

Analysis of Phenolic, Flavonoid Content, and Antioxidant Activity of Combination of Pumpkin Leaves (*Cucurbita moschata* Durch.) and Aibika Leaves (*Abelmoschus manihot* L.) Fractions

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Abstract

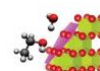
This study aims to determine the total phenolic and flavonoid content of ethyl acetate fractions from pumpkin (*Cucurbita moschata* Durch.) and aibika (*Abelmoschus manihot* L.) leaves, and to evaluate the antioxidant activity of their combined fractions. Analysis of total phenolics and flavonoids content was carried out using UV-Vis spectrophotometry with gallic acid (phenolics) and quercetin (flavonoids) as standards. Antioxidant potential testing was carried out using the DPPH method. The results showed that the phenolics content of the ethyl acetate fraction of PL and AL was 60.81 and 56.47 mgEAG/g, respectively, while the total flavonoids content of the ethyl acetate fraction of PL and AL was 64.54 and 55.07 mgEK/g, respectively. The IC_{50} value of the combination of ethyl acetate fractions of PL and AL with a ratio of 3:2 was 4.686 μ g/mL, while the single ethyl acetate fractions of PL and AL were 7.040 and 7.549 μ g/mL, respectively. This indicates that increasing antioxidant activity can be done by combining fractions from two or more types of plants.

Keywords: total phenolic content; total flavonoid content; antioxidant; *Cucurbita moschata* Durch leaves; *Abelmoschus manihot* L. leaves

1 Introduction

Degenerative diseases are a group of chronic diseases characterized by progressive decline in tissue or organ function due to long-term cell damage. These diseases include cardiovascular disease, diabetes mellitus, cancer, hypertension, and neurodegenerative diseases such as Alzheimer's and Parkinson's. According to the World Health Organization, non-communicable diseases (NCDs) cause approximately 41 million deaths annually, or approximately 74% of total global deaths. Cardiovascular disease accounts for approximately 17.9 million deaths, cancer for approximately 9.3 million, chronic respiratory disease for 4.1 million, and diabetes for approximately 2 million deaths per year [1]. This data indicates that degenerative diseases are a growing global health problem due to changes in lifestyle, diet, exposure to pollution, and an aging population. One of the main factors contributing to the development of degenerative diseases is oxidative stress [2].

Oxidative stress occurs when the amount of reactive oxygen species (ROS) exceeds the capacity of the body's antioxidant system. ROS are reactive molecules that have unpaired electrons so they easily react with important biomolecules such as lipids, proteins, and DNA [3]. ROS can be formed endogenously through normal metabolism in mitochondria during cellular respiration, or exogenously due to exposure to ultraviolet radiation, cigarette smoke, heavy metals, pesticides, and environmental pollution. Some common types of ROS include: Superoxide radicals ($O_2^{\bullet-}$), Hydrogen peroxide (H_2O_2), Hydroxyl radicals ($\bullet OH$), Singlet oxygen ($^1O_2$). The mechanism of ROS formation begins with the partial reduction of oxygen in the mitochondrial electron transport chain. Oxygen that receives one electron will form a superoxide radical. Next, the superoxide radical undergoes a dismutation reaction to become hydrogen peroxide by the enzyme superoxide dismutase (SOD). Hydrogen peroxide can then react with transition metal ions such as Fe^{2+} through the Fenton reaction,



producing highly reactive and toxic hydroxyl radicals [4]. Uncontrolled increases in ROS can cause lipid peroxidation of cell membranes, protein denaturation, DNA damage, and activation of inflammatory pathways and apoptosis. This damage contributes to atherosclerosis in heart disease, insulin resistance in diabetes mellitus, genetic mutations in cancer, and neuronal degeneration in Alzheimer's and Parkinson's diseases [2].

To protect the body from oxidative damage, antioxidants capable of neutralizing free radicals are needed. Antioxidants work by donating electrons to free radicals, stabilizing the molecules and preventing further cell damage. The body possesses endogenous antioxidant systems such as glutathione, catalase, and superoxide dismutase, but under conditions of severe oxidative stress, additional exogenous antioxidants from food or medicinal plants are required [3]. In recent decades, natural or plant-based antioxidants have received significant attention due to their relative safety, ease of access, and broad biological activity. Plants are known to contain various secondary metabolites with high antioxidant activity, such as phenolics and flavonoids.

Phenolics are the largest secondary metabolites in plants. Phenolics compounds in plants can be phenols, phenolics acids, tannins, and flavonoids [5]. The presence of hydroxyl groups ($-OH$) and double bonds ($C=C$) in phenolics and flavonoids is an important part of their antioxidant capacity.

The human body can naturally produce antioxidants. However, when the number of free radicals exceeds the number of antioxidants in the body, free radicals will attack body tissues. In this condition, the body needs antioxidants from external sources. Natural sources of antioxidants from external sources can be obtained from plants such as pumpkin (*Cucurbita moschata* Durh.) and aibika (*Abelmoschus manihot* L.) [6,7]. These two plants have been widely used as medicinal and food ingredients [8,9].

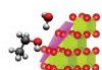
Pumpkin leaves (PL) and aibika leaves (AL) extract each have antioxidant activity [10,11]. This is due to the presence of bioactive compounds such as flavonoids, phenolics, and terpenoids [12]. To increase antioxidant activity, plant extracts or fractions can be combined. Several previous studies have combined plant extracts to increase antioxidant activity, such as

soursop leaves (*Annona muricata*) and guava leaves (*Psidium guajava*) [13], as well as avocado leaves (*Persea americana*) and guava leaves (*Psidium guajava*) [14]. The combination of two or more types of plants containing antioxidants is expected to produce higher antioxidant power due to their synergy [15]. However, to date there has been no research report that specifically evaluates the antioxidant activity of the combination of PL and AL fractions. Existing studies generally still focus on testing the antioxidant activity of single extracts from each plant, without examining the effects of combinations between fractions, especially the ethyl acetate fraction. Furthermore, studies on the interaction of bioactive compounds from the two plants in the form of combined fractions are still very limited, so the mechanisms of potential synergism and increased free radical scavenging capacity are not yet clearly understood.

The combination approach at the fraction level is considered superior to the combination of crude extracts because it can produce better specificity and reproducibility. Fractionation can reduce the complexity of the extract matrix, allowing for more focused and controlled scavenging of compounds with similar polarity, particularly semi-polar compounds in the ethyl acetate fraction, such as flavonoids and phenolic compounds. Based on this, the research hypothesis proposed is that the combination of ethyl acetate fractions from PL and AL will exhibit higher antioxidant activity than either fraction alone, presumably due to the potential synergistic interaction between the phytochemical constituents within the two fractions.

This study analyzed the levels of key bioactive compounds, specifically phenolics and flavonoids. This analysis is important because these two classes of compounds contribute primarily to antioxidant activity through hydrogen or electron donation mechanisms, neutralizing free radicals.

Total phenolic content (TPC) can be determined using the Folin-Ciocalteu method, while total flavonoid content (TFC) is generally analyzed using the aluminum chloride colorimetric method. Both methods are based on UV-Vis spectrophotometry, which allows compound quantification based on absorbance at specific wavelengths. The use of UV-Vis spectrophotometry is relevant because it is simple,



rapid, sensitive, and capable of providing reproducible results for evaluating phytochemical composition at the fraction level. Furthermore, phenolic and flavonoid content data can be used to correlate bioactive compound content with antioxidant activity values, allowing for a more comprehensive analysis of structure-activity relationships.

In addition to TPC and TFC analysis, antioxidant activity was also evaluated using UV-Vis spectrophotometry. This testing can be performed using several redox-based methods, such as DPPH (2,2-diphenyl-1-picrylhydrazyl). The principle of this method is to measure the change in absorbance due to a decrease in free radical intensity, which is then used to determine the percentage inhibition or IC_{50} value.

Thus, the combination of TPC and TFC analysis and antioxidant activity evaluation using UV-Vis spectrophotometry provides a comprehensive approach to understanding the contribution of bioactive compounds and testing the potential synergism of the combination of the two fractions more accurately and measurably.

2 Method

The equipment used includes glassware (pyrex), blender, maceration jar, porcelain dishes, water bath, stirring rod, rotary vacuum evaporator, oven, micropipette, analytical balance, filter paper, aluminum foil, cuvettes, and UV-Vis spectrophotometer.

Plant materials, namely PL and AL were collected from Kendari, Southeast Sulawesi, Indonesia (3°99'S, 122°52'E), at an altitude of approximately 50 m above sea level, leaves were harvested from mature plants in the morning and processed quickly, and botanical authentication was performed at the UHO Biology Laboratory to ensure traceability of research samples. The solvents and reagents used are distilled water, methanol, ethyl acetate, n-hexane, ethanol, aluminum chloride, sodium carbonate, potassium acetate, Folin Ciocalteu reagent, gallic acid, quercetin, and DPPH (Sigma-Aldrich).

2.1.1 Sample Preparation

Samples of PL and AL were sorted to remove impurities. They were then dried in the sun and blended into a powder.

2.1.2 Extraction and Fractionation

350 g of PL and AL powder were each soaked in methanol 3 times for 24 hours with a ratio of simplicia powder to solvent of 1:10 (w/v). The samples were stored in a tightly closed container, protected from direct light, at room temperature. Every 24 hours, stirring was carried out to increase contact between the solvent and the simplicia. Extraction was considered complete when the solvent from the third maceration was almost colorless compared to the first. Next, the obtained extract was concentrated using a rotary vacuum evaporator at 50 °C. The thick extract was dried in an oven at 40 °C.

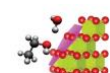
Each extract was fractionated using the partition method. A total of 40 g of methanol extract was diluted with 800 mL of distilled water and placed in a separating funnel. It was then partitioned using n-hexane and ethyl acetate solvents. The fractions obtained were concentrated using a rotary vacuum evaporator at 50 °C and an oven at 40 °C.

2.1.3 TPC Analysis

Standard solutions of gallic acid were prepared with varying concentrations of 10, 20, 30, 40, and 50 ppm. Similarly, sample solutions were prepared by dissolving 10 mg of the sample in methanol to a volume of 10 mL.

Next, 1 mL was taken from each series of gallic acid standard solution and sample solution concentrations, and 0.4 mL of Folin-Ciocalteu reagent was added, shaken, and left for about 8 minutes. Then, 7% Na_2CO_3 solution was added, shaken until homogeneous, and distilled water was added to make up to 10 mL. Measurements were taken at a maximum wavelength of 750 nm in triplicate. A calibration curve was made showing the relationship between gallic acid concentration and absorbance [16].

The gallic acid calibration curve showed excellent linearity with a coefficient of determination (R^2) of 0.9987. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the response and the slope of the calibration curve, and were found to be 2.14 ppm and 6.50 ppm, respectively. The method precision was evaluated through repeated measurements and showed a percentage relative standard deviation (%RSD) of less than 2% across all replicates, indicating



acceptable method precision. The measurement wavelength was set at 750 nm based on preliminary wavelength scan results in the 700–800 nm range, and this condition is in accordance with the published Folin–Ciocalteu protocol for the determination of TPC [17].

2.1.4 *TFC Analysis*

A series of quercetin standard solution concentrations were prepared, namely 20, 40, 60, 80, and 100 ppm. Sample were prepared by taking 10 mg of sample dissolved in methanol to a volume of 10 mL.

For each series of standard and sample solution concentrations, 1 mL was taken and added to 3 mL of methanol, 0.2 mL of 10% aluminum chloride, and 0.2 mL of 1 M potassium acetate, then diluted to 10 mL with distilled water. An incubation time of 30 min after the addition of $AlCl_3$ was applied before absorbance measurements. The wavelength of 432 nm was chosen after initial optimization scans (400–500 nm). The quercetin calibration curve showed excellent linearity with a coefficient of determination (R^2) of 0.9991. These measurement conditions are in accordance with the method reported by da Silva et al. (2015) [18].

2.1.5 *Antioxidant Activity Testing*

Antioxidant activity testing was performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Concentrated extracts dissolved in methanol were prepared in five concentrations, namely 1, 2, 3, 4, and 5 ppm. A 1 mL sample solution of each concentration was taken, added to 3 mL of methanol, and 1 mL of 0.6 mM DPPH [19]. The mixture was then shaken until homogeneous and incubated for 30 minutes at 37°C in a dark room [20]. Absorbance measurements were performed using a UV-Vis spectrophotometer at a wavelength of 513 nm. For the control solution, the same steps were performed, replacing the sample solution with methanol. The absorbance values obtained were used to calculate the percentage of inhibition (%I) according to Eq. 1.

$$\%I = ((A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}) \times 100\% \quad (\text{Equation 1})$$

Then, a calibration curve was created by plotting the inhibition percentage (%I) as the y-axis against the sample concentration as the x-axis to obtain a linear regression equation. The linear regression equation is expressed in the form: $y = ax + b$ where y is the inhibition percentage, x is the sample concentration, a is the slope of the line (slope), and b is the intercept. The coefficient of determination (R^2) value was used to evaluate the linearity of the relationship between sample concentration and inhibition percentage. The IC_{50} value was determined by substituting the y value of 50 into the linear regression equation, then calculating the obtained x value as the concentration capable of inhibiting 50% of DPPH free radicals.

3 Result and Discussion

3.1 *Total Phenolic and Flavonoid Content*

Folin-Ciocalteu reagent is used in the analysis of TPC in samples. During the reaction, the phenolic-hydroxyl group reacts with the Folin-Ciocalteu reagent, forming a blue phosphotungstate-phosphomolybdate complex with an unknown structure that can be detected using a UV-Vis spectrophotometer. The blue color formed will become more intense in proportion to the concentration of phenolate ions formed, meaning that the higher the concentration of phenolic compounds, the more phenolate ions will reduce the heteropoly acid, resulting in a more intense blue color [16].

The TPC was determined based on the gallic acid standard curve. Gallic acid is a derivative of hydrobenzoate, which is a simple phenolic acid that is pure and stable. A standard curve was determined to find the relationship between the solution concentration and its absorbance value so that the sample concentration could be determined. Then, the absorbance value was entered into a linear regression of the standard curve. The TPC of each sample can be seen in Tables 1 and 2 below

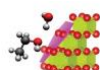


Table 1. Total Phenolics Content of PL Fraction

No.	Fractions	TPC (mean±SD) mg GAE/g
1.	Ethyl acetate	60.81±0.79
2.	n-hexane	43.19±1.22
3.	Water	41.33±1.23

Table 2. Total Phenolics Content of AL Fractions

No.	Fractions	TPC (mean±SD) mg GAE/g
1.	Ethyl acetate	56.47±0.20
2.	n-Hexane	36.90±0.25
3.	Water	32.04±0.21

Based on statistical analysis using one-way ANOVA followed by Tukey's test, a p value <0.05 was obtained, indicating that there were significant differences between the fraction groups regarding TPC. The Tukey test showed that the ethyl acetate fraction of PL and the ethyl acetate fraction of AL had TPC that were significantly different compared to the other fractions. Based on the data, both ethyl acetate fractions showed a higher TPC. This is thought to be due to the semipolar nature of ethyl acetate as a solvent that is able to extract phenolics more optimally. Phenolics are generally semipolar because they have hydroxyl groups in their aromatic structure, so they are more soluble in semipolar solvents than nonpolar or very polar solvents. Therefore, the use of ethyl acetate produces fractions that are richer in phenolics,

which have the potential to contribute to biological activity, especially antioxidant activity [20].

In the analysis of TFC, the sample solution is added with AlCl_3 reagent, which can form a complex between aluminum chloride and the ketone group on the C-4 atom and the hydroxyl group on the C-3 or C-5 atom adjacent to the flavone and flavonol groups, causing a shift in wavelength towards the visible range [21]. This reaction produces a more yellow color of the solution, and the addition of potassium acetate are intended to maintain the wavelength in the visible region. The TFC was calculated using a calibration curve equation with quercetin as the standard. The following are tables 3 and 4 which show the TFC of each fraction of PL and AL.

Tabel 3. Total Flavonoid Content of PL Fractions

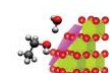
No.	Fractions	TFC (mean±SD) mg QE/g
1.	Ethyl acetate	64.54±0.25
2.	n-Hexane	42.49±0.52
3.	Water	17.58±0.22

Table 4. Total Flavonoid Content of AL Fractions

No.	Fractions	TFC (mean±SD) mg QE/g
1.	Ethyl acetate	55.07±0.39
2.	n-Hexane	40.09±0.55
3.	Water	15.77±0.28

The data above shows that the ethyl acetate fraction of PL and the ethyl acetate fraction of AL also have higher total flavonoids content compared to other fractions. This may be because flavonoids are also semipolar in nature, so they tend to be attracted to semipolar solvents such as ethyl acetate. Based on the results of statistical

analysis using one-way ANOVA followed by Tukey's test, it also produced a p value <0.05, which indicates that there is a significant difference between the fraction groups regarding the total flavonoid content.



3.2 Antioxidant Activity

Antioxidant activity testing can be performed using the DPPH method. The DPPH test serves as an effective initial screening for antioxidants in various sample compounds. When antioxidant compounds interact with DPPH, the antioxidants reduce the DPPH radicals, resulting in a color change from dark purple to yellow, which can be assessed quantitatively [22]. The color change corresponds to the concentration of antioxidants in the tested solution, thus providing accurate data on the antioxidant capacity of a sample. The color

change causes a change in absorbance at the maximum wavelength of DPPH, thereby determining the free radical scavenging activity value expressed as the Inhibition Concentration 50% (IC₅₀).

The IC₅₀ value is the effective sample concentration required to attenuate 50% of the total DPPH. The smaller the IC₅₀ value, the greater the activity. The antioxidant activity of PL and AL fractions can be seen in Table 5 and Table 6

Table 5. IC₅₀ Value of PL Fractions

No.	Fractions	IC ₅₀ (mean±SD) µg/mL
1.	Ethyl acetate	7.040±0.20
2.	n-Hexane	10.13±0.07
3.	Water	12.34±0.10
4.	Ascorbic acid	4.013±0.01

Tabel 6. IC₅₀ Value of AL Fractions

No.	Fractions	IC ₅₀ (mean±SD) µg /mL
1.	Ethyl acetate	7.549±0.008
2.	n-hexane	11.87±0.040
3.	Water	13.32±0.780
4.	Ascorbic acid	4.013±0.010

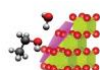
Based on Tables 5 and 6, each fraction of PL and AL showed strong antioxidant activity with an IC₅₀ value ≤50 [23]. PL and AL fractions have antioxidant activity because they contain phenolics and flavonoids. The ability of phenolics compounds as antioxidants is related to the presence of OH groups directly bound to aromatic hydrocarbon rings. Phenolics compounds could donate hydrogen atoms, thereby reducing DPPH free radicals to a more stable form. The more hydroxyl groups a phenolics compound has, the greater its antioxidant activity [24].

The data also showed that the ethyl acetate fraction had a lower IC₅₀ value (greater antioxidant activity) compared to the other fractions. This may be because ethyl acetate is a semi-polar solvent, where phenolic and flavonoid groups are more attracted to acetate solvents due to their similar polarity. Phenolic and flavonoid compounds have a very close relationship with antioxidant activity

because both can donate electrons or hydrogen atoms to neutralize free radicals. The higher the phenolic and flavonoid content of a natural product, generally the higher its antioxidant activity. Pearson correlation coefficients between TPC and IC₅₀, and between TFC and IC₅₀, in the three fractions of the two plant species. Preliminary analysis showed a strong negative correlation between

TPC and IC₅₀ ($r = -0.97$, $p < 0.05$), indicating that phenolics are the main contributors to antioxidant activity in these fractions. The correlation between TFC and IC₅₀ ($r = -0.91$, $p < 0.05$) also indicates a significant role of flavonoids, although slightly less dominant.

The ethyl acetate fraction of each sample was tested for antioxidant activity and compared between the single form and the combination. The results are presented in Table 7.



Tabel 7. IC₅₀ Value of Ethyl Acetate Fractions Combination from PL and AL

No.	Sample	IC ₅₀ (mean±SD) µg/mL
1.	Ethyl acetate fraction of PL (A)	7.040±0.014
2.	Ethyl acetate fraction of AL (B)	7.549±0.091
3.	Fraction combination A:B = 1:1	7.518±0.050
4.	Fraction combination A:B = 2:3	5.305±0.015
5.	Fraction combination A:B = 3:2	4.686±0.009
6.	Ascorbic acid	4.013±0.010

Based on the results of statistical analysis using one-way ANOVA followed by Tukey's further test, $p < 0.05$ was obtained, indicating that there was a significant difference between the treatment groups in the IC₅₀ value. The results of the Tukey test showed that the combination of ethyl acetate fractions of PL and AL with a ratio of 3:2 was significantly different compared to the single fractions or other ratio combinations. Based on Table 7, the 3:2 ratio combination had the lowest IC₅₀ value, thus indicating the highest antioxidant activity. A lower IC₅₀ value indicates a stronger ability to ward off free radicals. The significant difference in IC₅₀ value between the single fraction and the combination is thought to be influenced by differences in the distribution and interaction of secondary metabolite compounds, such as phenolics and flavonoids, which act as antioxidants. The combination of the two fractions allows for a synergistic effect between bioactive compounds, thereby increasing antioxidant capacity compared to the use of a single fraction.

The Combination Index (CI) using the Chou-Talalay method showed that CI = 0.72 for an A:B ratio of 3:2, formally classifying this interaction as synergistic (CI < 1). The synergistic interaction arises from the complementary hydrogen donor capacity of quercetin derivatives from *A. manihot* and lutein/β-carotene from *C. moschata* likely acting at different stages of the radical chain reaction, providing a broader spectrum of radical scavenging activity. Our 33.4% increase in IC₅₀ (from 7,040 to 4,686 µg/mL) compares favorably with the 18.7% increase reported by Wicaksono and Ulfah (2017) for the soursop–guava combination [13], suggesting that the ethyl acetate fraction approach offers superior synergistic potential.

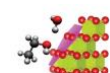
4 Conclusion

Based on the research results, the ethyl acetate fraction of PL (*C. moschata* Durh) and AL (*A.*

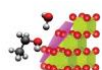
manihot L.) showed the highest total phenolic content (TPC) and total flavonoid content (TFC) at 60.81 and 56.47 mg GAE/g and 64.54 and 55.07 mg QE/g, respectively, which correlated with very strong antioxidant activity. The combination of fractions at a 3:2 ratio provided optimal antioxidant activity with an IC₅₀ value of 4.686 µg/mL, indicating a synergistic effect between the bioactive components. These results confirm that solvent polarity plays an important role in the selectivity of phenolic and flavonoid extraction, especially in the ethyl acetate fraction. These findings demonstrate the potential of plant fractions as a source of natural antioxidants for nutraceutical development. Further research is needed to identify the bioactive compounds causing synergism, assess antioxidant activity in vivo, and evaluate their toxicity and safety aspects.

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