 nm and an absorbance value of 0.688. The IC_{50} values of ethanol extract (EECR), ethyl acetate (EAECR), and n-hexane (NECR) derived from *Citrus reticulata* Blanco are 31.971, 41.617, and 66.078 ppm, respectively, as presented in Table 5. From the test results, ethanol and ethyl acetate extracts of *Citrus reticulata* Blanco fruit peel are categorized as strong antioxidants because these are in the range of 10-50 ppm, while n-hexane extract is categorized as a moderate, strong antioxidant in the range of 50-100 ppm. The ABTS approach is predicated upon the radical-reducing capacity exhibited by ABTS. The principle of this method is the interaction of antioxidants with ABTS radical cations. It could absorb blue-green chromophores because there is an oxidation reaction of ABTS with potassium persulfate due to the addition of antioxidants. The evaluation of antioxidants by the ABTS method is dependent on the perceptible decrease in the intensity of the blue hue caused by the interaction between antioxidants and ABTS, resulting in its reduction (Abramovič et al., 2017; Fidrianny, 2013b, 2013a; Sridhar & Charles, 2019). Both experiments used a positive control (comparison) of vitamin C, due to its potent ability to mitigate or prevent the formation of free radicals. Researchers commonly utilize vitamin C as a positive control, especially when evaluating antioxidant activity (Fowler et al., 2019; Koomson, 2018; Roshanak, 2016).

Based on the IC_{50} value, the ABTS value demonstrates a stronger value than the DPPH method. However, when considering the treatment procedure and methodology, it can be observed that the DPPH approach exhibits greater stability and relatively simple compared to the ABTS method, because the ABTS solution requires multiple treatments, which increases the error factor (Abramovič et al., 2017; Sridhar & Charles, 2019).

CONCLUSION

The concentrations of phenolic compounds in the ethanol, ethyl acetate, and n-hexane extracts obtained from the skin of *Citrus reticulata* Blanco fruit were 142.02, 74.60, and 57.17 mgGAE/g, respectively. Similarly, the quantities of flavonoid compounds in the ethanol, ethyl acetate, and n-hexane extracts were found to be 45.96, 40.22, and 38.54 mgQE/g, respectively. The antioxidant activity of *Citrus reticulata* Blanco fruit peel extracts, obtained using ethanol, ethyl acetate, and n-hexane solvents, was evaluated using the DPPH and ABTS methods. The ethanol extract exhibited a strong antioxidant activity, while the ethyl acetate and n-hexane extracts showed a moderate antioxidant activity. However, it is worth noting that the ethyl acetate extract demonstrated a strong antioxidant activity when assessed using the ABTS method, whereas the DPPH method placed it in the moderate-strong/medium category. The antioxidant activity of the test extract is directly correlated with the total phenolic and flavonoid

contents. The greater the cumulative phenolic and flavonoid concentration, as measured by the IC_{50} value, the greater the antioxidant activity. This research suggests that the *Citrus reticulata* Blanco fruit peel may be a valuable source of natural antioxidant compounds. These findings shed light on the potential use of fruit peel in development of pharmaceutical preparations and health supplements with antioxidant benefits. Additional investigation is required to ascertain the specific bioactive constituents accountable for the notable antioxidant efficacy shown in the ethanol extract derived from the peel of *Citrus reticulata* Blanco fruit. The utilization of chromatographic methodologies, such as high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) might facilitate the identification of these chemicals. Furthermore, the utilization of FRAP (Ferric Reducing Antioxidant Power) or ORAC (Oxygen Radical Absorbance Capacity) techniques can yield further insights into the antioxidant efficacy of *Citrus reticulata* Blanco fruit peel extract. Further exploration can be conducted to assess the pharmacological activity of *Citrus reticulata* Blanco fruit peel, as well as to develop formulations based on ethanol extract for use as health supplements and active ingredients in certain pharmaceutical preparations.

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CONFLICT OF INTEREST

All authors declared no conflict of interest in this research.

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