

# Determination of Total Flavonoid Content and Antioxidant Activity of Ethyl Acetate Extracts From Yellow Squash (*Cucurbita Moschata* Duch) Using The Dpph Method

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**Abstract.** Indonesia is one of the plant-producing countries that has abundant natural resource potential. Free radicals are molecules that are highly reactive to attract other electrons in the body, causing damage to biomolecules. Antioxidants function to ward off free radicals by donating one electron to oxidant compounds so that the activity of these oxidant compounds can be inhibited. Antioxidants can be sourced from natural ingredients or synthesizers. One of the natural antioxidants is yellow pumpkin. The compound content of yellow pumpkin fruit is  $\beta$ -carotene, vitamin C, phytosterols, zeaxanthin, selenium, and linoleic acid which play a role in antioxidant activity. The antioxidant results in yellow pumpkin extract were obtained with an  $IC_{50}$  of 30.75, which is in the very strong category. Flavonoids are secondary metabolite compounds of the phenol group that are widely found in plants. Based on the above background, a study was conducted to determine the total flavonoid content and test the antioxidant activity of the ethyl acetate extract of yellow pumpkin fruit (*Cucurbita moschata* Duch). This study was an experimental study that included phytochemical screening, moisture content test, total flavonoid level test, and antioxidant activity test. The results showed that ethyl acetate extract of yellow pumpkin fruit contains secondary metabolites of alkaloids, flavonoids, tannins, and saponins. The average moisture content was 5.15%, the result of the total flavonoid content of ethyl acetate extract in yellow pumpkin was 3.109 mg QE/g extract, and the antioxidant activity was included in the category of strong antioxidant activity using the DPPH method, with an  $IC_{50}$  value of 78.20 ppm. Ethyl acetate extract from yellow pumpkin fruit is effective in extracting flavonoid secondary metabolite compounds, the total flavonoid content in ethyl acetate extract in yellow pumpkin is 3.109 mg QE/g extract, with antioxidant activity obtained an  $IC_{50}$  value of 78.20 ppm including the category of strong antioxidant activity.

**Keywords:** Antioxidant, Ethyl Acetate Extract, Total Flavonoid Content, Yellow Pumpkin (*Cucurbita moschata* Duch)

## INTRODUCTION

Indonesia is a major agricultural producer with abundant natural resources. Free radicals are unpaired molecules that are unstable and highly reactive, attracting electrons from other molecules in the body and potentially causing cellular damage (Soedarini & Nugrahedi, 2019). Free radicals can attack healthy body cells, causing them to lose their structure and function (Asih *et al.*, 2022). To prevent damage caused by free radicals, antioxidants are needed to inhibit the formation of free radicals (Arnanda & Nuwatda, 2019).

Antioxidants are active compounds that neutralize free radicals by donating one of their electrons to oxidizing compounds, thereby inhibiting the activity of those oxidizing compounds. Antioxidants can be derived from natural sources or synthesized. The use of synthetic antioxidants has negative effects on human health, including damage to internal organ function and toxicity. Therefore, the use of natural antioxidants which are typically found in all plants is recommended to avoid the negative effects of excessive synthetic antioxidant use (Pratiwi *et al.*, 2024).

One plant that produces natural antioxidants is the yellow squash (Lismawati *et al.*, 2021). Yellow squash is a plant native to tropical climates that has become increasingly popular in Indonesia as a natural food source rich in antioxidants (Sukohar *et al.*, 2022). Several studies indicate that squash has potential as a natural antioxidant that can be used to treat diabetes and cardiovascular diseases. The potassium content in yellow squash helps regulate the body's metabolism, while vitamin C, vitamin A, minerals, and carbohydrates provide essential nutrients, making it safe for daily consumption. Research indicates that yellow squash skin possesses high antioxidant potential (Ali *et al.*, 2023).

The compounds found in yellow squash including  $\beta$ -carotene, vitamin C, phytosterols, zeaxanthin, selenium, and linoleic acid contribute to antioxidant activity in the human body. The vitamin C content in yellow squash also supports carotenoid activity through a mechanism that protects cells from oxidative damage (Erwiyani *et al.*, 2022). The antioxidant activity of yellow squash extract yielded

an  $IC_{50}$  value of 30.75, while ascorbic acid had an  $IC_{50}$  value of 18.43, classifying them as very strong antioxidants (Lismawati *et al.*, 2021).

Flavonoids are phenolic secondary metabolites commonly found in higher plants. These 2-phenyl-benzyl- $\gamma$ -pyrone derivatives are synthesized via the phenylpropanoid pathway and possess a phenolic backbone. Flavonoids are found in various parts of plants, such as fruits, seeds, fruit peels, roots, stems, and flowers; they contribute to color, flavor, and aroma, protect plants from environmental stress and UV-Vis radiation, and possess antimicrobial properties. The bioactive components of flavonoids hold potential in the health sector as antioxidants, anti-inflammatory agents, antimutagens, and anticarcinogens (Ilham *et al.*, 2024). Yellow pumpkin flesh contains polar flavonoids that can be extracted using ethyl acetate as a solvent (Erwiyani *et al.*, 2022). Ethyl acetate has low toxicity and is semi-polar, making it capable of extracting secondary metabolites ranging from nonpolar to polar (Putri, 2023).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was chosen because it is simple, fast, proven to be accurate, reliable, practical, and can be reacted directly (Aryanti, 2021). The mechanism of action of antioxidant compounds involves the ability of phenolic groups to bind free radicals by donating a hydrogen atom, thereby producing a phenoxyl radical through a hydrogen atom transfer (HAT) reaction with DPPH; the resulting phenoxyl radical is subsequently stabilized through resonance effects (Aji *et al.*, 2023).

The principle behind the DPPH method for testing antioxidant activity is the reaction between the antioxidant compound and DPPH, which is reduced and undergoes a decrease in color intensity from purple to yellow or clear; this decrease in intensity is measured using a UV-Vis spectrophotometer.

Based on the background described above, the researchers were interested in conducting a study to determine the total flavonoid content and test the antioxidant activity of ethyl acetate extract from yellow squash (*Cucurbita moschata* Duch).

## METHODS

This study employed a quantitative experimental design, including phytochemical screening, moisture content testing, total flavonoid content testing, and antioxidant activity testing. The population and sample consisted of yellow squash (*Cucurbita moschata* Duch) obtained from Temulus Village, Mejobo Subdistrict, Kudus Regency, Central Java. Non-probability sampling was used. This study was conducted from June to July 2025 at the Chemistry Laboratory of the Cendekia Utama Kudus Institute of Health Technology, the Microbiology Laboratory of the Cendekia Utama Kudus Institute of Health Technology, and the Pharmaceutical Technology Laboratory of the Cendekia Utama Kudus Institute of Health Technology.

### Equipment and Materials

The equipment used included a distillation flask (Pyrex), a moisture balance, brown bottles, a condenser (Pyrex), a water bath (Mettler), test tubes (Herma), test tube racks, a thermometer (GEA), beakers (Herma), measuring cylinders (Herma), volumetric pipettes (Pyrex), micropipettes (DragonLab), droppers (Onemed), Petri dishes (Pyrex), volumetric flasks (Pyrex), a UV-Vis spectrophotometer-Vis (Imprezza), quartz cuvettes (Hellma), stirring rods (Iwaki), spatulas (OMM), horn spoons, watch glasses (Normax), analytical balances (Ohaus), 40-mesh sieve, and a blender (Waring type 8010 Bu). The materials used in this study were yellow pumpkin pulp from Temulus Village, Mejobo Subdistrict, Kudus Regency; ethyl acetate; p.a. ethanol (Brataco); 0.1 M sodium acetate (Arkito); 1% aluminum chloride (Drysol); distilled water (Sari Kimia Raya), acetic acid (Emsure), quercetin (Aldrich), and DPPH (2,2-diphenyl-1-picrylhydrazyl).

### Moisture Content Test

The moisture content test was conducted using a moisture balance. The instrument was prepared, the sample was placed inside, the moisture analyzer lid was closed, and the reading was recorded once a constant value was reached.

### Phytochemical Screening

#### Alkaloid Identification

##### Mayer's Reagent

Place 0.5 grams of the sample into a test tube, add 2 drops of 2N HCl, and then add 2 drops of

Dragendrof's reagent; if an orange to reddish-brown precipitate forms, this indicates the presence of alkaloids (Budiman *et al.*, 2024) .

#### **Bouchardat Reagent**

Place 0.5 grams into a test tube, add 2 drops of 2N HCl, and then add 2 drops of Bouchardat's reagent; if a brown to black precipitate forms, this indicates the presence of the alkaloid (Budiman *et al.*, 2024) .

#### **Dragendrof Reagent**

Place 0.5 grams in a test tube, add 2 drops of 2N HCl, and then add 2 drops of Dragendrof's reagent; if an orange to reddish-brown precipitate forms, this indicates the presence of alkaloids (Budiman *et al.*, 2024) .

#### **Flavonoid Identification**

A total of 0.5 g of ethyl acetate extract from yellow squash was heated with 5 mL of distilled water

##### **Wilstater Test**

Weigh 1 mL of the filtrate from the ethyl acetate extract of yellow squash into a dish, add 1 mL of distilled water, then add magnesium powder and 2 drops of concentrated HCl, and shake the mixture. A visible change the formation of an orange color indicates the presence of flavone compounds, whereas the formation of a dark red color indicates the presence of flavonol compounds (Susiloningrum & Indrawati, 2020)

##### **Bate-Smith Test**

Weigh 1 mL of the ethyl acetate filtrate from the yellow squash into a dish, add 1 mL of distilled water, then add a few drops of concentrated HCl, and heat the mixture on a water bath. The sample changed color from dark red to purple. The formation of this dark red to purple color indicates the presence of anthocyanidin-type flavonoids (Susiloningrum & Indrawati, 2020) .

##### **10% NaOH Test**

A 1-mL aliquot of the ethyl acetate extract of yellow squash was weighed into a dish and mixed with 1 mL of distilled water; then, a few drops of 10% NaOH solution were added until a color change occurred. The resulting color change indicates the presence of phenolic compounds (Susiloningrum & Indrawati, 2020) .

##### **Tannin Identification**

Add 1–2 drops of FeCl<sub>3</sub> reagent to 0.05 grams of ethyl acetate extract from yellow squash; if a blue or blackish-green color appears, this indicates the presence of tannins (Budiman *et al.*, 2024) .

##### **Identification of Saponins**

A total of 0.05 grams of ethyl acetate extract from yellow squash fruit is placed in a test tube, then 5 drops of hot water are added; allow to cool, then shake vigorously for 10 seconds; if foam forms that lasts for 10 minutes and reaches a height of 1 cm and does not disappear when a drop of 2N HCl is added, then the sample is positive for saponins (Budiman *et al.*, 2024).

##### **Determination of Total Flavonoid Content**

###### **Preparation of a Quercetin Standard Solution**

Weigh 25 mg of quercetin standard and dissolve it in 25 mL of analytical-grade ethanol to obtain a concentration of 1000 ppm. the 1000 ppm quercetin stock solution was diluted to a series of 100 ppm solutions by pipetting 1 mL and then adding 10 mL of analytical-grade ethanol; and the 100 ppm quercetin stock solution was further diluted to a series of solutions at concentrations of 10 ppm, 15 ppm, 20 ppm, 25 ppm, and 30 ppm by pipetting 1 mL, 1.5 mL, 2 mL, 2.5 mL, and 3 mL, respectively, and then diluting each with 10 mL of p.a. ethanol (Evita *et al.*, 2022) .

###### **Determination of the Maximum Wavelength of Quercetin**

To determine the maximum wavelength of quercetin, 1 mL of a 60 ppm quercetin solution was placed in a 10-mL volumetric flask, followed by the addition of 0.1 mL of 10% AlCl<sub>3</sub>, 0.1 mL of 1 M sodium acetate, and ethanol up to the mark, then homogenized. Measure the absorbance at the maximum wavelength range of 400–800 nm (Yeti & Yuniarti, 2021) .

**Determination of Operating Time**

Determination of Operating Time: Pipette 4 mL of a 10 ppm quercetin solution into a 10 mL volumetric flask ( $C = 40 \mu\text{g/mL}$ ), add 0.1 mL of  $\text{AlCl}_3$  10%, 0.1 mL of 1 M sodium acetate, then add ethanol up to the mark, and measure the quercetin operating time for 60 minutes at a wavelength of 400–800 nm (Yeti & Yuniarti, 2021).

**Preparation of a Quercetin Calibration Curve**

A series of solutions with varying concentrations was prepared using quercetin as the standard reference. A series of solutions with concentrations of 10 ppm, 15 ppm, 20 ppm, 25 ppm, and 30 ppm was prepared. A total of 1 mL of quercetin solution from each concentration was added to a 10-mL volumetric flask, followed by 0.1 mL of 10%  $\text{AlCl}_3$ , 0.1 mL of sodium acetate, and p.a. ethanol up to the mark. The mixture was homogenized and allowed to stand for the time specified in the operating time at room temperature. Absorbance was measured at the previously optimized maximum wavelength (Yeti & Yuniarti, 2021).

**Determination of Total Flavonoids in Ethanol Extracts of Yellow Squash Fruit**

Weigh 10 mg of the ethyl acetate extract of yellow squash and place it in a 10-mL volumetric flask; then add p.a. ethanol up to the mark to obtain a concentration of 1,000 ppm. A 1 mL aliquot of each solution was pipetted into a 10-mL volumetric flask, followed by the addition of 0.1 mL of 10%  $\text{AlCl}_3$ , 0.1 mL of sodium acetate, and ethanol p.a. up to the mark. The mixture was homogenized and then allowed to stand for the time specified in the operating time. Absorbance was measured using a UV-Vis spectrophotometer at the optimized wavelengths.

**Antioxidant Testing Using the DPPH Method****Preparation of the DPPH Solution**

A total of 19.71 mg of DPPH powder was dissolved in 100 mL of p.a. ethanol to obtain a 0.5 mM DPPH solution (Fatmawati *et al.*, 2023).

**Preparation of the DPPH Blank Solution**

Preparation of the DPPH blank solution was based on the following method: 0.5 mL of 0.5 mM DPPH was added to 5 mL of p.a. ethanol and shaken until homogeneous (Fatmawati *et al.*, 2023).

**Determination of the Maximum Wavelength of the DPPH Solution**

The absorbance of the 0.5 mM DPPH solution was measured using a UV-Vis spectrophotometer at wavelengths ranging from 400 to 800 nm. This wavelength range was selected based on the absorption of the DPPH color complex, which is dark purple (Fatmawati *et al.*, 2023).

**Operating Time (OT)**

OT was determined by adding 0.5 mL of a 0.5 mM DPPH solution to 5 mL of p.a. ethanol up to the mark on a 5-mL volumetric flask. The absorbance of the solution was measured at the maximum wavelength obtained at 1-minute intervals from 0 to 60 minutes until a stable absorbance was achieved (Susiloningrum, 2021).

**Preparation of Quercetin Solution**

Weigh 25 mg of quercetin standard and dissolve it in 25 mL of p.a. ethanol up to the mark, then shake the solution until homogeneous to obtain a concentration of 1000 ppm (Fatmawati *et al.*, 2023). Solutions with concentrations of 20, 22, 24, 26, and 28 ppm were prepared from the 1000 ppm solution by pipetting 0.1 mL, 0.11 mL, 0.12 mL, 0.13 mL, and 0.14 mL, respectively, then diluted to 5 mL with p.a. ethanol, followed by the addition of p.a. ethanol up to the 5 mL mark on the volumetric flask; the solution was then shaken until homogeneous (Fatmawati *et al.*, 2023).

**Preparation of a 1000 ppm Sample Stock Solution**

A total of 50 mg of ethyl acetate extract was placed in a 50-mL volumetric flask, then analytical-grade ethanol was added up to the mark, and the solution was shaken until homogeneous (Fatmawati *et al.*, 2023).

**Preparation of Sample Series Solutions at 20, 40, 60, 80, and 100 ppm**

Solutions with concentrations of 20, 40, 60, 80, and 100 ppm were prepared by pipetting 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, and 0.5 mL, respectively, then diluting to 5 mL with p.a. ethanol, followed by adding p.a. ethanol up to the mark, then the solution was shaken until homogeneous (Fatmawati *et al.*,

2023).

### Measurement of Free Radical Scavenging Activity Using the DPPH Method

Ethyl acetate extracts of yellow squash were dissolved at concentrations of 20, 40, 60, 80, and 100 ppm, respectively. For each concentration, 2 mL was taken and 0.5 mL of 0.5 mM DPPH reagent solution was added in a volumetric flask. The mixture was shaken until homogeneous and incubated for the operating time. Absorbance was measured at the wavelength corresponding to the maximum absorption of DPPH (Fatmawati *et al.*, 2023).

## RESULTS AND DISCUSSION

### Moisture Content Test of Ethyl Acetate Extract from Yellow Squash

Moisture content is a parameter used to determine water residue (Sambode *et al.*, 2022). The moisture content test aims to measure the water content in the extract and establish a minimum limit for the amount of water in the material; moisture content is a critical factor in determining the quality and stability of an extract. The requirement for concentrated extracts is that the moisture content fall within the range of 5–30% (Voight, 1994). Moisture content was determined using a *moisture balance*. The results of the moisture content determination for the ethyl acetate extract of yellow squash are shown in Table 4.1.

Table 1: Moisture Content Test Results

| Replication   | Moisture Content (%) | Requirement (%) | Remarks |
|---------------|----------------------|-----------------|---------|
| Replication 1 | 5.10%                | 5–30            | Meets   |
| Replication 2 | 5.30%                | 5–30            | Meets   |
| Replication 3 | 5.07%                | 5–30            | Meets   |
| Average       | 5.15%                | 5–30            | Meets   |

The moisture content test results showed that replication 1 yielded 5.10%; replication 2, 5.30%; and replication 3, 5.07%. The average moisture content obtained in this study was 5.15%; this result indicates that the moisture content of the crude drug meets the requirements for a good concentrated extract, which is 5–30%, thereby preventing the possibility of the extract becoming contaminated by mold, fungi, and bacteria. Determining moisture content is related to extract purity because the higher the percentage of water content in a material, the more susceptible the extract is to degradation and spoilage caused by microbial growth (Sambode *et al.*, 2022). Conversely, low moisture content can make the extract hard or dense and difficult to process further because it is hard to dissolve (Wijaya & Rissa, 2024).

### Phytochemical Screening

Phytochemical screening aims to identify secondary metabolites in the extract under study. Phytochemical screening was performed qualitatively using color reactions with specific reagents (Vifta & Advistasari, 2018). The results of the phytochemical screening of the ethyl acetate extract of yellow squash, as shown in Table 4.2:.

Table 1 Data on Phytochemical Screening Results

| Phytochemical Screening | Reagent                   | Color Change                | Test Results | Remarks                    |
|-------------------------|---------------------------|-----------------------------|--------------|----------------------------|
| <b>Alkaloid Test</b>    |                           |                             |              |                            |
| a. Mayer Test           | 2N HCl + Mayer            | No white precipitate formed | (+)          | Does not contain alkaloids |
| b. Bouchardat Test      | 2N HCl + Bouchardat       | Brown precipitate           | (+)          | Contains alkaloids         |
| c. Dragendroff Test     | 2N HCl + Dragendroff      | No orange precipitate forms | (-)          | Does not contain alkaloids |
| <b>Flavonoid Test</b>   |                           |                             |              |                            |
| a. Wilstater Test       | Concentrated HCl + Mg     | Orange                      | (+)          | Contains Flavonoids        |
| b. Bate-Smith Test      | Concentrated HCl + heated | Dark red                    | (+)          | Contains Flavonoids        |
| c. 10% NaOH Test        |                           | Yellow                      | (+)          | Contains Flavonoids        |

|                    |                              |                |     |                   |
|--------------------|------------------------------|----------------|-----|-------------------|
| <b>Tannin Test</b> | FeCl <sub>3</sub>            | Blackish green | (+) | Contains Tannins  |
| Saponin Test       | Hot distilled water +<br>HCl | Foam forms     | (+) | Contains saponins |

Based on the results of the phytochemical screening above, the ethyl acetate extract of yellow squash fruit contains alkaloids, flavonoids, tannins, and saponins.

#### a. Alkaloid test

The alkaloid test was conducted to determine the presence or absence of alkaloids in the ethyl acetate extract of yellow squash. Testing of secondary metabolites in the ethyl acetate extract of yellow squash showed that the sample tested positive for alkaloids using the *Bouchardat* and *Mayer* reagents. The sample tested negative for alkaloids using the *Dragendorff* reagent. According to Rachmawati & Suriawati (2019), the addition of 2 N HCl is intended to extract the alkaloid compounds from the extract; since alkaloids are basic, the addition of an acid such as HCl forms salts, causing the alkaloids to separate from other plant cell components that were co-extracted by distributing them into the acidic phase.

This is consistent with the research by Rustina & Tasminatun (2016), who reported that the ethyl acetate extract of yellow squash contains alkaloids, in the *Mayer* reagent, the nitrogen atom of the alkaloid bonds with the potassium metal ion in potassium tetraiodomercurate(II), forming a potassium-alkaloid precipitate and causing a color change; whereas in the *Dragendorff* reagent, the nitrogen atom of the alkaloid does not bond with the potassium metal ion in potassium tetraiodobismuthate, so no potassium-alkaloid precipitate is formed

#### b. Flavonoid Test

Testing of secondary metabolites in the ethyl acetate extract of yellow squash indicated that the sample tested positive for flavonoids using the *Wilstatter*, *Bate-Smith*, and NaOH reagents. The *Wilstatter* test was performed by adding concentrated HCl and magnesium (Mg) powder to the extract, followed by heating to produce an orange-colored complex. This orange-colored complex is formed by the reduction of the benzopyrone ring present in the flavonoid structure. Based on the above results, the ethyl acetate extract of yellow squash tested positive for flavonoids due to the formation of an orange color. This is consistent with the research by Susiloningrum & Sari (2021), who noted that the formation of the orange color is due to the presence of flavone compounds.

The *Bate-Smith* test was performed by adding concentrated HCl to the extract and then heating it. The addition of *concentrated* HCl serves to hydrolyze the flavonoids by breaking down the O-glycosyl group into its aglycone. The heating process is intended to accelerate the hydrolysis reaction. Based on the results of the phytochemical screening, the ethyl acetate extract of yellow squash tested positive for flavonoids, as indicated by a color change to red. This is consistent with the research by Islamiyati *et al.* (2024), which states that the formation of a red color indicates the presence of anthocyanin-class flavonoids.

The flavonoid test using a 10% NaOH reagent was conducted by adding the ethyl acetate extract of yellow squash to 10% NaOH. 10% NaOH acts as a basic catalyst that causes the decomposition of flavonoid compounds, specifically chrysin, into acetophenone molecules (Ferdian & Rizki, 2021). Based on the research results, the ethyl acetate extract of yellow squash tested positive for flavonoids, as indicated by a color change to yellow. This aligns with the study by Islamiyati *et al.* (2024), which states that the formation of a yellow color indicates the presence of phenolic flavonoid

#### c. Tannin Test

The tannin test aims to determine the presence or absence of tannins in the ethyl acetate extract of yellow squash using the FeCl<sub>3</sub> reagent. The addition of FeCl<sub>3</sub> is intended to detect the presence of phenolic groups, indicated by a color change to blackish green. The blackish-green color change occurs due to the formation of a complex between phenolic compounds and Fe<sup>3+</sup> ions in FeCl<sub>3</sub> (Islamiyati *et al.*, 2024).

The results of this study indicate that the ethyl acetate extract of yellow squash fruit tested positive for tannins, as indicated by the blackish-green color change. This is consistent with the study by Rustina & Tasminatun (2016), which reported that the ethyl acetate extract of yellow squash fruit contains tannins.

#### d. Saponin Test

The saponin test was conducted to determine the presence or absence of saponin compounds in the ethyl acetate extract of yellow squash. The formation of foam is caused by the glycosides present in saponins, which hydrolyze into glucose and produce foam when shaken. The results of the saponin test indicate that the ethyl acetate extract of yellow pumpkin fruit contains saponin compounds. This is consistent with the study by Islamiyati *et al.* (2024), which states that a sample is considered positive for saponins if consistent foam forms for 10 seconds after the sample is shaken.

### Determination of Total Flavonoid Content

#### Determination of the Maximum Wavelength

Determining the maximum wavelength in the total flavonoid content assay aims to identify the wavelength that exhibits the highest absorbance, where a flavonoid complex forms with the  $\text{AlCl}_3$  reagent, indicating the most effective wavelength for measuring flavonoid levels in the sample under study. Absorbance is measured at a maximum wavelength of 400–800 nm (Yeti & Yuniarti, 2021). The results of the maximum wavelength determination for total flavonoid content are shown in the following table:

| Table 3 Results of the determination of the maximum wavelength |            |
|----------------------------------------------------------------|------------|
| Maximum Wavelength (nm)                                        | Absorbance |
| 446.0                                                          | 0.217      |
| 444.0                                                          | 0.258      |
| 442.0                                                          | 0.241      |
| 440.0                                                          | 0.235      |

The maximum wavelength measurement using a UV-Vis spectrophotometer to determine total flavonoid content was obtained at a wavelength of 444 nm with an absorbance of 0.258. Consistent with the results of a study by Pujiastuti & Zeba (2021), the maximum wavelength for total flavonoid content was 444 nm with the highest absorbance value of 0.296.

#### a. Determination of Operating Time

*Operating time* is the optimal time used for absorbance readings after the flavonoid compounds react with the  $\text{AlCl}_3$  reagent; it determines the incubation time for the sample solution. Absorbance measurements were performed at a maximum wavelength range of 400–800 nm (Yeti & Yuniarti, 2021). The results of the *operating time* determination for total flavonoid content are shown in the following Table.

| Table 4 Operating Time Results |       |
|--------------------------------|-------|
| Minute                         | Data  |
| 21                             | 0.503 |
| 22                             | 0.503 |
| 23                             | 0.503 |
| 24                             | 0.503 |

The *operating time* was determined using a spectrophotometer with a quercetin concentration of 60 ppm; measurements were taken at 1-minute intervals for 60 minutes at a wavelength of 440 nm. A stable absorbance was obtained at the 23rd minute, which was used to determine the incubation time for the samples. Consistent with the results of the study by Pujiastuti & Zeba (2021), the *operating time* for total flavonoid content was found to be similar to that in the present study, ranging from the 21st to the 25th minute.

#### b. Determination of the Quercetin Calibration Curve

The analysis of total flavonoid content is a measurement of the total flavonoids contained in a sample (Sari *et al.*, 2021). Quercetin was used as a reference standard at concentrations of 10, 15, 20, 25, and 30 ppm. Quercetin was used as the standard because it is a flavonol-type flavonoid that has a keto group

on the C-4 atom and hydroxyl groups on the adjacent C-3 and C-5 atoms. The results of the quercetin calibration curve measurements are shown in the following Table

**Table 5** Quercetin Calibration Curve Measurement Results

| Concentration (ppm) | Absorbance | Linear Regression Equation |
|---------------------|------------|----------------------------|
| 10                  | 0.351      | $y = 0.0111x + 0.2368$     |
| 15                  | 0.401      |                            |
| 20                  | 0.455      |                            |
| 25                  | 0.510      |                            |
| 30                  | 0.573      |                            |

The methods used were colorimetry and UV-Vis spectrophotometry. The reagent  $\text{AlCl}_3$  was used to detect adjacent hydroxy and keto groups and ortho-hydroxy groups.  $\text{AlCl}_3$  causes a shift in the ultraviolet spectrum of flavonoids; the principle behind the aluminum chloride method for determining flavonoid content is the formation of a complex between aluminum chloride and the keto group at the C-4 atom and the hydroxyl group at the adjacent C-3 or C-5 atom of the flavone and flavonol groups (Sari *et al.*, 2021).

A calibration curve was prepared to calculate the flavonoid content in the sample based on the absorbance measured using the calibration equation. To determine the total flavonoid content, quercetin standard solutions were used at concentrations of 10 ppm, 15 ppm, 20 ppm, 25 ppm, and 30 ppm. Absorbance measurements were taken at a wavelength of 444 nm, followed by incubation for 23 minutes. The absorbance values obtained were, in order: 0.351; 0.401; 0.455; 0.510; 0.573.

The results of the study on the total flavonoid content of the ethyl acetate extract of yellow squash, measured using a UV-Vis spectrophotometer, yielded a linear regression equation based on the quercetin calibration curve:  $y = 0.0111x + 0.2368$ , with a correlation coefficient ( $r$ ) of 0.9982. The  $R^2$  value was excellent, approaching 1. The standard curve equation for quercetin was used as a reference to determine the concentration of total flavonoids in the sample extracts.

### c. Results of Total Flavonoid Content Determination

The total flavonoid content of the ethyl acetate extract of yellow squash was calculated using linear regression from the previously measured quercetin standard curve. The results of the quercetin equivalence and total flavonoid content are shown in the following Table.

**Tabel 6** Results of the Determination of Total Flavonoid Content in Ethyl Acetate Extracts of Yellow Squash (*Cucurbita moschata* Duch)

| Concentration (ppm) | Replication | Absorbance | Equivalent Content (ppm) | Total Flavonoid Content (%) |
|---------------------|-------------|------------|--------------------------|-----------------------------|
| 1,000               | 1           | 0.569      | 29.927                   | 2.992                       |
|                     | 2           | 0.578      | 30.738                   | 3.073                       |
|                     | 3           | 0.599      | 32.630                   | 3.263                       |
| Mean $\pm$ SD       |             |            | 31.098                   | 3.109                       |

Measurements of the total flavonoid content in the ethyl acetate extract of yellow squash were replicated three times, yielding absorbance values of 0.569, 0.578, and 0.599, respectively. The absorbance values of the ethyl acetate extract of yellow squash were used to calculate the total flavonoid content, and the sample absorbance values were averaged. The calculations yielded the following average total flavonoid content for the ethyl acetate extract of yellow squash: 2.992% for replication 1, 3.073% for replication 2, and 3.263% for replication 3. The average total flavonoid content of the ethyl acetate extract of yellow squash was 3.109%, which equates to 3.1 grams of flavonoids per 100 grams of extract (31.09 mg QE/g extract). This indicates that the ethyl acetate extract of yellow squash contains bioactive flavonoids and antioxidants that can be utilized in pharmaceutical applications.

### Antioxidant Activity Test

The antioxidant activity test aims to determine whether a sample possesses antioxidant activity or not. DPPH is a dark-colored crystalline powder consisting of stable free radical molecules (Soedarini & Nugrahedi, 2019). Antioxidant activity was determined quantitatively using the DPPH method, measured

with a UV-Vis spectrophotometer. The principle of the DPPH method is to measure color fading caused by the presence of antioxidants capable of neutralizing free radical molecules (Wulan *et al.*, 2019). According to Aryanti *et al.* (2021), the DPPH method was chosen in this study because DPPH free radicals can react directly with samples possessing antioxidant activity, and the measurement is highly sensitive.

#### a. Determination of the DPPH Maximum Wavelength

The determination of the maximum wavelength aims to identify the maximum measurement sensitivity, indicated by the highest absorbance value. To determine the maximum wavelength, a 0.5 mM DPPH solution was used, and its absorbance was measured with a UV-Vis spectrophotometer within the 400–800 nm spectral range (Fatmawati *et al.*, 2023). The results of the maximum wavelength determination are shown in the following Table.

**Table 2** Results of Maximum Wavelength Determination

| Maximum Wavelength (nm) | Absorbance |
|-------------------------|------------|
| 476.0                   | 0.780      |
| 474.0                   | 0.785      |
| 472.0                   | 0.788      |
| 470.0                   | 0.786      |
| 468.0                   | 0.782      |

The maximum wavelength was measured at 472 nm with an absorbance of 0.788. This is consistent with the findings of Anggraini *et al.* (2018), who reported that the maximum wavelength of DPPH ranges from 400 to 800 nm.

#### b. Determination of Operating Time

The purpose of determining *the operating time* is to identify the time required to measure a compound; *the operating time* is determined when a stable absorbance is obtained (Harahapi & Ridwanto, 2023). *The operating time* was determined at the maximum wavelength. The results of *the operating time* determination are shown in the following Table.

**Table 8** Operating Time Results

| Minute | Data  |
|--------|-------|
| 34     | 0.596 |
| 35     | 0.596 |
| 36     | 0.596 |
| 37     | 0.596 |
| 38     | 0.596 |

In this study, *the operating time* was determined to be at the 36th minute, because stable absorbance was observed from the 34th to the 38th minute, and after the 38th minute, instability occurred, indicating that the compound had decomposed. The *operating time* obtained was used for the incubation process of the samples. The *operating time* obtained in this study is consistent with that reported by Rizki *et al.* (2022), who also observed stable absorbance at the 36th minute.

#### c. DPPH Blank Determination

The DPPH blank determination serves to measure or correct the DPPH absorbance in the absence of a sample or active substance, in order to obtain accurate antioxidant activity measurements and ensure that the measured absorbance stems purely from changes in DPPH resulting from interaction with the antioxidant, rather than from the solvent used (Andriani *et al.*, 2025).

**Table 9** DPPH Blank Solution Results

| Replication | Results | Average |
|-------------|---------|---------|
| 1           | 0.761   | 0.761   |
| 2           | 0.763   |         |
| 3           | 0.760   |         |

The absorbance results for the DPPH blank, used as a negative control in this study, were 0.761 for replication 1, 0.763 for replication 2, and 0.760 for replication 3. The average DPPH blank value was 0.761.

#### d. Antioxidant Activity Test Using Quercetin as a Reference Standard

The antioxidant activity test using quercetin as a reference standard aims to compare the antioxidant activity of the samples with the known antioxidant activity of quercetin. According to Yadi & Pertiwi (2024), antioxidant activity is categorized as very strong with an  $IC_{50}$  value  $< 50$  ppm, strong with an  $IC_{50}$  value of 50–100 ppm, moderate with an  $IC_{50}$  value of 100–150 ppm, weak with an  $IC_{50}$  value of 151–200 ppm, and very weak with an  $IC_{50}$  value of  $>200$  ppm. The  $IC_{50}$  value is the concentration of the sample solution required to inhibit 50% of free radical activity (Vifta *et al.*, 2019). The lower the  $IC_{50}$  value, the stronger the antioxidant activity (Syafitri *et al.*, 2020). The results of the absorbance measurements of the quercetin reference standard are shown in the following Table.

**Table 3** Results of Quercetin's Antioxidant Activity

| Replication   | Concentration (ppm) | Absorbance | % Inhibition | $IC_{50}$ | Average |
|---------------|---------------------|------------|--------------|-----------|---------|
| Replication 1 | 20                  | 0.690      | 9.329        | 25,216    |         |
|               | 22                  | 0.587      | 22.864       |           |         |
|               | 24                  | 0.416      | 45.335       |           |         |
|               | 26                  | 0.355      | 53.335       |           |         |
|               | 28                  | 0.214      | 71.879       |           |         |
| Replication 2 | 20                  | 0.645      | 15.243       | 26,096    | 24.986  |
|               | 22                  | 0.549      | 27.858       |           |         |
|               | 24                  | 0.483      | 36.530       |           |         |
|               | 26                  | 0.396      | 47.963       |           |         |
|               | 28                  | 0.286      | 62.417       |           |         |
| Replication 3 | 20                  | 0.596      | 21.681       | 23,648    |         |
|               | 22                  | 0.436      | 43.706       |           |         |
|               | 24                  | 0.339      | 55.453       |           |         |
|               | 26                  | 0.241      | 68.331       |           |         |
|               | 28                  | 0.215      | 71.747       |           |         |

The results of the DPPH assay for the antioxidant activity of the quercetin reference standard were as follows: replication 1, 25.216 ppm; replication 2, 26.096 ppm; and replication 3, 23.648 ppm. The average  $IC_{50}$  value obtained for quercetin was 24.986 ppm, which falls into the “very strong antioxidant” category because the  $IC_{50}$  value is  $< 50$  ppm. Consistent with the study by *et al.* (2021), quercetin is classified as a “very strong antioxidant” with an  $IC_{50}$  value of 25.94 ppm.

#### e. Antioxidant Activity Test of the Sample

The antioxidant activity test of the ethanol extract of yellow squash was conducted to determine whether the sample contained antioxidants. When DPPH radicals react with antioxidants, a color change from purple to yellow occurs, and a reduction process takes place due to the presence of a hydrogen donor from the substrate, resulting in a reduction of the purple color in DPPH (Soedarini & Nugrahedi, 2019).

The results of the DPPH assay for the antioxidant activity of the ethyl acetate extract of yellow squash were as follows: replication 1, 81.82 ppm; replication 2, 76.80 ppm; and replication 3, 75.98 ppm. The average  $IC_{50}$  value obtained was 78.20 ppm, which falls into the “strong antioxidant” category, as  $IC_{50}$  values in this range are 50–100 ppm. According to Yadi & Pertiwi (2024), antioxidant activity is classified as “strong” with  $IC_{50}$  values of 50–100 ppm. In line with the research by Yeda *et al.* (2015), who reported that the ethyl acetate extract of yellow squash is a strong antioxidant with an  $IC_{50}$  value of 84.79 ppm. The results of the absorbance measurements of the ethyl acetate extract of yellow squash at a maximum wavelength of 472 nm are shown in the following Table.

**Table 41** Results of Antioxidant Activity of Ethyl Acetate Extracts of Yellow Squash

| Replication   | Concentration (ppm) | Absorbance | % Inhibition | $IC_{50}$ | Average |
|---------------|---------------------|------------|--------------|-----------|---------|
| Replication 1 | 20                  | 0.650      | 14.586       | 81.82     | 78.20   |
|               | 40                  | 0.578      | 24.047       |           |         |
|               | 60                  | 0.469      | 38.370       |           |         |
|               | 80                  | 0.356      | 53.219       |           |         |
|               | 100                 | 0.286      | 62.417       |           |         |

| Replication      | Concentration (ppm) | Absorbance | % Inhibition | IC <sub>50</sub> | Average |
|------------------|---------------------|------------|--------------|------------------|---------|
| Replication<br>2 | 20                  | 0.616      | 19.053       | 76.8             |         |
|                  | 40                  | 0.532      | 30.091       |                  |         |
|                  | 60                  | 0.448      | 41.130       |                  |         |
|                  | 80                  | 0.349      | 54.139       |                  |         |
|                  | 100                 | 0.300      | 60.578       |                  |         |
| Replication<br>3 | 20                  | 0.648      | 14.848       | 75.98            |         |
|                  | 40                  | 0.558      | 26.675       |                  |         |
|                  | 60                  | 0.452      | 40.604       |                  |         |
|                  | 80                  | 0.348      | 54.270       |                  |         |
|                  | 100                 | 0.277      | 63.600       |                  |         |

Based on the results of antioxidant activity measurements of quercetin solutions and ethyl acetate extract of yellow squash, it was shown that as the solution concentration in ppm increased, the resulting absorbance values decreased. This is because higher solution concentrations lead to more antioxidant compounds acting as hydrogen or electron donors to DPPH radicals, causing a color change in DPPH that results in a decrease in absorbance. The higher the solution concentration, the greater the percentage inhibition obtained. The results from three replicates of the quercetin reference solution and the yellow squash ethyl acetate extract test solution yielded a linear regression equation with a good correlation coefficient (r) approaching 1 (Putri *et al.*, 2023).

Based on the results obtained, the ethyl acetate extract of yellow squash fruit has an average IC<sub>50</sub> of 84.79 ppm, classified as having strong antioxidant activity, while quercetin, as the reference standard, has an IC<sub>50</sub> of 25.49 ppm, classified as having very strong antioxidant activity. It can be concluded that the ethyl acetate extract of yellow squash fruit has a lower antioxidant activity value compared to quercetin as its reference standard, because quercetin is a very strong natural antioxidant with high purity; it belongs to the flavonol class of flavonoids, which possess a keto group at C-4 and a hydroxyl group at either the C-3 or C-5 position adjacent to the flavone and flavonol moieties, making it effective in neutralizing free radicals (Chandra *et al.*, 2022).

## CONCLUSION

The ethyl acetate extract from yellow squash (*Cucurbita moschata* Duch) is effective in extracting flavonoid secondary metabolites. The total flavonoid content in the ethyl acetate extract of yellow squash is 3.109 mg QE/g of yellow squash ethyl acetate extract. The ethyl acetate extract of yellow squash exhibits antioxidant activity as determined by the DPPH method, with an IC<sub>50</sub> value of 78.20 ppm, which falls within the category of strong antioxidant activity.

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