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Extraction of Flavonoid from Rice Straw: The Study of Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Antibacterial Activity

Siti Lestari[1a]*, Tri Sunaryo[2a] and Rizky Ibnufaatih Arvianto[3b]

Abstract: The combustion of rice straw results in air pollution and a decrease in soil fertility. This research aims to extract flavonoids from rice straw and evaluate their antibacterial properties. The extraction of flavonoids from rice straw was conducted using Soxhlet, reflux, and maceration methods. These extraction techniques were subsequently compared based on the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). The optimal extraction method for rice straw involved ethanol solvents with concentrations of 0%, 25%, 70%, and 96%. This same optimal method was used to extract rice straw at various time intervals, specifically 12 hours, one day, and three days. A comparative analysis of ethanol concentration and extraction duration was performed based on the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). The rice straw extract underwent the disc diffusion method for antibacterial tests against gram-positive (Staphylococcus aureus) and gram-negative (Escherichia coli) bacteria. The antibacterial efficacy of rice straw extract was evaluated based on its ability to inhibit the growth of these microorganisms. The maceration method proved to be the most effective for extracting rice straw due to its significantly greater total flavonoid content (TFC) value compared to the reflux and Soxhlet extraction methods. Increasing the ethanol concentration from 0% to 96% and extending the extraction time from 12 hours to 3 days led to a proportional increase in total phenolic content (TPC) and TFC. Ethanol at a concentration of 96% was the most appropriate solvent for extracting rice straw due to its highest total flavonoid content (TFC) value relative to other ethanol concentrations. A three-day extraction period was optimal, yielding a significantly higher TFC value than 12-hour and one-day extractions. Furthermore, it is noteworthy that rice straw extract exhibits greater inhibition of gram-negative bacteria (Escherichia coli) than gram-positive bacteria (Staphylococcus aureus). The antibacterial activity of rice straw extract correlated with the flavonoid content in the sample.

Keywords: *Rice straw, phenolic, flavonoid, anti-bacterial.*

1. Introduction

Indonesia is an agrarian nation where various agricultural products, including rice, cassava, and corn, are cultivated. Indonesia is the world's third-largest rice producer, behind China and India (Indonesian Ministry of Home Affairs, 2004). The elevated rice production levels result in a proportional increase in waste generation. In 2021, rice production was estimated to have reached 55.27 million tons of GKG, marking a rise of 620.42 thousand tons or 1.14 percent compared to the 2020 rice production figure, which stood at 54.65 million tons of GKG (BPS-Indonesian Statistics, 2021). The rise in rice production results in a growth in agricultural waste, notably straw. Generally, this waste is collected and subsequently burned, thereby contributing to air pollution through the emission of several hazardous gases, including sulfur dioxide (SO_2), carbon monoxide (CO), volatile organic compounds (VOC), polycyclic aromatic hydrocarbons (PAHs), as well as various greenhouse gases, such as methane (CH_4), nitrous oxide (N_2O), and carbon dioxide (CO_2) (Gadde et al., 2009; Romasanta et al., 2017). The increase in greenhouse gases leads to global warming and Earth's climate change (Gadde et al., 2009). Burning straw also results in a decline in soil quality characterized by the loss of essential nutrients and organic matter, the depletion of soil-fertilizing bacteria, and an increased vulnerability to soil erosion (Kumar et al., 2019). Rice straw waste must be used carefully to prevent further pollution.

Rice straw contains secondary metabolites in the form of phenolic compounds, particularly flavonoids (Elzaawely et al., 2017; Karimi et al., 2014). These phenolic compounds show potent antibacterial activity. The extract from *Crataegus azarolus L. var. Aronia callus* contained high levels of flavonoids. These compounds showed antibacterial activity against both gram-positive and gram-negative bacteria, including *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (NCIMB 8853), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853), *Micrococcus luteus* (NCIMB 8166), and *Salmonella typhimurium* (Bahri-Sahloul et al., 2014). Bergamot seed extract (Citrus bergamia Risso) contains flavonoids that give it antibacterial capabilities against gram-positive bacteria, including Listeria innocua, Bacillus subtilis, Staphylococcus aureus, and Lactococcus lactis (Mandalari et al., 2007). Mandarin orange seed extract (Citrus reticulata Blanco) contains the highest concentration of flavonoids. These compounds show antibacterial activity against *Bacillus subtilis* and *Klebsiella pneumoniae* (Balaky et al., 2020). Numerous previous studies have demonstrated the strong antibacterial properties of flavonoid compounds.

The flavonoid extraction process is influenced by several factors.

Authors information:
[a]Nursing Department, Politeknik Kesehatan Kementrian Kesehatan Surakarta, Jl. Letjen Sutoyo Mojosongo Jebres Surakarta 57127 INDONESIA. E-mail: lestaristi68@gmail.com[1]; tri_sunaryo@yahoo.com[2]
[b]Petrochemical Industry Technology Process Study Program, Petrochemical Industry Politechnic Banten, Jalan Raya Karang Bolong, Cikoneng, Anyar, Kab. Serang, Banten 42166, INDONESIA. E-mail: rizky.ibnufaatih@poltek-petrokimia.ac.id[3]
*Corresponding Author: lestaristi68@gmail.com

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Extraction methods

Flavonoid extraction methods can be conducted through reflux, Soxhlet, or maceration. Reflux involves extraction with a solvent at its boiling point for a specified time, maintaining a constant solvent volume due to the presence of a condenser (Chua et al., 2016). Soxhlet extraction shows similarities to reflux. A Soxhlet flask is placed between the condenser and the round-bottom flask, acting as a solid sample holder. This extraction method is used for non heat-resistant samples, preventing direct contact between the solid sample and boiling solvent during extraction (Redfern et al., 2014). Meanwhile, maceration involves immersing the material in a solvent without or with minimal heating (Rabiu & Haque, 2017; Tambun et al., 2021).

Solvent Concentration

Solvent concentration is related to solvent polarity, and flavonoids should exhibit similar polarity to the solvent (Yusof et al., 2020). Furthermore, concentration reflects the quantity of solvent molecules, and an increase in the number of particles results in a higher frequency of collisions during the extraction process (Cui et al., 2021).

Extraction Time

Extraction time is correlated with dwelling time, as a longer extraction time leads to a prolonged period of solvent contact with the sample during extraction (Dewi et al., 2019).

The total phenolic content (TPC) analysis in samples was performed using the Folin-Ciocalteu method. Phenolic compounds react with the Folin-Ciocalteu reagent under alkaline conditions to produce a blue complex compound (Blainski et al., 2013). Meanwhile, the colorimetric method was employed to analyze the total flavonoid content (TFC) of the sample (Martono et al., 2019; Shraim et al., 2021). This method used aluminum chloride (AlCl3), which forms stable complex compounds with flavonoid compounds. AlCl3 creates a stable complex compound by interacting with the ketone group at C4 and the OH group at C3 or C5 (Martono et al., 2019). AlCl3 also forms an acid-labile complex with the orthodihydroxyl group on the flavonoid ring (Martono et al., 2019). This complex compound causes a shift in wavelength toward visible light so that the solution changes color to yellow (Fadillah et al., 2007; Martono et al., 2019). The absorbance of the color changes in the sample can be measured using a UV-visible spectrophotometer at specific wavelengths. This absorbance value is directly proportional to the total phenolic and flavonoid content in the sample (Mekonnen & Desta, 2021; Zhu et al., 2010).

The present study focused on isolating flavonoids from rice straw using the procedures of reflux, Soxhlet, or maceration. The optimal flavonoid extraction method was determined based on the Total Flavonoid Content (TFC) and Total Phenolic Content (TPC). The solvent concentration and extraction duration influence on TPC and TFC was assessed using the selected extraction method. Subsequently, using the disc diffusion method, rice straw extracts with varying solvent concentrations were assessed for their antibacterial activity against Staphylococcus aureus (gram-positive) and *Escherichia coli* (gram-negative). The antibacterial efficacy of the rice straw extract was evaluated based on the clear zone diameter against these bacteria.

2. Materials and Methods

Material

In this study, various materials were employed, such as 96% ethanol, distilled water, sodium bicarbonate, gallic acid, rice straw, glass wool, Whatman paper, *Staphylococcus aureus* and *Escherichia coli cultures*, Muller-Hinton medium, Nutrient Agar, and Nutrient Broth.

Methods

Straw Powder Production

A kilogram of rice straw underwent an 8-hour drying procedure conducted in direct sunlight. The drying process involved enveloping the rice straw with a black mesh. After a drying duration of 8 hours, the straw was sliced into small pieces.

Total Flavonoid Content (TFC) Analysis

Determination of Standard Curve

The samples' flavonoid content was quantified using quercetin as the standard. A mixture was prepared by combining 10.0 mg of quercetin, 0.3 mL of a 5% sodium nitrite solution, 0.6 mL of a 10% aluminum chloride solution, and 2 mL of a 1 M sodium hydroxide solution in a glass beaker. The resulting quercetin solution was diluted with distilled water to a final volume of 10 mL using a volumetric flask. The quercetin stock solution, obtained at a concentration of 1000 ppm, was diluted to concentrations of 0.5 ppm, 1 ppm, 2 ppm, 5 ppm, 10 ppm, 25 ppm, 50 ppm, 75 ppm, and 100 ppm, as shown in Table 1. Subsequently, the absorbance of each solution was measured using a UV-Vis spectrophotometer at a wavelength of 510 nm. The linearity equation was obtained from a standard curve plotting absorbance versus concentration. This equation was used to determine the flavonoid content in the extract samples.

Table 1. Dilution of quercetin stock solution (1000 ppm)

Concentration (ppm)	0.5	1	2	5	10	25	50	75	100
Stock solution (mL)	0.005	0.010	0.020	0.050	0.100	0.250	0.500	0.750	1.000
Aquades (mL)	9.995	9.990	9.980	9.950	9.900	9.750	9.500	9.250	9.000
Final volume (mL)	10	10	10	10	10	10	10	10	10

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TFC Determination of Rice Straw Extract

The colorimetric assay measured the total flavonoid content (Shraim et al., 2021). Two milliliters of a sodium hydroxide solution (1 M) and two milliliters of a 10% aluminum chloride solution were added to 0.2 milliliters of rice straw extract and 0.3 milliliters of 5% sodium nitrite. The extract solution was then diluted using a measuring flask until it reached a final volume of 10 mL. Subsequently, a UV-Vis spectrophotometer was used to measure the extract solution's absorbance at a wavelength of 510 nm. Using a linear equation, we determined the total flavonoid concentration of the rice straw extract.

Total Phenolic Content (TPC) Analysis

Determination of Standard Curve

A glass beaker combined 10 mg of gallic acid, 0.5 mL of Folin-Ciocalteu reagent, 1.5 mg of 20% sodium carbonate, and 7.5 mL of distilled water (aquabides). The resulting gallic acid solution was heated in a water bath at 40 °C for 20 minutes and rapidly cooled on ice. The original stock solution of gallic acid (1000 ppm) was subsequently diluted to various concentrations ranging from 0.5 ppm to 100 ppm. The absorbance of each of these dilutions was measured using a UV-Vis spectrophotometer, with readings taken at a wavelength of 760 nm. A standard curve established the linearity equation by plotting absorbance against concentration. This linear equation provides an essential basis for calculating the phenolic content in the extract samples.

Table 2. Dilution of gallic acid stock solution (1000 ppm)

Concentration (ppm)	0.5	1	2	5	10	25	50	75	100
Stock solution (mL)	0.005	0.010	0.020	0.050	0.100	0.250	0.500	0.750	1.000
Aquades (mL)	9.995	9.990	9.980	9.950	9.900	9.750	9.500	9.250	9.000
Final volume (mL)	10	10	10	10	10	10	10	10	10

TPC Determination of Rice Straw Extract

In a glass beaker, 0.2 mL of rice straw extract, 0.5 mL of Folin-Ciocalteu reagent, 1.5 mL of 20% sodium carbonate, and 7.5 mL of distilled water (aquabides) were combined. The extract solution was adjusted to a final volume of 10 mL using a volumetric flask. Subsequently, the absorbance of the extract solution was measured using a UV-Vis spectrophotometer at a wavelength of 760 nm. The total flavonoid content within the extract solution was determined employing the linearity equation.

Effect of Extraction Method on TPC and TFC

Reflux Extraction

In total, 50 grams of rice straw were placed into a round-bottom flask. 300 mL of 70% ethanol was added to the round-bottom flask. The rice straw underwent reflux for a duration of 2 hours at 70℃. Following this step, the filtrate and residue were separated using a Buchner vacuum filter. The filtrate was evaporated using a distillator to remove the ethanol solvent. The resulting rice straw extract was then analyzed for TFC (Total Flavonoid Content) and TPC (Total Phenolic Content).

Soxhlet Extraction

A Soxhlet flask was filled with fifty grams of rice straw. The round-bottom vial was subsequently filled with 300 mL of 70% ethanol. The Soxhlet extraction was conducted for a period of two hours. The sample was then filtered using a Buchner vacuum filter. The ethanol solvent was removed by evaporating the resulting filtrate with a distillator. Ultimately, the rice straw extract was analyzed for TFC (Total Flavonoid Content) and TPC (Total Phenolic Content).

Maceration

For 15 hours at room temperature, fifty grams of rice straw were submerged in 300 mL of 70% ethanol. The filtrate and residue were separated using a Buchner vacuum filter after 24 hours. The ethanol solvent was subsequently removed from the filtrate by evaporating it with a distillator. Ultimately, the rice straw extract was analyzed for TFC (Total Flavonoid Content) and TPC (Total Phenolic Content).

Effect of Ethanol Concentration and Extraction Time on TPC and TFC

The extraction procedure was carried out according to the optimum method specified in section 2.2.4 of the paper. Ethanol concentrations were adjusted to 0%, 25%, 70%, and 96%. The extraction times were set to durations of 12 hours, one day, and three days. An investigation was conducted on each rice straw extract, which varied in ethanol concentrations and extraction times, to determine its Total Flavonoid Content (TFC) and Total Phenolic Content (TPC).

Anti-bacterial Activity Test

Testing was performed to evaluate the antibacterial activity of the most effective rice straw extraction technique, as outlined in section 2.2.4. Antibacterial tests employed *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive) bacterial strains. A single dose of culture from Nutrient Agar (NA) was suspended in Nutrient Broth (NB) medium and incubated for 24 hours. In a petri dish, 1 mL of Nutrient Broth (NB) culture was mixed with 9 mL of Mueller Hinton (MH) medium. The mixture was agitated and left undisturbed briefly to allow solidification. Paper discs soaked in 1000 mg of chloroform were placed evenly within the solidified medium. The plate was incubated at 37 ℃ for 24 hours, and measurements were taken by assessing the Inhibitory Zone Diameter.

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3. Result and Discussion

Total Phenolic Content (TPC) Analysis

The standard curve for total phenolic analysis is depicted in Figure 1. The graph demonstrates that the concentration of gallic acid is directly proportional to the absorbance (Mayerhöfer & Popp, 2019). The structure of the linear equation is as follows: y = 0.0081x + 0.0005 (where "x" and "y" represent the phenolic concentration and absorbance, respectively). Therefore, the phenolic concentration in straw extract can be calculated using equation (1).

$$\text{Concentration} = \frac{\text{Absorbance}}{0{,}0081} - 0{,}0005 \qquad (1)$$

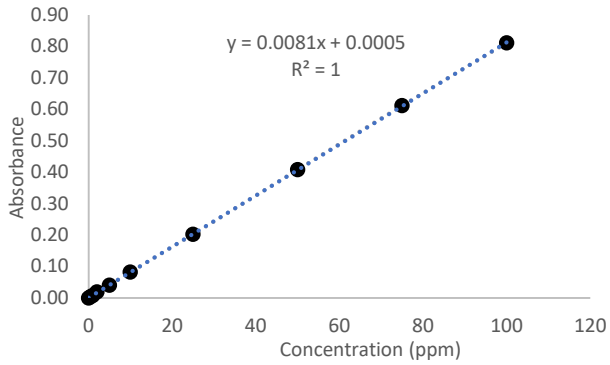

Figure 1. Standard Curve of TPC Analysis

Phenolics were compounds characterized by aromatic rings that could bind one or more hydroxyl groups (Tsao, 2010; Vuolo et al., 2019). Numerous studies have demonstrated that gallic acid is the reference for quantifying total phenolics in various samples (Blainski et al., 2013; Elzaawely et al., 2017; Karimi et al., 2014). When gallic acid and Folin-Ciocalteu reagent were combined in an alkaline environment, they formed a blue complex compound (Martono et al., 2019). The addition of Na_2CO_3 into the sample created an alkaline environment. Na_2CO_3 enabled the dissociation of protons in gallic acid, forming gallic acid phenolate ions (Karimi et al., 2014; Martono et al., 2019). Subsequently, the hydroxyl group within the gallic acid phenolate ions reacted with the Folin-Ciocalteu reagent, resulting in the formation of a blue molybdenum-tungsten complex (Abdelkader et al., 2014; Agbor et al., 2014; Martono et al., 2019). The gallic acid phenolate ion acted as a reducing agent in this reaction, converting Mo^{6+} to Mo^{5+}. Simultaneously, molybdenum oxidizes the hydroxyl group within the gallic acid phenolate ion, transforming it into a ketone group (Shi et al., 2022). The process for the formation of the molybdenum-tungsten complex was illustrated in Figure 2.

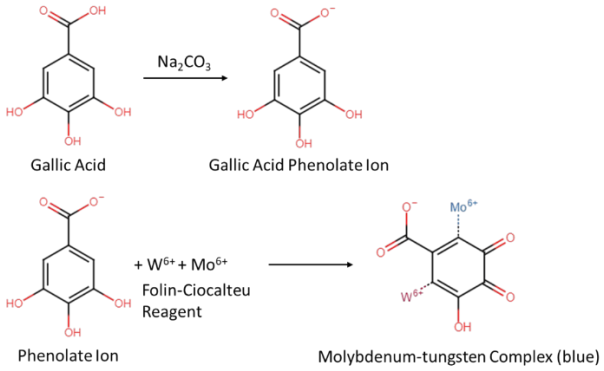

Figure 2. Formation of molybdenum-tungsten complex (Martono et al., 2019; Shi et al., 2022).

The absorbance of the blue molybdenum-tungsten complex was measured using a UV-visible spectrophotometer (Zhu et al., 2010). The absorbance is directly proportional to the concentration of the substance (Mayerhöfer & Popp, 2019). Gallic acid was prepared at different concentrations to create a standard curve (Bhaigyabati et al., 2015). A linear equation was derived from this curve, which was then used to determine the phenolic compound content in rice straw extract.

Total Flavonoid Content (TFC) Analysis

The total flavonoid concentration in rice straw extract was quantified using the same methods applied for the total phenolic analysis, using quercetin as the reference compound. Figure 3 presents the standard curve for the analysis of total flavonoids. The plot shows the direct relationship between the concentration of quercetin and the absorbance, as cited in the literature (Mayerhöfer & Popp, 2019). The result of the regression equation is y = 0.0032x - 0.0026 (“x” and "y” respectively represent the flavonoid concentration and absorbance). Therefore, the concentration of flavonoids in the straw extract could be calculated utilizing equation (2).

$$\text{Concentration} = \frac{\text{Absorbance}}{0{,}0032} + 0{,}0026 \qquad (2)$$

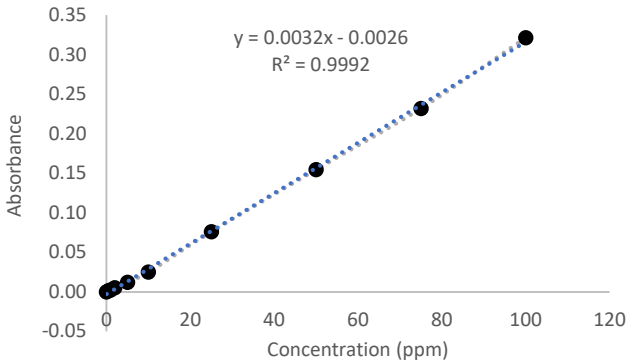

Figure 3. Standard Curve of TFC Analysis

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A series of investigations have shown that quercetin is a flavonoid that can be used to determine the total flavonoid content (Alide et al., 2020; Bhaigyabati et al., 2015; Chun et al., 2003; Rammohan et al., 2019). Figure 4 illustrates the formation of an Al(III)-quercetin complex resulting from the interaction between AlCl3 and quercetin in an alkaline environment. The complex formed by Al(III) with quercetin is shaded yellow (Rammohan et al., 2019).

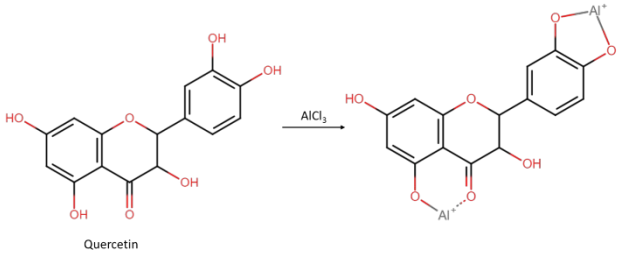

Figure 4. Formation of the Al(III)-quercetin complex (Martono et al., 2019)

Effect of Extraction Method on TPC and TFC

A comparison of extraction methods for rice straw was conducted to ascertain the most efficient technique for flavonoid extraction, as measured by TPC and TFC values. The employed methods included Soxhlet extraction, reflux, and maceration, using a 70% ethanol solvent. Figure 6 shows that maceration yielded a substantially higher flavonoid content than Soxhlet or reflux methods. These results were consistent with the research conducted by Vifta et al. (2019). The extraction of Clitoria ternatea flowers using the maceration method produced a TFC value of 53.127 mg QE/g. This value was significantly higher than that obtained through reflux extraction (24.527 mg QE/g) and Soxhlet (21.06 mg QE/g) methods (Vifta et al., 2022). Maceration was conducted at room temperature without heating, whereas reflux and Soxhlet extractions were performed at the boiling point of ethanol using heating. According to the literature, flavonoids are polyphenolic compounds easily degraded by heat exposure (Anukam et al., 2014; Vifta et al., 2022). Several studies also support this finding. The extraction of flavonoids from garlic using ethanol as a solvent exhibited a decrease in TFC from 414.98 ± 20.16 mg QE/g to 69.10 ± 6.03 mg QE/g as the extraction temperature rose from 25 ℃ to 150 ℃ (Alide et al., 2020). Flavonoids undergo degradation with increasing extraction temperatures. A reduction in flavonoid content also occurred during the black rice extraction process, decreasing from 450 mg/100g to 350 mg/100g due to elevating the extraction temperature from 40 ℃ to 100 ℃ (Lang et al., 2019). The decline in TFC can be attributed to the degradation or damage of flavonoids with increasing temperature (Gao et al., 2022).

The phenolics extracted through reflux exhibit the highest values compared with those obtained through Soxhlet and maceration techniques (Figure 6). Some literature suggests that phenolic compounds have higher thermal degradation thresholds than flavonoids. On the other hand, reflux allows the sample to be subjected to more intense heat than Soxhlet or maceration. Thus, reflux extraction causes the release of phenolic compounds due to heat breakdown of flavonoid compounds. In grape seed flour samples, phenolic compounds degraded when exposed to temperatures above 180 ℃, whereas flavonoids began to degrade at 120 ℃ (Ross et al., 2011). The high TPC and low TFC values observed during reflux extraction indicate the extraction of other phenolic compounds, including tannins, stilbenes, phenolic acids, and lignin. These compounds possess a higher thermal degradation threshold than flavonoids (Tambun et al., 2021; Widiyastuti et al., 2020). Figure 7 illustrates that flavonoids were transformed into various phenolic compounds when exposed to high temperatures (Chaaban et al., 2017). As a result, the TPC obtained through the reflux method typically exceeded that obtained through maceration.

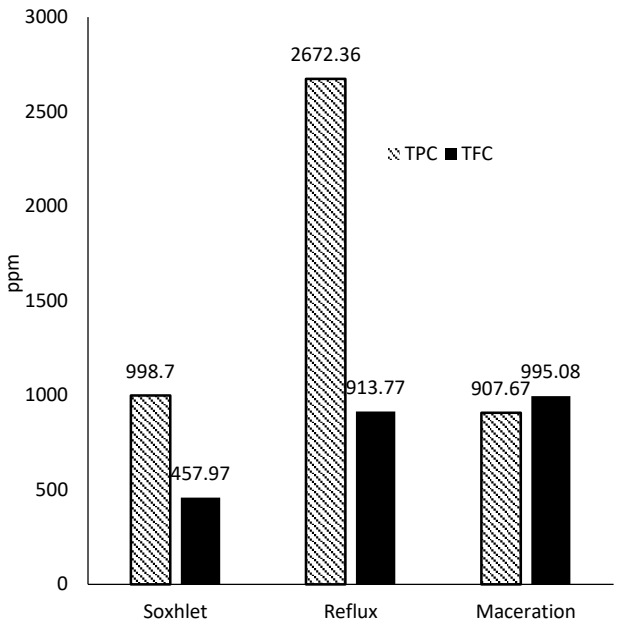

Figure 4. TPC and TFC of rice straw extracts using various methods

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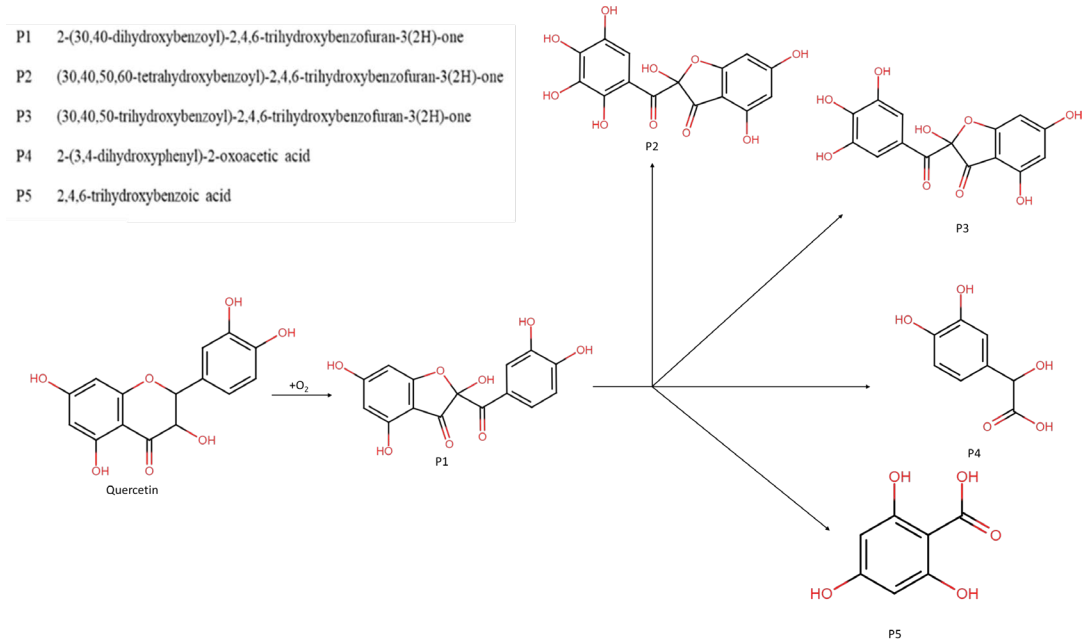

Figure 5. Scheme of the reaction of flavonoid degradation into other phenolics (non-flavonoids) (Chaaban et al., 2017)

The color of the rice straw extract is depicted in Figure 7. Existing literature indicates that flavonoid compounds typically exhibit a pale yellow hue (Iwashina, 2015; Stavenga et al., 2021). The maceration method for extracting rice straw results in a pale yellow color, confirming the presence of the highest flavonoid content. This finding is further supported by the higher TFC observed in macerated rice straw extract compared to reflux and Soxhlet extractions, as illustrated in Figure 5. The increasing darkness observed from maceration to reflux and Soxhlet extraction indicates an elevated concentration of other phenolic compounds (non-flavonoids) (Ge et al., 2021). This result aligns with Figure 5, which shows the highest TPC value for reflux compared to Soxhlet and maceration.

(a) (b) (c)

Figure 6. The color of rice straw extract: (a) soxhlet, (b) reflux, and (c) maceration

According to the literature, flavonoids were recognized as active compounds with antibacterial properties (Biharee et al., 2020; Cushnie & Lamb, 2005; Donadio et al., 2021). Consequently, the selection of an effective extraction method hinged on achieving the highest TFC. As depicted in Figure 5, maceration emerged as the most effective method for extracting rice straw. The optimization of ethanol concentration and contact time during the rice straw extraction process was carried out using the maceration method.

Effect of Ethanol Concentration on TPC and TFC

A comparison of ethanol concentrations was conducted to determine the optimal solvent concentration for extracting flavonoids, which were recognized for their antibacterial properties. Ethanol concentrations were varied at 0%, 25%, 70%, and 96% to evaluate the effect of solvent concentration on TPC and TFC. Notably, 96% ethanol yielded the highest TPC (1288.56 ppm) and TFC (1000.16 ppm), as shown in Figure 8. Consequently, the TPC and TFC exhibited a proportional decline with the reduction in ethanol concentration. According to the literature, the increase of ethanol concentration from 20% to 80% in the *Malaysian Propolis* extraction raised the TFC value from 0.010 ± 0.19 mg QE/ml to 0.034 ± 0.1875 mg QE/ml. TPC also rose from 1,456 ± 0.0025 mg GAE/ml to 8,898 ± 0.008 mg GAE/ml (Yusof et al., 2020). Comparable findings in other studies indicate that increasing the ethanol content from 50% to 70% in the extraction

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of A. bilimbi resulted in an increase in TFC (62.74 ± 1.16 to 64.81 ± 1.85 mg RTE/g) and TPC (103.79 ± 3.19 to 119.47 ± 1.76 mg GAE/g) (Rahardhian et al., 2019). An increase in ethanol content led to a decrease in the polarity of the solvent. In contrast, flavonoids have relatively low polarity (Wang et al., 2022). As a result, flavonoids were more readily dissolved and extracted when the ethanol concentration was high (Rahardhian et al., 2019; Wang et al., 2022; Yusof et al., 2020). The extraction process was enhanced by the solvent's rapid penetration of the solid matrix of the sample, which was aided by the similarity in polarity (Yusof et al., 2020). Furthermore, the increase in concentration resulted in more ethanol particles in the solution. A higher concentration of ethanol particles led to a greater frequency of contacts between the sample and the solvent (Cui et al., 2021).

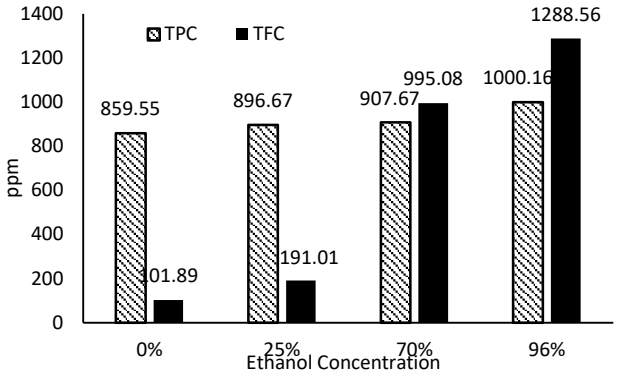
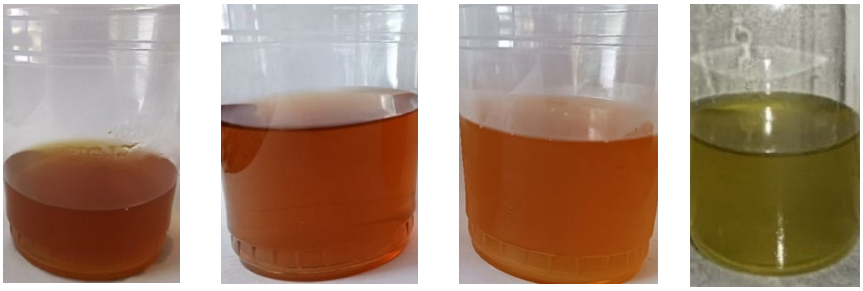


Figure 7. TPC and TFC of rice straw extracts with various ethanol concentrations

Differences in ethanol concentration influenced the color of rice straw extract, as shown in Figure 9. A decrease in color darkness occurred in direct proportion to the increase in ethanol concentration, ranging from 0% to 98% (shifting from dark yellow to pale yellow). This result provided evidence of increased flavonoid content (Iwashina, 2015; Stavenga et al., 2021), as illustrated in Figure 8. Conversely, the dark color of the rice straw extract indicated a high non-flavonoid phenolic content (Ge et al., 2021).



(a) (b) (c) (d)

Figure 8. TPC and TFC of rice straw extracts with various ethanol concentrations

The ethanol concentration was directly proportional to the flavonoid content. Flavonoids have been demonstrated to possess the ability to disrupt bacterial cell walls (Xie et al., 2014). Therefore, 96% ethanol is the most appropriate solvent for rice straw extraction.

Effect of Time Extraction on TPC and TFC

A comparative analysis of extraction time was conducted to determine the most effective period for obtaining flavonoids from rice straw. Extraction times were adjusted to 12 hours, one day, and three days. The data shown in Figure 10 demonstrates that extending the extraction time resulted in an increase in total phenolic content (TPC) and total flavonoid content (TFC). This result is similar to the literature by Diniyah et al. (2023), where increasing the extraction time of *Mucuna pruriens L.* at room temperature from 2 hours to 6 hours caused an increase in TPC (20.79 mg QE/100 g to 26.02 mg QE/100 g) and TFC (0.77 mg QE/100 g to 1.09 mg QE/100 g) (Diniyah et al., 2023). The extended extraction time increases the contact duration between the sample and the solvent (Dewi et al., 2019; Diniyah et al., 2023).

Extending the extraction time resulted in a shift towards a pale yellow color in the extracted solution. These findings indicated increased flavonoid concentration within the extract (Iwashina, 2015; Stavenga et al., 2021). Conversely, the shortest extraction period (12 hours) yielded a darker hue due to the high total phenolic content (TPC), as depicted in Figure 13 (Ge et al., 2021).

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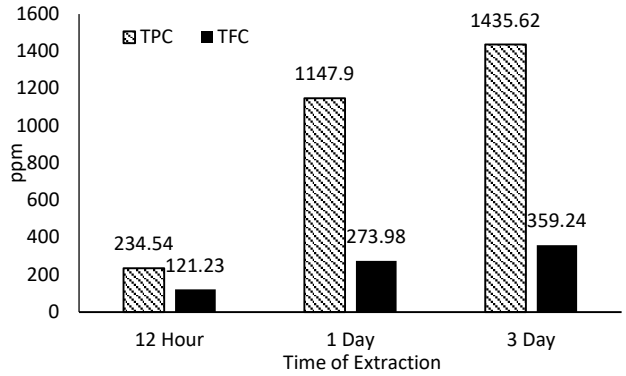

Figure 9. TPC and TFC of rice straw extract with various extraction times

(a) (b) (c)

Figure 10. The color of rice straw extract varies depending on the duration of the extraction process: (a) 12 hours, (b) 1 day, (c) 3 days

Anti-bacterial Activity

Flavonoids are phenolic compounds that exhibit antibacterial capabilities through various mechanisms. Numerous studies have demonstrated that flavonoids can impede energy metabolism, disrupt cytoplasmic membrane function, and inhibit nucleic acid synthesis (Biharee et al., 2020; Donadio et al., 2021; Górniak et al., 2019; Xie et al., 2014). Additionally, flavonoids show the ability to reduce adhesion and biofilm formation, regulate membrane permeability, affect porins on cell membranes, and decrease pathogenicity, which are all critical for bacterial proliferation (Biharee et al., 2020; Donadio et al., 2021; Xie et al., 2014).

Antibacterial testing was performed in this investigation with both gram-negative and gram-positive microorganisms. Gram-negative bacteria are enclosed by a lipopolysaccharide outer membrane and a thin peptidoglycan cell wall, whereas gram-positive bacteria have a thicker peptidoglycan layer and lack an outer membrane (Silhavy et al., 2010). Specifically, *Escherichia coli* served as the representative gram-negative bacterium, and *Staphylococcus aureus* was chosen as the gram-positive counterpart.

The extraction procedure was performed with ethanol concentrations of 96% (sample A), 70% (sample B), and 25% (sample C). Antibacterial testing was conducted by measuring the presence of clear zones (inhibitory zones). Figure 12 shows clear zones were observed in the *Escherichia coli* and *Staphylococcus aureus* tests during the first and second repetitions. These clear zones provided evidence of the antibacterial activity of the rice straw extract. Sample A, shown in Figure 13, exhibited the greatest clear zone width for *Escherichia coli* and *Staphylococcus aureus*. This result is related to the high ethanol content (96%). As shown in Figure 8, 96% ethanol contains the greatest flavonoid concentration (1288.56 ppm), indicating that sample A has better antibacterial effects than samples B and C. These results are consistent with previous studies by Bacon et al. (2017), where increasing the percentage of methanol solvent from 75% to 95% in *jalapeño pepper* extractions improves antibacterial activity against Escherichia coli (leading to a larger clear zone width from 7.4 to 8 mm) (Bacon et al., 2017). The clear zone diameter increased from 9 mm to 12 mm and from 10 mm to 13 mm, respectively, indicating that increasing the methanol concentration from 7.5 mg/ml to 15 mg/ml in the extraction of *Dipcadi viride (L.)* also led to enhanced antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (Al Farraj et al., 2020). Table 3 presents the classification of clear zones based on the literature. Sample A, classified as "very strong" and "strong," significantly reduced the development of Staphylococcus aureus and Escherichia coli, as shown in Figure 13.

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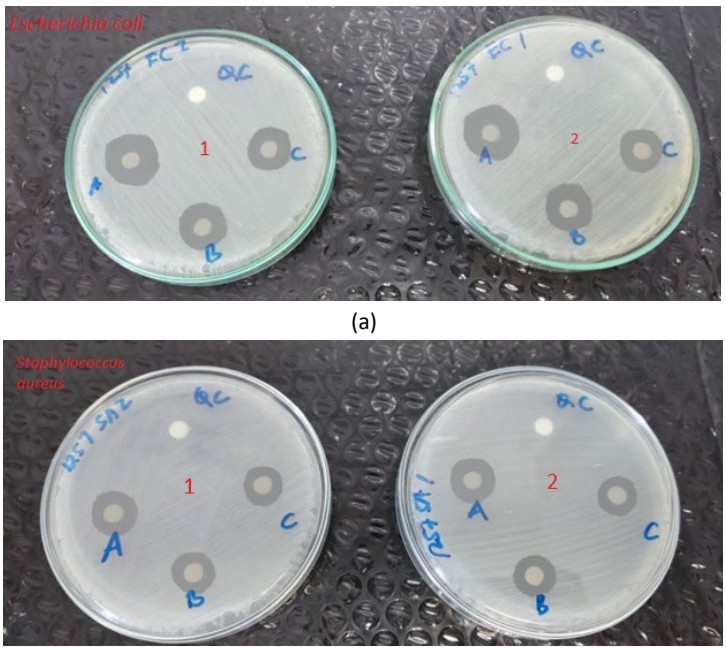

(a)

(b)

Figure 11. Antibacterial test of rice straw extract: (a) *Escherichia coli* dan (b) *Staphylococcus aureus*

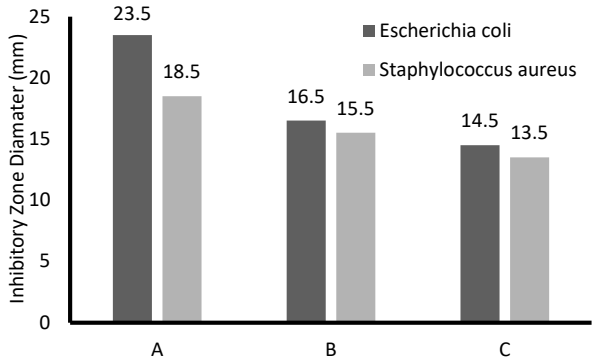

Figure 12. Average Inhibitory Zone Diamater for each straw extract

Table 3. Classification of Inhibitory Zone Diameter (Indriani et al., 2020)

Inhibitory Zone Diamater (mm)	Category
>20	Very Strong
10-20	Strong
5-10	Intermediate
<5	Weak

Figure 12 illustrates that flavonoids extracted from rice straw tend to display superior antibacterial activity against gram-negative bacteria (*Escherichia coli*) compared to gram-positive bacteria (*Staphylococcus aureus*). Several research studies show that flavonoids derived from bergamot orange seeds exhibit stronger antibacterial effects against gram-negative bacteria (*Escherichia coli*, *Pseudomonas putida*, *Salmonella enterica*) than gram-positive bacteria (*Listeria innocua*, *Bacillus subtilis*, *Staphylococcus aureus*, *Lactococcus lactis*) (Mandalari et al., 2007). A similar trend is observed in the flavonoid activity derived from the leaves of Bugis Ginseng (*Talinum paniculatum Gaertn.*). The efficacy of this extract in inhibiting gram-negative bacteria (*Escherichia coli*) is demonstrated by a clear zone measurement of 7.27 mm, which exceeds its impact on gram-positive bacteria (*Staphylococcus aureus*) at 7.04 mm (Emelda et al., 2021).

4. Conclusion

The study findings indicate that maceration is the most efficient approach for extracting flavonoids from rice straw compared to Soxhlet or reflux extraction methods. A direct correlation was observed between the increase in ethanol content and the corresponding rise in TPC and TFC. Significantly, the TPC and TFC values were higher following a three-day extraction period than 12 hours or 1 day. Comparative antibacterial testing demonstrated that the straw extract had greater inhibitory effectiveness against *Escherichia coli* than *Staphylococcus aureus*. The antibacterial activity exhibited a clear association with the TFC of the rice straw extract.

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