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EFFECTS OF FERMENTATION TIME AND EXTRACTION SOLVENT ON ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC AND FLAVONOID CONTENT OF PHENOLIC EXTRACT FROM ORANGE (*Citrus reticulata*) PEEL

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ABSTRACT: The rise in consumption of oranges worldwide causes an increase in orange peel, which accounts for 30 – 35% of the orange's weight. Phenolic compounds found in orange peel are conjugated with the cell wall components. The utilization of solid-state fermentation by *Aspergillus niger* can release bound phenolic compounds from the cell wall, thereby hence increases phenolic compounds extraction. This study aims to determine the effects of the fermentation time of orange peel using *A. niger* and extraction solvents (methanol, ethanol, acetone) on the yield of orange peel extract, total phenolic content, total flavonoid content, antioxidant activity, and hesperidin content of orange peel extract. The results showed that all the parameters performed best after 3 days of fermentation. The extract yield, total phenolic content, and total flavonoid content were 34.1% (dry weight), 51.01 mg GAE/g extract, and 14.75 mg QE/g extract, respectively. Moreover, the highest antioxidant activity (IC_{50} value) and hesperidin content found in orange peel were 184.32 ppm and 27.47 mg/g (dry weight) using 80% ethanol. Furthermore, the utilization of deep eutectic solvent as a biodegradable and environmentally benign substitute for organic solvents was investigated, proving that the alternative mixture of choline chloride and ethylene glycol is competitive.

ABSTRAK: Peningkatan penggunaan oren di seluruh dunia menyebabkan peningkatan sisa dalam bentuk kulit oren, iaitu 30 – 35% daripada beratnya. Sebatian fenolik yang terdapat dalam kulit oren berkonjugasi dengan komponen dinding sel. Penggunaan penapaian keadaan pepejal oleh *Aspergillus niger* boleh membebaskan sebatian fenolik terikat dari dinding sel dan seterusnya meningkatkan hasil pengekstrakan sebatian fenolik. Kajian ini bertujuan untuk mengetahui pengaruh variasi dalam masa penapaian dan pemilihan pelarut terhadap hasil ekstrak kulit oren, jumlah kandungan fenolik, jumlah kandungan flavonoid, aktiviti antioksidan, dan kandungan hesperidin ekstrak kulit oren. Hasil menunjukkan bahawa selepas 3 hari penapaian semua parameter menunjukkan prestasi terbaik. Hasil ekstrak, jumlah kandungan fenolik dan jumlah kandungan flavonoid masing-masing adalah 34.1% (berat kering), 51.01 mg ekstrak GAE/g dan 14.75 mg ekstrak QE/g. Selain itu, aktiviti antioksidan tertinggi (nilai IC_{50}) dan kandungan hesperidin yang terdapat dalam kulit oren ialah 184.32 ppm dan 27.47 mg/g menggunakan 80% etanol. Tambahan pula, penggunaan *deep eutectic solvent* sebagai plearut yang mesra alam dan dapat terdegradasi telah dikenalpasti dan terbukti bahawa campuran alternatif kolin klorida dan etilena glikol adalah kompetitif.

KEYWORDS: *Antioxidant activity, Aspergillus niger, hesperidin, orange peel, solid-state fermentation*

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1. INTRODUCTION

Citrus is an annual plant that bears fruit throughout the year and can grow in both tropical and subtropical climates. The most common type of citrus variety sold in the market is the orange (*Citrus reticulata*), and the total worldwide production is 104 million tons per year [1]. The food industry generally uses oranges to produce canned fruit, jam, jellies, and juice, which results in significant by-product production that accounts for around 50% of the overall weight. The percentage of peel in one orange is around 30 – 35%, making it one of the major wastes generated throughout this production [2]. The proper utilization of fruit waste is a critical issue since the amount produced each year threatens to contaminate the soil, water, and ecosystem. This waste is a significant problem for the environment because the acidic nature of the orange peel will lower the soil pH [3] causing a reduction in the solubility of oxygen in the soil, which can reduce soil fertility.

Numerous studies have been conducted to investigate approaches for increasing the value of the productive chain through the valorization of agro-industrial residue and acquiring marketable products [4-8]. Valorization of these wastes becomes attractive since the waste composition may be a source of bioactive substances, including polyphenols, sugars, essential oils, pigments, enzymes, and pectin [9]. These compounds could potentially be utilized in the agriculture sector, flavor and fragrance companies, food and beverage companies, and pharmacies as nutraceuticals. Orange (Citrus reticulata) is a popular fruit as it has significant effects such as antimutagenic, anti-inflammatory, antioxidant, antitumor, anti-atherosclerotic, and antibacterial [10]. As a result, the conversion of orange peel waste into value-added products has recently drawn more attention [2, 9, 11, 12]. Orange peel consists of an outer part (flavedo), which is rich in essential oils and carotenoids, and an inner part (albedo), which is rich in lignocellulose, pectin, and phenolics [13]. Phenolics are compounds mostly found in plants that have hydroxyl groups and are produced in response to environmental stress to counter its effects. As a result, phenolic compounds function as antioxidants, antimicrobials, and anti-inflammatory [14, 15]. The total phenolic content of orange peel ranges from 1.39 to 1.85 mg GAE/100 g dry biomass [16, 17].

Orange peel contains lignocellulose, which is the main ingredient in plant cell walls consisting of hemicellulose, cellulose, and lignin [18]. Phenolic compounds generally exist in a conjugated form with cell wall components. Extracts of phenolic compounds can be obtained by providing pre-treatment in fermentation. Solid state fermentation is an alternative method that can release cell-wall bound bioactive compounds by degrading the components of the cell wall to facilitate contact of the solvent with the compound to be isolated [19]. Aspergillus niger is one of the fungi that can be utilized in the fermentation process. This is because A. niger produces the enzymes cellulase, xylanase, and ligninase, which break down lignocellulosic materials to facilitate the release of phenolic chemicals [20, 21]. In recent studies, no initial treatment was given in the form of fermentation. Therefore, in this study, fermentation was carried out using A. niger to degrade lignocellulose in orange peel to increase extraction yield, total phenolic content, total flavonoid content, antioxidant activity, and hesperidin content in orange peel extract.

Extraction is an important step in isolating, identifying, and quantifying phenolic compounds in plants. In this study, the maceration extraction method used ethanol, methanol, and acetone as organic solvents. Maceration is the simplest extraction method for isolating polyphenolic compounds from plant materials [22]. Many previous studies reported the content of phenolic compounds in orange peel from different origins and varieties. In the study of Safdar et al. [23], the yield of Kinnow orange peel (C. reticulata L.) phenolic compound

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extracts using ethanol, methanol, and acetone was 18.46%, 14.5%, and 8% respectively. Whereas in the study of Hegazy & Ibrahium [24], the yield of orange peel phenolic compound extracts using ethanol, methanol, and acetone solvents was 27.96%, 28.32%, and 18.21%, respectively.

However, large-scale consumption of organic solvents can harm the environment and pose health risks to humans [25]. Thus, the idea of extracting valuable molecules from biomass using green solvents is increasingly being considered. Deep eutectic solvent (DES) is a promising alternative for organic solvents as they are low cost, renewable, environmentally friendly, non-toxic, and biodegradable [26]. A previous study by Xu et al. [27] showed that choline chloride: levulinic acid: n-methyl urea outperformed several organic solvents such as methanol, ethanol, and n-butanol in polyphenols extraction from citrus peel waste. This study aims to use various solvents, ranging from organic solvents to choline chloride-based DES to extract and recover phenolic compounds from orange peel waste. The extraction capabilities of different types of solvents are examined.

2. MATERIALS AND METHODS

2.1. Materials

Fresh oranges (*Citrus reticulata*) used in this study were obtained from plantations in Ciwidey, West Bandung, West Java, Indonesia. The commercial culture of *A. niger* was obtained from the Nanobio Laboratory. Analytical grade hesperidin (98%) used as a standard compound in this study was obtained from Markherb. Ethanol, methanol, acetone, ethylene glycol, lactic acid, sodium hydroxide (NaOH), potassium sulfate (K_2SO_4), copper (II) sulfate ($CuSO_4$), hydrochloric acid (HCl), boric acid (H_3BO_3), aluminum chloride ($AlCl_3$), sulfuric acid (H_2SO_4), nitric acid (HNO_3), acetic acid (CH_3COOH), sodium carbonate (Na_2CO_3), Folin-Ciocalteu reagent, and gallic acid were obtained from Merck, while DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) was from Sigma. Choline chloride was from MaxLab, Quercetin was from Nitrakimia, magnesium sulfate ($MgSO_4$) was from Pudak Scientific, potassium phosphate (KH_2PO_4) was from Kimia Market, yeast extract was from HiMedia, and calcium chloride ($CaCl_2$) was from Pharmapreneur store.

2.2. Preparation of orange peel

The fresh oranges were peeled off, and the peel was washed with water and divided into tiny (1×1 cm) pieces. The whole orange peel was then dried in an oven at 60°C for 24 hours until the moisture content was 15%, followed by crushing using a dry miller (Miyako blender BL-152GF 300 W) and then sieved using a 60-mesh sieve to obtain a size of 0.25 mm [28].

2.3. Proximate analysis of orange peel

Proximate analysis was applied to determine the moisture, protein, lipid, carbohydrate, and ash content of orange peel samples used in this study. The proximate analysis of the samples was performed with three repetitions, and a gravimetric approach was applied to measure the moisture content. The protein content was determined using the Kjeldahl method, whereas the lipid content was assessed using the Soxhlet method. The samples were dried in a furnace at 600°C for 6 hours to determine the ash content, while the carbohydrate content was determined using the by-difference approach.

2.4. Solid-state fermentation of orange peel using *A. niger*

The solid-state fermentation was carried out under aseptic conditions using trays, with each tray containing 250 g orange peel mixed with a salt solution containing 3.4 g yeast extract, 0.09 g $CaCl_2.2H_2O$, 1.3 g KH_2PO_4, 6.6 g glucose, and 3.3 g $MgSO_4.7H_2O$ in 100 g of orange peel. Subsequently, distilled water was added to the mixture with a ratio of 1:2 (v/w) [20, 21]. Following that, a 3:20 fungus-to-substrate ratio was used to inoculate *A. niger*, which had a cell count of 4 x 10^8 CFU/g, and the fermentation was carried out for 7 days at 27°C, 76% humidity, and 0 W/m^2 light intensity [29].

2.5. Determination of *A. niger* growth curve

The growth curve of *A. niger* was determined based on the daily measured dry weight of the biomass [30]. Approximately 7 g (m_1) of a fresh fermented mixture was dried at 60°C for 4 hours, and after that, the dried biomass was weighed again (m_2). A mixture of acetic acid solution (80%) and pure nitric acid reagent was then added to the fermented mixture with a ratio of 1:10 and then incubated for 30 minutes in a water bath at a temperature of 55°C and a stirring speed of 120 rpm followed by treatment with acid and then dried once more in an oven set at 60°C for 4 hours (m_3). The dry biomass and specific growth rate of *A. niger* were calculated using Eq. (1) and Eq. (2), respectively.

$$\text{Dry biomass } \left(\frac{g}{g}\right) = \frac{m_3\,(g) - m_2\,(g)}{m_1(g)} \quad (1)$$

$$\mu = \frac{lnX_t - lnX_0}{\Delta t} \quad (2)$$

where μ is the growth rate (hour-1), X_t is the amount of dry biomass at the end of the exponential phase (g), X_0 is the amount of dry biomass at the beginning of the exponential phase (g), and Δt is the difference in observation time or the length of the logarithmic phase.

2.6. Determination of lignocellulosic content

The lignocellulosic content in the biomass was determined using the method of Chesson-Datta [31]. Approximately 4 g of dried orange peel sample (m_1) were mixed with 200 mL of distilled water, followed by 2 hours of refluxing at 100°C before filtration. The filtrate was then washed using 300 mL of hot water and dried in an oven at 105ºC for approximately 2 hours, and the weight remained constant (m_2). After that, the residue was added to 300 mL of 1 N H_2SO_4 and refluxed for 2 hours at 100°C, followed by filtration. The residue was then washed with 300 mL of hot water, dried in an oven at 105ºC for 2 hours, and then weighed (m_3). Next, the dry residue was soaked in 10 mL of 72% H_2SO_4 for 4 hours at room temperature (27ºC). After that, 300 mL of 1 N H_2SO_4 was added, followed by reflux at 100°C for 2 hours before being filtered. The residue was washed again with 300 mL of hot water, dried in an oven at 105ºC for 2 hours, and then weighed (m_4). Lastly, the residue was placed in a furnace set at 600ºC to measure the amount of ash (m_5). The determination of lignocellulosic content was carried out 3 times for each fermentation time. The levels of hemicellulose, cellulose, and lignin can be determined by Eq. (3), (4), and (5).

$$Hemicellulose(\%) = \frac{m_2\,(g) - m_3(g)}{m_1(g)} \times 100 \quad (3)$$

$$Cellulose(\%) = \frac{m_3(g) - m_4(g)}{m_1(g)} \times 100 \quad (4)$$

$$Lignin(\%) = \frac{m_4(g) - m_5(g)}{m_1(g)} \times 100 \quad (5)$$

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where m_1 is the initial mass of dried orange peel, m_2 is the mass of dried orange peel after refluxing using distilled water, m_3 is the mass of dried orange peel using reflux using 1 N H_2SO_4, m_4 is the mass of dried orange peel after immersion using 72% H_2SO_4 and reflux using 1 N H_2SO_4 N, m_5 is the ash of orange peel. The lignocellulose content of orange peel was calculated on the first, third, fifth, and seventh days of fermentation.

2.7. Preparation of Deep Eutectic Solvent

Three different DES were prepared following the procedure described by Dai [32]. Choline chloride was used as a hydrogen bond acceptor (HBA), while glycerol, ethylene glycol, or lactic acid were used as hydrogen bond donors (HBD). The compounds were weighed using an analytical balance (New Classic MS, Mettler Toledo) with an accuracy of ± 0.0001 g according to the required molar ratio. Subsequently, HBA and HBD mixtures at appropriate ratios were stirred and heated at 50ºC until a homogeneous transparent liquid was formed. The complete list of DES abbreviations and organic solvents utilized in this study is provided in Table 1.

Table 1. Deep Eutectic Solvents and organic solvents used for orange peel extraction

Solvent	Molar ratio	Water content (%)	Abbreviation
Choline chloride: Glycerol	1:2	10	ChCl: Gly
Choline chloride: Lactic acid	1:1	10	ChCl: LA
Choline chloride: Ethylene glycol	1:2	10	ChCl: EG
Acetone	-	20	Acetone
Ethanol	-	20	Ethanol
Methanol	-	20	Methanol

2.8. Extraction of orange peel

10 grams of dried orange peel were placed inside an Erlenmeyer flask and added to each organic solvent and DES sample with a solid-liquid ratio of 1:10 (w/v). Extractions were conducted in three replicates at 60ºC with 400 rpm stirring speed for 2 hours using an incubator shaker. An ultrasound-assisted extraction at room temperature **(**27°C**)** for 30 minutes with a sample-solvent ratio of 1:20 (w/v) was carried out as an initial screening step to select the DES for further experimentation [27]. The empty flask was weighed using an analytical balance (m1) to calculate the yield extract. Subsequently, the organic solvents were evaporated using a rotary vacuum evaporator to obtain a crude extract. The flasks containing the orange peel extract were weighed to determine the orange peel extract (m_2). The yield of the orange peel extract was determined 3 times for each fermentation time. The yield of the orange peel extract was calculated using Eq. (6). Following that, the samples were filtered using Whatman No. 1 paper and stored at 4°C in dark tubes for further analysis.

$$Yield\ extract\ (\%) = \frac{m_2(g) - m_1(g)}{m_3(g)} \times 100 \quad (6)$$

2.9. Determination of hesperidin content

The hesperidin content in the orange peel extract was identified using high-performance liquid chromatography (HPLC). An Agilent 1100 LC system (Agilent, Santa Clara, CA, USA) equipped with a Zorbax SB-C18 reversed-phase column (150 × 4.6 mm, 5 µm) was used. The mobile phase consisted of isocratic elution with a low-pressure gradient using double-distilled water HPLC grade and methanol HPLC grade (65:35). The flow rate was set at 1.0 mL/min, and the column temperature was maintained at 40°C. The injection volume was 20 µl, and peaks were monitored at 280 nm. As much as 6 points dilutions of the standard solution were

made by diluting the hesperidin standard compound in HPLC methanol with a range of 2.5 – 100 ppm. Before any injection, both the standard and samples were filtered through a 0.22 µm membrane filter. To quantify hesperidin content, retention times of hesperidin were 98% as the standard was considered, and extracts were identified based on the retention times and order of elution with respect to standards [33].

2.10. Determination of Total Phenolic Content

The Folin-Ciocalteu method was used to calculate the total phenolic content of the extracts, and gallic acid was chosen as the reference standard for the calibration curve. Briefly, 100 mg of gallic acid was dissolved in 1 L of 80% ethanol before being diluted to produce solutions with concentrations of 2, 8, 20, 32, 64, and 90 ppm. After establishing the standard curve, the mixture containing 2.5 mL of 10% Folin-Ciocalteau reagent and 2.5 mL of 7.5% Na_2CO_3 was added to 0.5 mL of extracts. The mixture was then vortexed until uniform and incubated for 30 minutes in the dark. A UV/Vis spectroscopy with a 765 nm wavelength was used to measure the absorbance, and the results were represented as mg of gallic acid equivalent (GAE) per gram of orange peel [23].

2.11. Determination of Total Flavonoid Content

The total flavonoid content in this study was measured using the calorimetry test adapted from Chandra [27]. The quercetin solution was made by weighing 5 mg of quercetin that had been dissolved in 1 mL of ethanol (5000 ppm). With the quercetin solution diluted, concentrations of 5, 10, 15, 20, 25, 30, and 40 ppm were obtained. Approximately 2 mL of the dilution solution was then added to 2 mL of 2% $AlCl_3$ prior to incubation for 60 minutes at room temperature. The analysis was done using UV-Vis spectroscopy, with the wavelength used to measure absorbance 420 nm. Then, 2 mL of a 5000-ppm orange extract and 2 mL of 2% $AlCl_3$ were incubated at room temperature **(**27°C**)** for 60 minutes. The value of the total flavonoid content was calculated using the standard curve equation that had been obtained, and the results were expressed in mg QE/g extract [34].

2.12. Determination of antioxidant activity

The DPPH free radical scavenging capacity of the orange peel extract was determined as described by Molyneux [35]. A solution of 50 µM DPPH was prepared in ethanol. The mixture containing 1 mL of the diluted sample and 4 mL of 50 µM DPPH solution was then homogenized using a vortex and incubated for 30 minutes in a dark room. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 514 nm for each solvent extraction. The antioxidant activity was determined by Eq. (7).

$$Antioxidant\ activity = DPPH\ Scavenging\ activity\ (\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (7)$$

2.13. Statistical analysis

Statistical analysis carried out in this study included one-way ANOVA, post-hoc testing through Duncan's test, and correlation testing between research parameters through Pearson Coefficient Correlation and P-values using SPSS Statistics 26 software. The one-way ANOVA test ($P < 0.05$) aimed to determine the significance of the results obtained from the extraction of phenolic compounds, whereas post-hoc testing aimed to determine whether there were significant differences between data from one group to another. The correlation testing aimed to determine the level of closeness of the relationship between research parameters [30].

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3. RESULTS AND DISCUSSIONS

3.1. Proximate analysis of orange peel and growth of *A. niger*

The proximate analysis was carried out to determine the moisture, ash, protein, lipid, and carbohydrate contents of the orange peel, and the results are shown in Table 2.

Table 2. Moisture, ash, protein, lipid, and carbohydrate content of orange peel

Content	This study	References
Moisture (%)	84.63 ± 0.75	75 – 90 [10]
Ash (%)	2.23 ± 0.03	2 – 4 [31]
Protein (%)	6.27 ± 0.97	6 – 9 [31]
Lipid (%)	2.43 ± 0.15	2 – 6 [31]
Carbohydrate (%)	4.43 ± 0.43	7.13 [29]

The moisture, ash, protein, and lipid content of the orange peel investigated in the study are within the range of previous values reported by de la Torre et al. [36] and Bruinhorst et al. [37]. Slight variations in the carbohydrate content may be due to the differences in geographical locations and meteorological conditions [38].

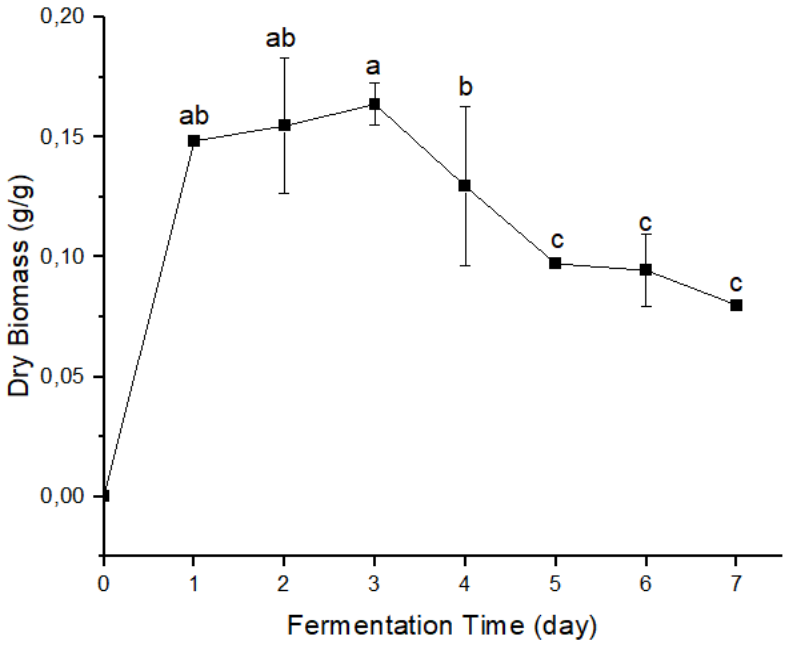

Figure 1. Growth curve of *A. niger* based on dry weight.

The specific growth rate of A. niger found in this study was 0.002 hour-1. From the growth curve of A. niger as shown in Figure 1, it can be observed that the growth increased considerably from the first day up to the third day of fermentation before then slowed down throughout the remaining fermentation period due to the decrease in nutrients supporting the growth of A. niger present in the fermentation medium [38]. These results are in line with the results obtained by Kuivanen et al. [39], which found that A. niger grew best on an orange peel substrate between 2 to 4 days.

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3.2. Effects of fermentation on biodegradation of lignocellulose

The lignocellulose content of the orange peel investigated in this study is shown in Table 3. A. niger is capable of decomposing cellulose and hemicellulose into saccharides to be used as an organic carbon source for metabolic growth [19]. Consequently, there is a clear relationship between the decrease in lignocellulosic content and the increase in fungal biomass. The weight of the dry biomass increased during the exponential phase while the lignocellulosic content sharply dropped. According to Augustine et al. [40], the fungus was particularly active in manufacturing its extracellular enzymes throughout the early to late exponential phases. This indicates that A. niger actively grew and produced many cellulolytic enzymes from day 1 to day 3 to digest complex polysaccharides like lignocellulose and use them as a source of organic carbon [41]. The percentage of degradation during the fermentation period is shown in Table 4 and Figure 2.

Table 3. Lignocellulose content of orange peel without fermentation treatment

Component	Content (%)
Hemicellulose	27.67 ± 0.84
Cellulose	11.43 ± 1.25
Lignin	7.19 ± 0.57

Table 4. Lignocellulose degradation of orange peel during fermentation

Fermentation time (day)	Percentage of degradation (%)		
	Hemicellulose	Cellulose	Lignin
1	28.17	3.76	16.79
3	40.34	5.09	35.97
5	49.53	9.94	11.23
7	62.56	46.63	39.97

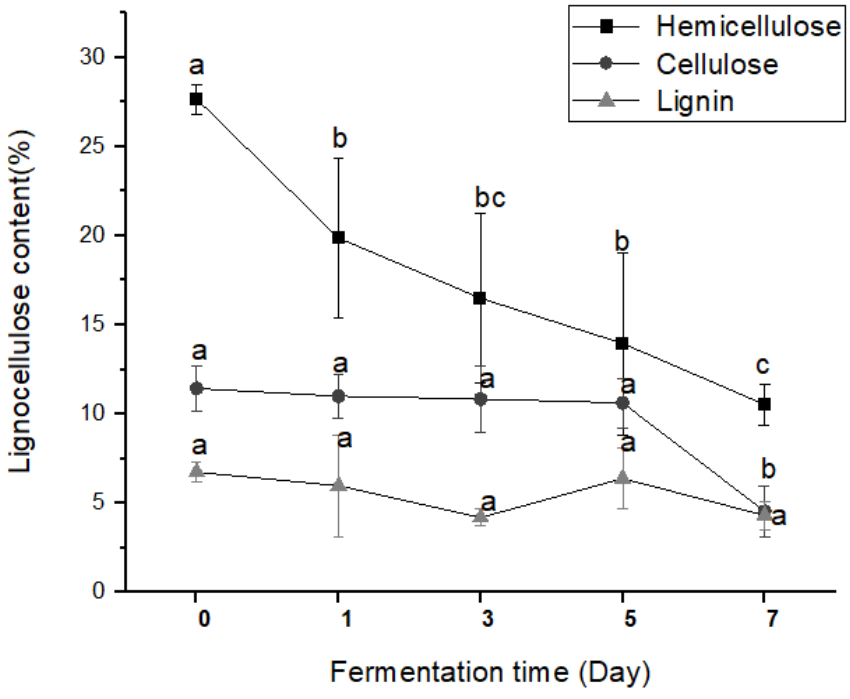

Figure 2. Lignocellulose content of orange peel on variations in fermentation time.

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Initially, the hemicellulose, cellulose, and lignin content were 27.67 ± 0.84, 11.43% ± 1.25, and 7.19% ± 1.25, respectively. All components exhibited a decreasing profile, and after 7 days of fermentation, the hemicellulose, cellulose, and lignin eventually dropped to 62.56%, 46.63%, and 39.97%, respectively. This may highlight that A. niger is able to degrade lignocellulose due to the presence of xylanase, cellulase, α-amylase, and β-glucosidase that can selectively and efficiently hydrolyze the lignocellulosic matrix in plant cell walls [42].

3.3. Effects of fermentation time and extraction solvent on the yield of orange peel extract

Unfermented (day 0) and fermented orange peel samples (1, 3, 5, and 7 days) were extracted using different organic solvents (80% ethanol, 80% methanol, and 80% acetone), and the results are shown in Figure 3. The yield of orange peel extract following A. niger fermentation tends to be higher than the yield of orange peel extracts without fermentation. For unfermented biomass, the highest yield (33.1%, dry weight) of orange peel extract was obtained when the extraction was carried out using 80% methanol, followed by 80% ethanol and 80% acetone. This value is higher than the orange peel extract of 27.3% for 30 minutes using alkaline hot water [43].

The maximum yield of 34.1% was achieved on the third day of fermentation using 80% methanol, while the lowest yield (17.1%, dry weight) was obtained when the extraction was carried out using 80% acetone. Santos da Silveira, et al. [44] assert that the conjugate bonds between phenolic chemicals and polysaccharides in plant cell walls can be broken by the cellulolytic enzyme complex produced by A. niger, and that this can boost the yield of phenolic extracts. As a result, phenolic compounds may become more soluble in the organic solvents used in the extraction process. The quantity of orange peel extract did, however, start to decline after the third day of fermentation. This is a result of A. niger biomass's diminished capacity to release enzymes because of its slower growth after the fourth day of fermentation.

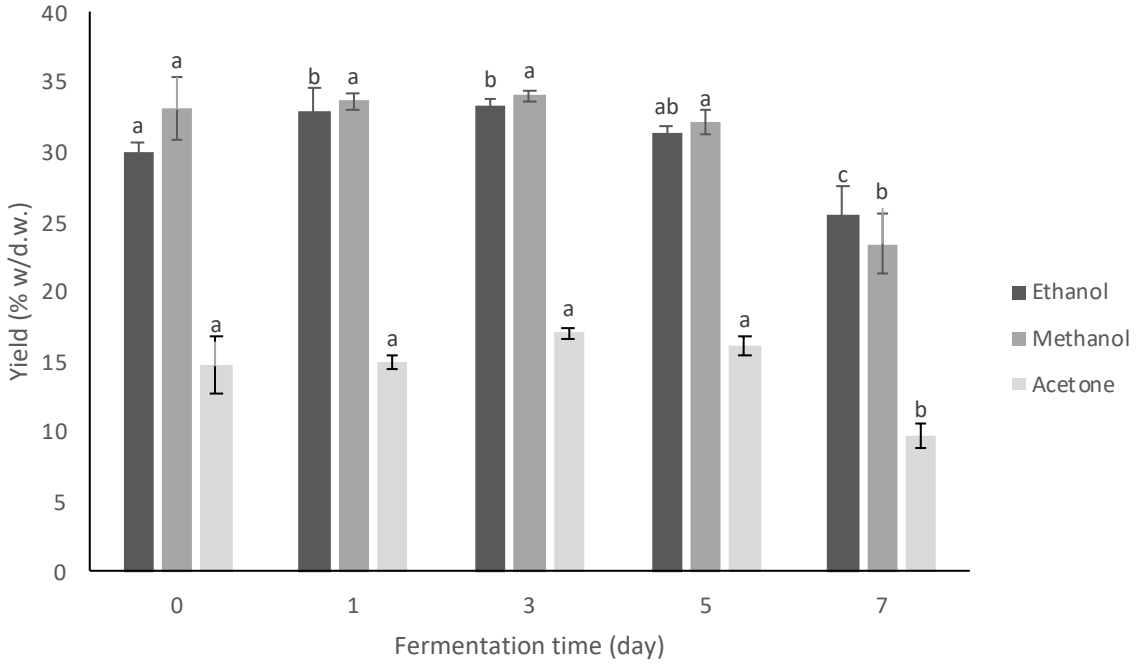

Figure 3. Yield of orange peel extract with variations of solvent ethanol 80%, methanol 80%, and acetone 80%.

The degree of polarity of the solvent plays an important role in determining the yield of orange peel extract. Since phenolic compounds are polar, they tend to dissolve in polar solvents

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[45]. At 20oC, methanol has a dielectric constant of 32.35, and ethanol has a dielectric constant of 25.00; both values are larger than the dielectric constant of acetone, which is 19.56 [46]. Accordingly, methanol is more polar than ethanol and acetone. The gain will rise in proportion to the solvent's polarity level. Because methanol has greater polar characteristics than ethanol and acetone, the yield of orange peel extract extracted using methanol solvent is higher.

3.4. Effects of fermentation time and extraction solvent on hesperidin content of orange peel extract

Figure 4 illustrates that the hesperidin content reached its highest point after three days of fermentation. The obtained value of the extraction yield, which peaks on the third day of fermentation, is in line with this result. These findings indicate that pre-treating orange peel extract with solid-state fermentation increases the amount of hesperidin by 1.67 times. These findings align with [47], who obtained hesperidin compound in the Geotrichum candidum fermented extract by 1.96 times greater than without fermentation when extracting flavonoids from orange peel. Meanwhile, the decrease in hesperidin content after the third to seventh day of fermentation may be caused by the conversion of hesperidin to hesperetin after the third day of fermentation [48]. Lee et al. [49] reported that Aspergillus sp. produces mannosidase, which releases the rhamnose sugar group from hesperidin and converts it into hesperetin-7-O-glucosidase which is then converted to hesperidin by the enzyme β-glucosidase.

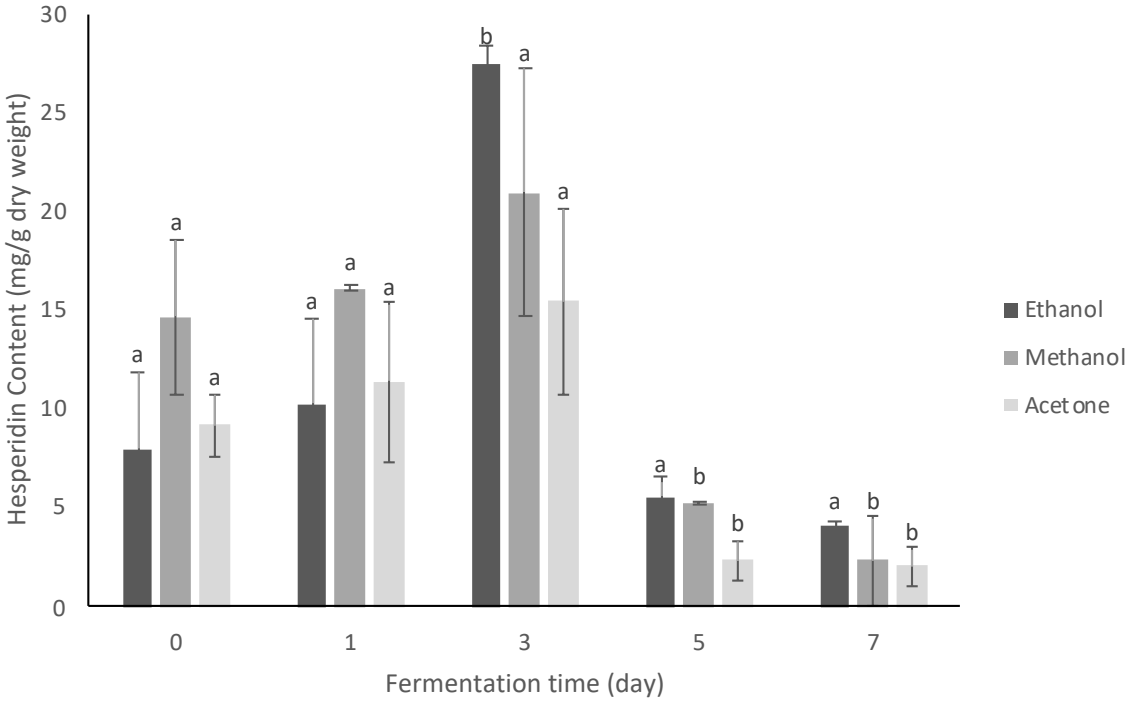

Figure 4. Hesperidin content in C. reticulata peel extract using ethanol 80%, methanol 80%, and acetone 80%.

Compared to methanol and acetone, the hesperidin content with ethanol as an extraction solvent has the highest value. This result is confirmed by Kim and Lim [50], which demonstrated that orange pomace (Citrus unshiu) extracted with ethanol had the highest hesperidin level, followed by acetone and methanol. In contrast, Samota, et al. [51] found that hesperidin content in orange peel extract (C. reticulata) using methanol as the solvent is higher than when ethanol was used. Different orange varieties, solvent concentrations, and extraction parameters such as temperature and time can all contribute to different results. In general,

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ethanol is a suitable extraction solvent for flavonoids and their glycosides, catechol, and tannins; methanol for phenolic acids and catechins; and acetone for high molecular weight polyphenols such as proanthocyanidins and tannins.

This study also carried out preliminary studies on utilizing deep eutectic solvents as a sustainable alternative to organic solvents. The initial step involved screening for glycerol, lactic acid, and ethylene glycol as three different sources of hydrogen bond donors of DES. In this screening, fermented orange peel from day 3 of fermentation was used for the DES extraction as the best result. Figure 5 depicts the results of the hesperidin content analysis.

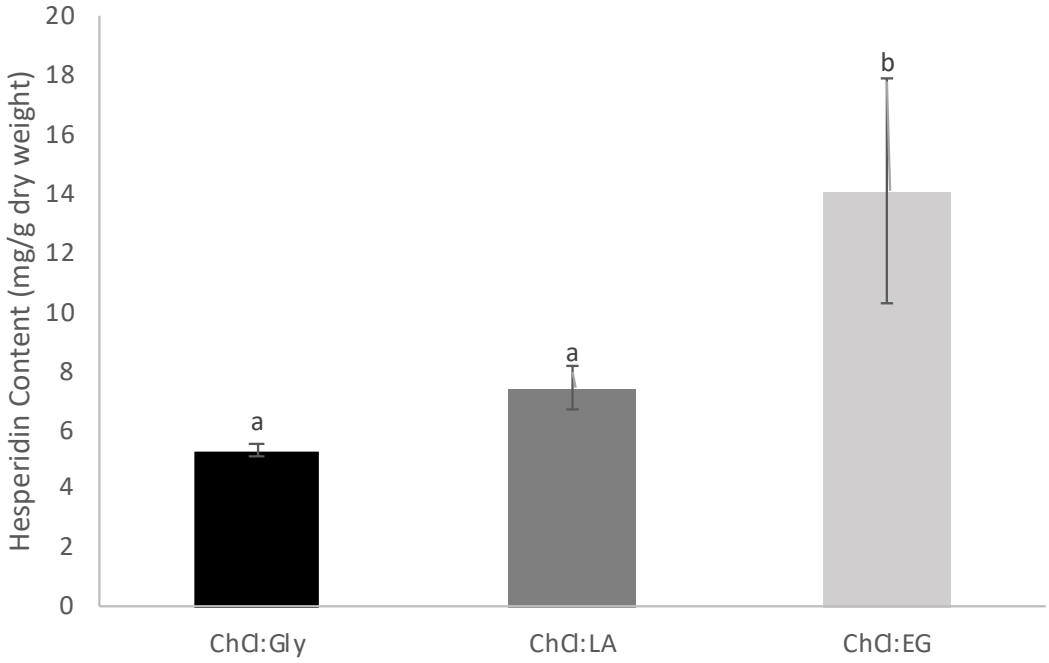

Figure 5. Screening of deep eutectic solvent for extraction of hesperidin from C. reticulata.

The DES produced the highest amount of hesperidin consisting of choline chloride and ethylene glycol, at 14.08 mg/g of dried fermented orange peel. As ChCl was used as HBA in all DES systems, the ultimate physicochemical properties of the used DES depend on the chemical structures of the HBD. In this study, the viscosity of ChCl: Gly, ChCl: LA, and ChCl: EG at 30ºC were 188 mPa.s, 508.17 mPa.s, and 35 mPa.s, respectively [27, 52, 53]. DES consists of ethylene glycol, creating a solvent with lower viscosity than lactic acid and glycerol as hydrogen bond donors. Low-viscosity DES is regarded as an effective extractants because low viscosities can facilitate the mass transfer of target chemicals to the DES solution [54]. These findings align with the previous results obtained by Xu et al. [27] all three types of DES followed the same trend when extracting freeze-dried orange peel. Hence, DES, which consists of choline chloride and ethylene glycol, was selected and further used to compare its performance with previously used organic solvents.

The extraction of hesperidin was conducted at 60°C for 120 minutes using an organic solvent. 80% ethanol extracted 27.47 mg/g of hesperidin under these conditions. In contrast, ChCl: EG could only extract 12.5 mg/g of the sample under the same conditions. However, using DES at a higher temperature of 70, the hesperidin yield continued to climb, reaching 17.6 mg/g, as indicated in Figure 6. A previous study [55] showed the potential of choline chloride-based DES utilization, which proved more effective than ethanol in extracting polyphenols

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from orange waste. Therefore, further optimization studies are necessary to explore the capacity of ChCl further: EG to extract hesperidin

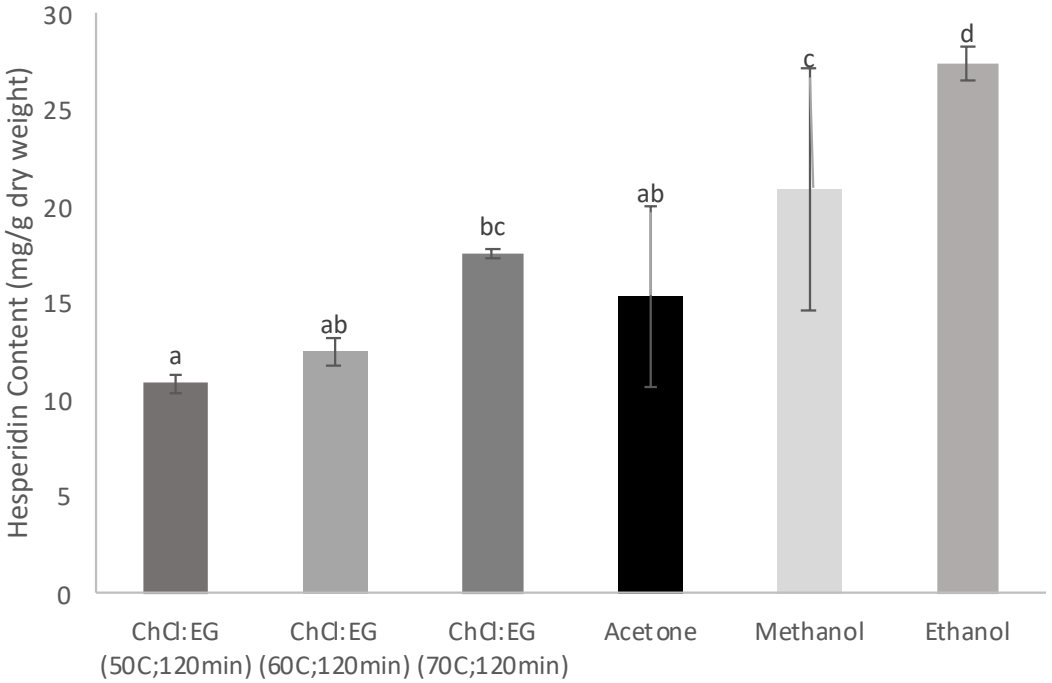

Figure 6. Comparison of hesperidin content using DES and organic solvents after three days of fermentation

3.5. Effects of fermentation time and extraction solvent on total phenolic and flavonoid content of orange peel extract

Figure 7 illustrates the effects of fermentation time and extracting solvent on the orange peel extract's total phenolic content and flavonoid content. The orange peel extract with 80% methanol, 80% ethanol, and 80% acetone for unfermented samples had a total phenolic content of 25.61 ± 1.77 mg GAE/g extract, 20.11 ± 2.04 mg GAE/g extract, and 38.23 ± 3.49 mg GAE/g and total flavonoid content of 3.63 ± 0.124 mg QE/g extract, 3.78 ± 0.22 mg QE/g extract, and 6.1 ± 0.97 mg QE/g extract, respectively. The trend of total phenol content during the fermentation period is strongly associated with the total flavonoid content, as shown in Figures 7(a) and 7(b). The overall concentration of flavonoids and phenols increased from the first to the third day of fermentation, then decreased from the fourth to the seventh day. The total phenol concentration and the total flavonoid content were highest on the third day of fermentation. Table 5 displays each solvent's TPC and TFC values, including the DES.

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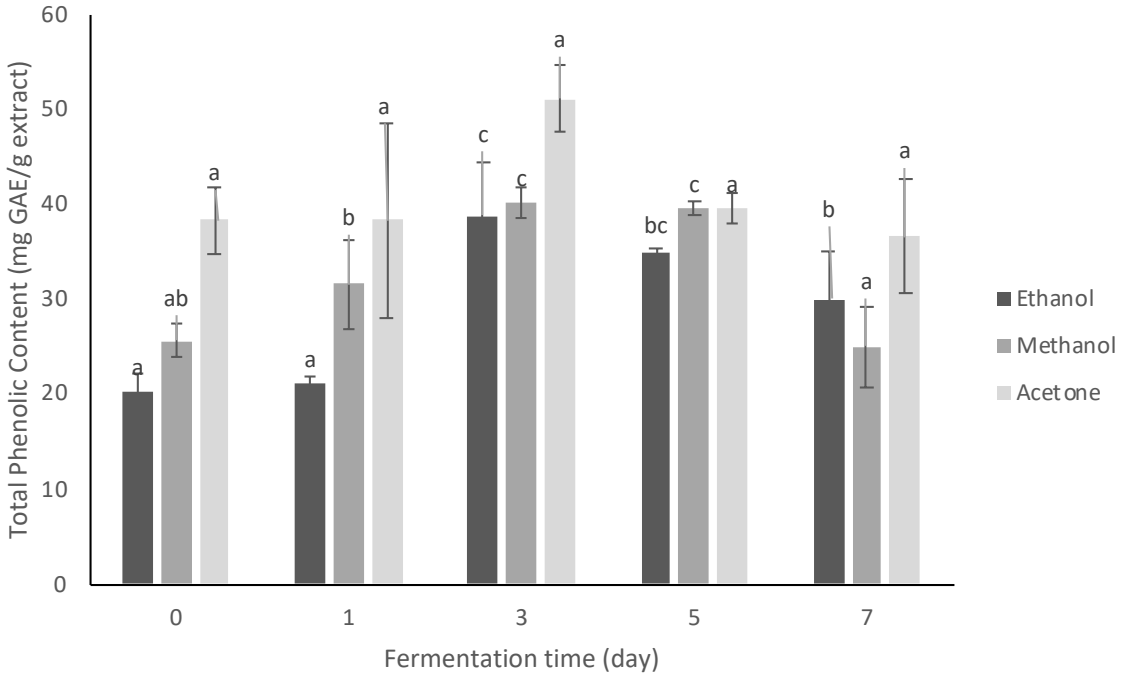
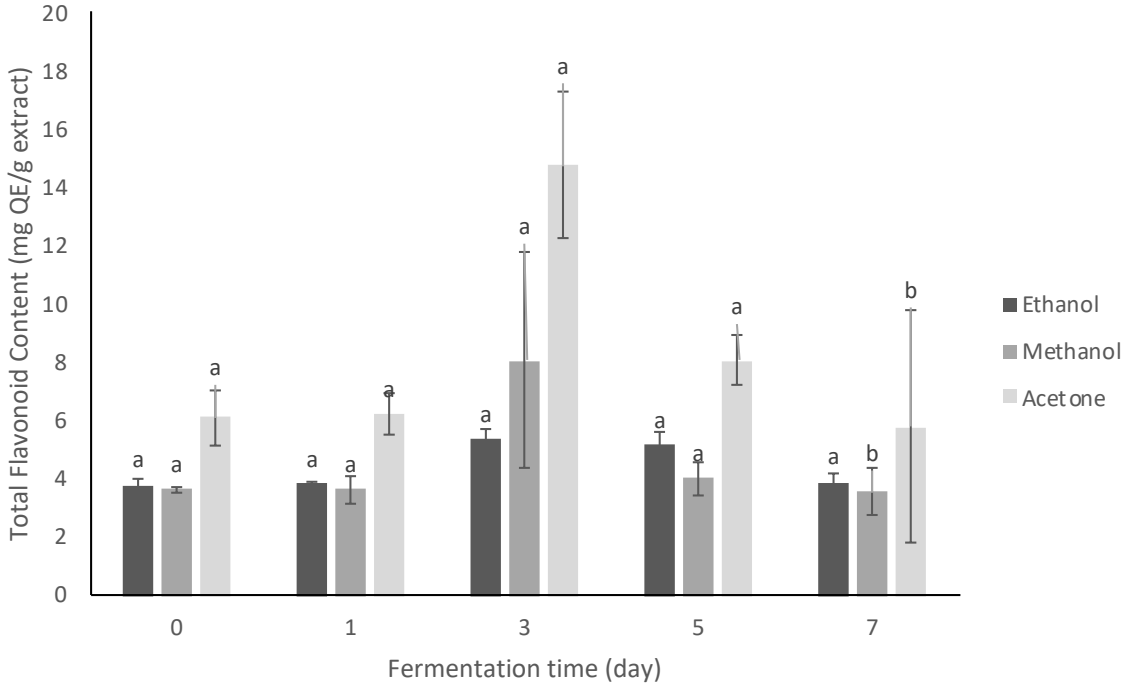


(a)



(b)

Figure 7. Total phenolic content (a) and total flavonoid content (b) of C. reticulata peel extract using different solvents.

Most phenols are in the conjugated form in plants, where one or more sugar residues are joined to hydroxyl groups. The amount of free phenolic in the orange peel, which was utilized as a substrate in the fermentation process, could be increased through the enzymatic hydrolysis of phenolic conjugated with enzymes that break down carbohydrates generated by filamentous fungal strains during solid-state fermentation. Cellulolytic enzymes that may break down cell

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walls containing recalcitrant such as lignocellulose are naturally produced by A. niger [56]. As a result, more phenolic compounds may be mobilized for solvent extraction, and more antioxidant compounds may be synthesized. The exponential phase was demonstrated in this study from day zero to its peak, the third day of fermentation. The fungus actively produces its extracellular enzymes and reproduces throughout the exponential phase. The fungal biomass grew at its maximum rate on the third day of fermentation, indicating that this is also the day when the fungi are actively establishing extracellular enzymes. The ability of β-glucosidase enzyme produced by Aspergillus sp. to hydrolyze phenolic glycosides and release free phenolic compounds can improve the extraction of phenolic compounds [57].

From the fourth to the seventh day of fermentation, the total phenolic and flavonoid content decreased, similar to that observed in a study by Torres-León, et al. [58] in which they used A. oryzae to brew barley for seven days. Due to the exponential phase of the fermentation process, the overall phenolic and flavonoid concentration may decrease. Following the exponential phase, the *A.niger* started to exhibit a decline in self-replication activity and a reduction in the synthesis of the enzyme β-glucosidase, which slowed the process of hydrolyzing phenolic glycosides to liberate phenolic compounds. The findings of this study are consistent with those of a study by Gulo, et al. [59], which found that orange peel extract contains more total flavonoids and phenolics in acetone than in ethanol or methanol. This demonstrates that acetone solvents are more effective than ethanol and methanol for extracting phenolic compounds. However, less polar aglycones such as isoflavones, flavonones, flavones, and flavonols tend to be more soluble in semi-polar solvents [60], while flavonoids that are attached to sugar prefer to dissolve in polar solvents.

Table 5. Total phenolic and flavonoid content on the third day of fermentation

Solvent	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
Ethanol	38.66	5.34
Methanol	40.02	8.08
Acetone	51.01	14.75
ChCl: EG	25.79	3.40

3.6. Effects of fermentation time and extraction solvent on antioxidant activity of orange peel extract

In this study, the antioxidant activity in orange peel extract samples was determined by determining the percentage of DPPH inhibition in the sample to determine the IC50 value. The IC50 value can indicate the sample concentration required to counteract 50% of the free radical content [61]. Figure 8 illustrates the effects of fermentation time and extracting solvent on the antioxidant activity of the orange peel extract. Without being given pre-treatment, the antioxidant activities of the orange peel extract using 80% ethanol, 80% methanol, and 80% acetone were 508.52, 828.73 ppm, and 371.41 ppm, respectively. The IC50 value decreased on the first and third day of fermentation and then increased on the fifth and seventh day. The lower the IC50 value, the lower the sample concentration needed to inhibit as much as 50% of the percent DPPH inhibition. The lower the IC50 value in an extract sample, the better the antioxidant activity of the sample because the dose or concentration required to inhibit 50% of the percent DPPH inhibition is smaller [62]. This trend shows consistency between antioxidant activity and total flavonoid, phenolic, and hesperidin content [57].

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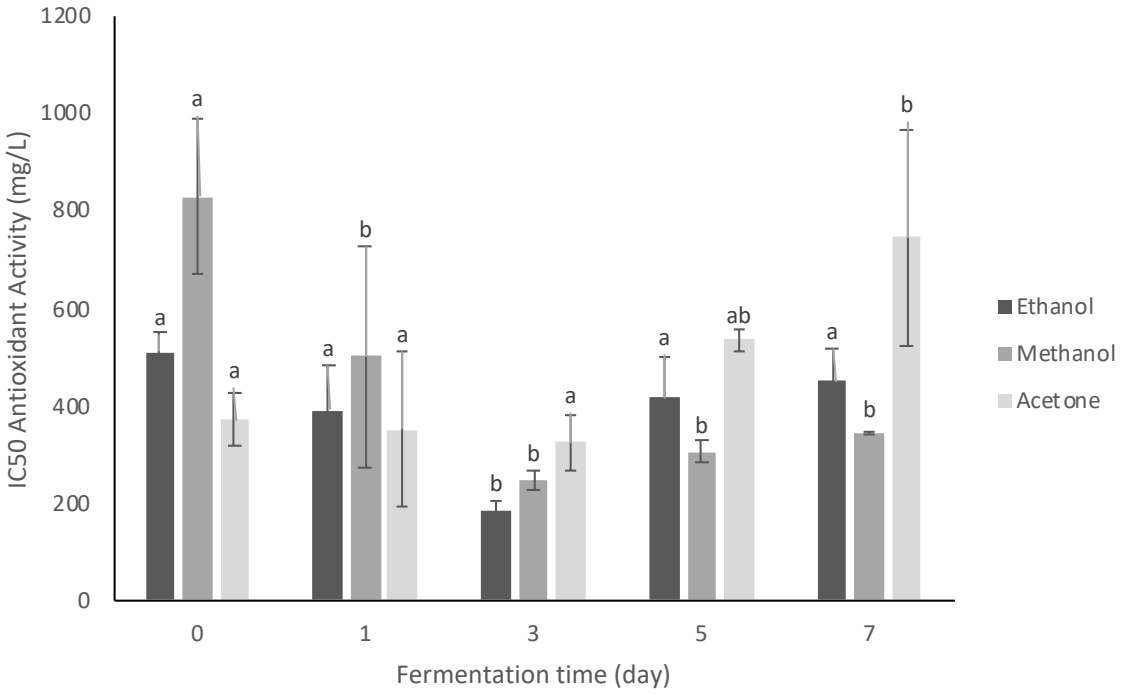

Figure 8. Antioxidant activity of C. reticulata peel extract using different solvents.

Phenolic compounds found in orange peel extract can act as antioxidant compounds so that they can increase the antioxidant activity of the extract [63] due to the presence of aromatic rings in the phenolic compound containing one or two hydroxyl groups, which can donate their hydrogen atoms to free radicals so that the product formation is more stable [64]. However, on the fifth and seventh days of fermentation, the IC50 value of the orange peel extract indicated that its antioxidant activity decreased. This was due to the decrease in extract yield on those days, so the phenolic compounds released were also reduced, thereby reducing the antioxidant activity produced.

Table 6 demonstrates that the orange peel extract utilizing 80% ethanol had the lowest value of IC50 among all the solvents, indicating that the antioxidant activity of the extract is stronger than that of the other solvents. This aligns with the research of Truong, et al. [65], where the ethanol and methanol extracts of S. buxifolia twigs exhibited a stronger antioxidant activity than acetone extracts. Flavonoids have the potential as antioxidants. However, the flavonoids in the acetone extract may be glycosides, making them less effective as antioxidant agents compared to their aglycone forms. This results in the antioxidant activity of the acetone extract being weaker than that of the ethanol extract and methanol extract.

Table 6. Antioxidant activity of orange peel extract on the third day of fermentation

Solvent	Antioxidant activity (IC_{50}, ppm)
Ethanol	184.32
Methanol	247.24
Acetone	323.95
ChCl: EG	269.30

4. CONCLUSION

The extraction of phenolic compounds using various solvent types was evaluated to extract phenolic compounds from orange peel waste. Hemicellulose, cellulose, and lignin content in the orange peel decreased due to the solid-state fermentation using A. niger. The yield of orange peel extract increased as the lignocellulose level decreased. The third day of fermentation with 80% methanol resulted in the highest yield of orange peel extract (34.1%). The total phenolic content, total flavonoid content, and antioxidant activity of the extract all increased due to the solid-state fermentation of orange peel using A. niger. The third day of fermentation led to the highest hesperidin content of orange peel extract, 27.47 mg/g dried fermented orange peel using 80% ethanol. In this study, hesperidin extraction was also performed using DES as a more sustainable alternative for organic solvents. The experiment showed that HBD in DES and ethylene glycol may extract hesperidin as effectively as organic solvents. A further study to explore the capacity of ChCl: EG as a green solvent for hesperidin extraction should also be investigated.

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