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Research Article

The Effect of Variations in Solvent Type on the Yield and TLC Profile of Flavonoid in *Cosmos caudatus* Leaf Extract

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ABSTRACT

Flavonoids are a group of polyphenols compounds that act as antioxidants. *Cosmos caudatus* leaves are thought to contain flavonoid compounds. Extraction of active compounds in plants can be influenced by the choice of solvent during the extraction process. The purpose of this study was to determine whether there is a significant difference between extraction with ethanol and methanol solvents in *Cosmos caudatus* leaves, and to test the presence of flavonoids in the extract using TLC. The extraction method used was maceration, with each extraction repeated twice with each solvent. The extraction ability of the two solvents is indicated by the extract yield value. The presence of flavonoids is indicated by an Rf value between 0.2 and 0.75. The results showed that there was no significant difference in the extract yield value of the two solvents, with a significance value of 0.5896. Therefore, both solvents are equally strong in extracting natural ingredients. However, both extraction results could not be confirmed as containing flavonoid compounds. This was because the difference in the Rf value of the sample and the Rf of the reference sample was greater than 0.05, thus the results were declared negative for flavonoids. This study demonstrates the importance of eluent selection in TLC testing. For flavonoid testing, the combination of chloroform and ethyl acetate should not be used.

Keywords: Polyphenols, Extraction, Maceration

1. INTRODUCTION

Cosmos caudatus leaves are commonly known as a food ingredient and can grow wild in rice fields. *Cosmos caudatus* leaves also have medicinal properties. This is due to the presence of active compounds, such as flavonoids [1]. Flavonoid compounds are secondary metabolites with varying phenolic structures and can be found in all plant organs and have the potential to have antioxidant and anti-inflammatory activity [2].

The use of *Cosmos caudatus* leaves as a raw material for medicine requires a process of extracting the active compounds from the leaves. Extraction is the process of separating one or more components from a homogeneous mixture using a liquid solvent as a separating agent

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using a specific method [3]. During the extraction process, careful attention is paid to the choice of solvent. Different solvent types affect the yield of the extract [4].

Several studies used *Cosmos caudatus* leaves for biological activity testing or preparation. The extraction process generally involves maceration with 96% and 70% ethanol as solvents [5–7]. Ethanol 96% is commonly used as an extraction solvent because it can dissolve compounds with various polarities, is relatively safe, resistant to microbial growth, and affordable [8]. Ethanol was chosen as a solvent because it is selective, non-toxic, neutral, and has good absorption ability. In addition, ethanol concentrations above 20% can inhibit mold growth. Ethanol is also miscible with water in various proportions, requires lower heat during the concentration process, and dissolves only a limited amount of interfering substances. Therefore, ethanol is used as a solvent because it is suitable for extracting phenolic compounds [9].

Methanol is an organic solvent that is often used to extract phenolic compounds, including flavonoids, so it is also widely used in the extraction process [10]. Methanol, with its higher polarity than ethanol, is more effective in extracting polar compounds [11]. Methanol is used as an extraction solvent because it almost completely dissolves the organic compounds present in the sample. Furthermore, it is volatile, making it easy to remove from the extract and is also capable of better dissolving phenolic compounds, resulting in higher levels in the extract [12]. However, it is important to consider the toxicity of methanol. The purpose of this study was to compare the extraction results of *Cosmos caudatus* leaves using 96% ethanol and methanol solvents by examining the yield and chemical profile of flavonoid compounds through TLC. This research is important because the type and concentration of solvent significantly affect the extraction results of bioactive compounds. Research on Opuntia stricta Haw fruit showed that a less polar solvent (80% acetone) produced the highest levels of polyphenols, flavonoids, and antioxidant activity, although the extract yield was the lowest [13]. However, another study on Zanthoxylum planispinum Var. Dintanensis leaves concluded that extraction with water resulted in a higher total flavonoid content compared to extraction using ethanol [14].

Studies have examined solvent variations in the extraction process of *Cosmos caudatus* leaves, but these studies focused on their effect on antioxidant activity [15]. Other studies have compared methanol and ethanol extracts of *Cosmos caudatus* leaves for their

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antioxidant properties [16]. However, no study has examined the effect of varying solvent types in *Cosmos caudatus* leaf extraction on yield and the presence of flavonoid compounds through TLC.

2. EXPERIMENTAL SECTION

2.1. Materials

The material studied was *Cosmos caudatus* leaves taken from Cepu City. Other materials used in this study were 96% ethanol (technical), methanol (technical), quercetin (Sigma-Aldrich), chloroform (Merck), ethyl acetate (Merck), and distilled water. In addition, several consumables were needed, including capillary tubes (Asian), filter paper (Whatman), TLC plates (Silica GF254), and aluminum foil (Total Wrap).

2.2. Instrumentation

The sample preparation process required an oven (Kirin), a blender (Philips), a basin, microscope (Binocular Olympus), glass objects, glass slides, and a 45-mesh sieve (RETSCH Test Sieve). The extraction process required the following equipment: a maceration vessel, a glass stirrer, a beaker (Herma), an analytical balance (MRC Lab), a measuring cylinder (Herma), rotary evaporator (IKA RV 10) and a glass funnel. The TLC test phase used a TLC chamber (Duran), a dropper, a measuring cylinder (Herma), a beaker (Herma), and a UV lamp (Spectroline).

2.3. Procedure

2.3.1. Sample Preparation

Five kilograms of fresh *Cosmos caudatus* leaves were oven-dried at 50°C for one day. The use of a temperature of 50°C is due to the optimal drying temperature for medicinal plants. Temperatures exceeding 50°C will reduce color quality, and temperatures below 50°C will reduce drying time and energy efficiency [17]. The dried leaves were then ground using a blender. Grinding dry leaves is very important because the grinding stage greatly influences the extraction yield and total phenolic content [18]. Apart from the grinding process, there are other factors that can increase the active compounds extracted, namely sieving [19]. The sieving process for the *Cosmos caudatus* leaf powder uses a 45-mesh sieve. A sufficient amount of *Cosmos caudatus* leaf powder was taken for microscopic testing.

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Microscopic testing is performed by placing the powder on a glass slide, adding a drop of clarifying reagent, and then covering it with a cover slip. Afterward, the powder is observed under a microscope and identified fragments are identified [20]. Clarifying reagents that can be used are chloral hydrate and distilled water. In this study, the clarifying reagent used was distilled water.

2.3.2. Extraction

200 grams of dried leaf powder was extracted by maceration. Maceration is a simple extraction method and is suitable for the extraction of thermolabile compounds, such as polyphenolic compounds [21,22]. One group of polyphenol compounds is flavonoid compounds, such as quercetin, kaempferol, catechin, and anthocyanin [23]. In this study, the solvents used were varied, namely 96% ethanol and methanol. Alcohols (ethanol and methanol) are universal solvents in solvent extraction for phytochemical investigation [24]. The maceration process was repeated twice for each solvent. The solvent required for each maceration process was 700 mL. Maceration was carried out for 3 days with daily stirring. After 3 days of maceration, the next stage was filtration. The filtrate obtained was evaporated using a rotary evaporator until the extract was dry. The dry extract obtained is then used to find the extract yield (Eq. 1). Extract yield is a measure of the efficiency of the solvent to extract specific components from the original material [25,26].

$$\text{Extract yield} = \frac{\text{extract weight}}{\text{weight of simplicia powder}} \text{ x } 100\% \qquad \text{(Eq. 1)}$$

2.3.3. TLC Test

The TLC test used an 8 x 3 centimeter TLC plate and 2 TLC plates were prepared. 1 gram of dry extract was taken and dissolved in each solvent during extraction as much as 3 mL. A quercetin solution was also prepared as a standard solution. In preparing the quercetin solution, 1 gram of quercetin powder was weighed twice and dissolved in 96% ethanol and methanol solvents. The mobile phase required in this TLC test used a combination of chloroform and ethyl acetate solvents with a ratio of 7:3 (self-optimization results). The eluent affects the Rf value. The more polar the eluent, the higher the Rf value [27]. The Rf value of flavonoid compounds is between 0.2 and 0.75 [28]. A difference between the sample Rf and the reference Rf of <0.05 indicates that the sample contains the desired compound. However,

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a difference between the sample Rf and the reference Rf of ≥0.05 indicates that the sample contains the desired compound [29]. The calculation of the Rf value can be seen in Eq.2 [28].

$$\text{Rf} = \frac{\text{the distance traveled by the compound from the point of origin}}{\text{the distance traveled by the solvent from the point of origin}} \qquad \text{(Eq.2)}$$

2.3.4. Data Analysis

The data obtained in this study included yield data and Rf values. Each research data was then analyzed using a T-test. This test was conducted to conclude whether there were significant differences or not from the results of the treatment variations. The treatment variation in this study was by varying the type of solvent during extraction. The T-test was conducted using Ms. Excel. The T-test is used to compare the mean values of two groups. The criteria used are: if the significance value is > 0.05, then Ho is accepted and Ha is rejected. Conversely, if the significance value is < 0.05, Ho is rejected and Ha is accepted [30].

3. RESULTS AND DISCUSSION

The obtained *Cosmos caudatus* leaf powder was examined microscopically to confirm its authenticity. Several characteristic fragments were found in the *Cosmos caudatus* leaf powder (Figure 1). Microscopic tests are carried out using a microscope to identify the characteristic fragments of each simple drug [31]. Microscopic examination of leaves generally shows the presence of identification fragments such as covering hairs, vascular bundles and lower epidermis with stomata [20].

(a) (b) (c)

Figure 1. Microscopic results; (a) Lower epidermis with stomata, (b) Vascular bundle with ladder-type thickening, and (c) Leaf mesophyll with covering hairs.

Microscopic examination results revealed several fragments, and these fragments matched the characteristic fragments of *Cosmos caudatus* leaves listed in the Indonesian Herbal Pharmacopoeia. This confirmed the authenticity of the tested samples, which were leaves from the *Cosmos caudatus* plant. The results of this microscopic test were less than optimal (not clear). This was due to the inaccurate choice of clarifying reagent (distilled water).

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Distilled water is more suitable for observing characteristic fragments in starch, while chloral hydrate is best used to observe fragments in plants other than starch. Chloral hydrate dissolves cell contents and intercellular substances, making them easier to observe [32]. This study intentionally used distilled water as a clarifying reagent to see how well this reagent could identify fragments in leaf powder. Based on these results, it is recommended that chloral hydrate be used for microscopic testing of leaf powder.

3.1 Extraction

This study aimed to determine whether varying the solvent type during the extraction process had an effect on the yield. The choice of extraction method used needs to be considered because it will affect how the active substance of the herbal medicine is broken down and keep the active substance stable, so that the therapeutic effect of the herbal medicine is maintained [12]. The extraction method used was maceration. The maceration method was chosen because this extraction method is the simplest [8]. The maceration method is particularly suitable for materials that cannot withstand high temperatures, and the equipment used is very simple and uncomplicated [33].

Figure 2. The color of the liquid extract of *Cosmos caudatus* leaves

The maceration method is a cold extraction method that uses a solvent to penetrate the plant cell walls and enter the cell cavities containing the active ingredients. This forces the active ingredients, which are concentrated solutions, out of the cells due to the difference in concentration between the active ingredient solution inside and outside the cells [34]. The maceration method was chosen for extraction because the leaves are soft and easily expand in the extraction liquid [9]. Two solvents were used: 96% ethanol and methanol. Each

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extraction process with each solvent type was repeated twice. The maceration process was carried out for three days. After three days of extraction, the liquid extract was obtained, as shown in Table 1. The color of the liquid extract from both extraction processes is the same, namely blackish green (Figure 2).

Table 1. Lliquid extract of *Cosmos caudatus* leaves

Sample	Replication (R)	Volume (mL)
96% ethanol extract	R1	315
	R2	325
Methanol extract	R1	335
	R2	330

The amount of solvent used for the extraction process changed at the end of the extraction, namely to around 300 mL (Table 1). This decrease in the amount of solvent is due to the volatile nature of all solvents used. High temperatures can cause loss of solvent [24]. The evaporation process is expected to occur during the stirring process. However, in all repetitions, it can be concluded that there was no significant difference in the amount of liquid extract. This is due to the results of the statistical test which showed a p value > 0.05 (p of 0.1548), meaning there was no significant difference between the 96% ethanol liquid extract and the methanol liquid extract.

The liquid extract was thickened using a rotary evaporator, producing a dry extract. After obtaining the dry extract of the *Cosmos caudatus* leaves, the yield for each extraction process was determined, as shown in Table 2.

Table 2. Yield of extraction

Sample	Replication	Weight of leaf simplicia powder (g)	Dry extract weight (g)	Yield (%)
96% ethanol extract	R1	200	15.76	7.88
	R2	200	18.45	9.22
Methanol extract	R1	200	17.53	8.76
	R2	200	18.5	9.25

The yields obtained in all extraction processes were not identical. However, statistical analysis of the two solvent types concluded that there was no significant difference in yield. This is indicated by a p-value > 0.05 (p-value of 0.5896). This is because ethanol and methanol solvents are universal solvents and are recommended for the extraction process [35]. Methanol and ethanol solvents are able to extract phenolic compounds in plants, especially

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phenolic compounds in the form of glycosides [34]. The yield of all extraction results complies with the quality requirements for extract yield in the Indonesian Herbal Pharmacopoeia, which is not less than 6.8% [36].

3.2 TLC Test

Thin layer chromatography (TLC) is a method of separating compounds in a sample by using an eluent (mobile phase) to move the compounds in the sample that are spotted on the stationary phase on the TLC plate [37]. Thin Layer Chromatography (TLC) is a simple, inexpensive method with high separation speed [32]. Thin-Layer Chromatography (TLC) is a simple and economical method commonly used in natural product analysis. This technique is effective for chemical and biological screening of herbal ingredients, and is also useful for monitoring the isolation of active compounds based on biological activity [38]. The TLC test results from the two extractions can be seen in Figure 2 and Table 3.

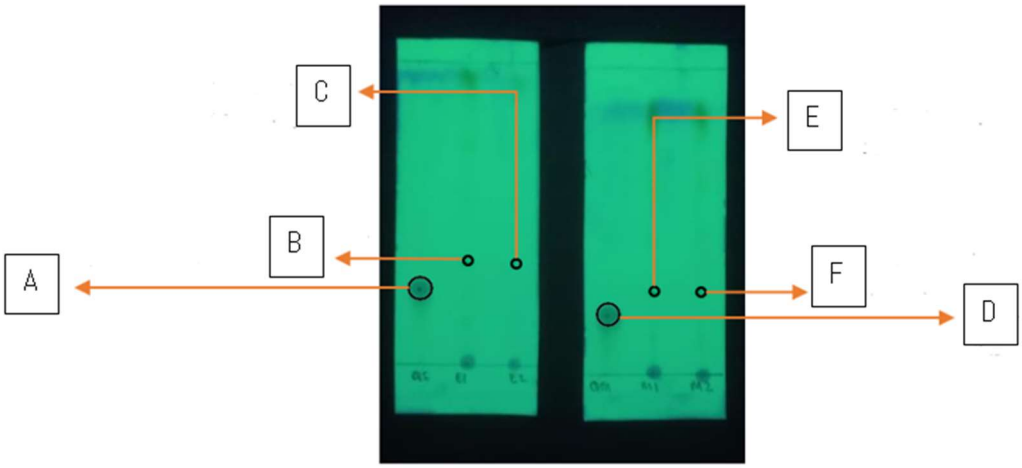

Figure 2. TLC test results; (A) quercetin in 96% ethanol solvent, (B) compound spots in 96% ethanol extract (R1), (C) compound spots in 96% ethanol extract (R2), (D) quercetin in methanol solvent, (E) compound spots in methanol extract (R1), (F) compound spots in methanol extract (R2).

The TLC test results showed a spot on the TLC plate. The spot on the TLC plate between the reference (quercetin) (Fig.2(A and D)) and the sample compound (Fig.2(B-C and E-F) was not significantly different. However, the color of the sample spot (extracted) was less distinct than the color of the reference spot. Indistinct TLC spot colors can be caused by the choice of eluent or mobile phase during the TLC test. Generally, the eluent used for TLC tests to identify the presence of flavonoid compounds is a solvent ratio of n-hexane and ethyl acetate with

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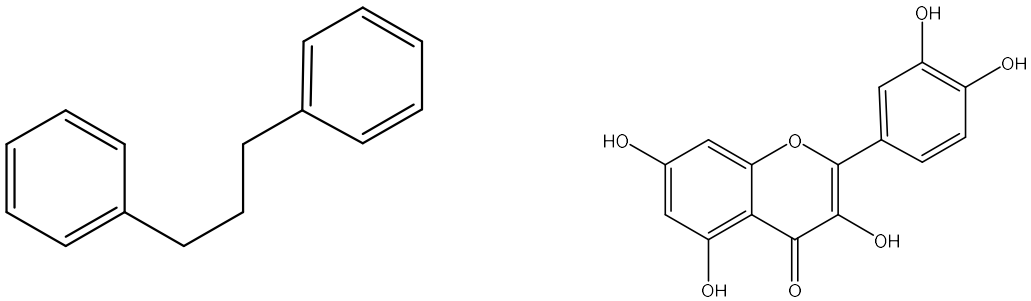
various ratios, one of which is 3:7 [34]. While in this study, the eluent ratio used was chloroform and ethyl acetate, namely 7:3. The choice of eluent, or mobile phase, in TLC is crucial. This is because the eluent, as a solvent, plays a crucial role in the efficiency of secondary metabolite extraction. Each class of secondary metabolite requires specific solvent properties [39]. This study used a combination of nonpolar and semipolar solvents, but the nonpolar solvent used was different, namely n-hexane was replaced with chloroform. Another difference lies in the solvent ratio. In the reference, the amount of semipolar solvent is greater than the nonpolar solvent, while in this study the reverse is true. This indicates that the flavonoid TLC test is better using a larger amount of semipolar solvent. Therefore, it is necessary to conduct TLC testing using a combination of chloroform and ethyl acetate 3:7 to determine whether the TLC spot color becomes clearer.

Quercetin was used as a reference because it is an example of a flavonoid compound [23]. Flavonoids are polyphenolic compounds consisting of 2 aromatic rings (rings A and B) connected by a 3-C chain that forms an oxygenated heterocyclic ring (ring C) and has a general structure of C6-C3-C6 [40]. A general depiction of the structure of flavonoid compounds can be seen in Figure 3 (a). The structure of the quercetin compound can be seen in the Figure 3 (b).



(a) (b)

Figure 3. (a) Flavonoid compound structure [34] and (b) Quercetin [41]

The TLC method is a separation method that can be qualitative or semi-quantitative. The semi-quantitative aspect of the TLC test lies in calculating the Rf value. The Rf value is the ratio of the distance traveled by a compound on the surface of the stationary phase divided by the distance traveled by the solvent as the mobile phase. Rf values vary depending on the nature of the eluent used and the compounds being separated. The separated compounds will be separated based on the distribution of the compounds in the stationary or mobile phase [42]. Table 3 shows that both extraction processes did not indicate the presence of flavonoid compounds. This is because the difference between the Rf value of the sample and the Rf of

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the reference sample is greater than 0.05. If the sample Rf value is ≤ 0.05, it is considered positive for the presence of the test compound; if it is > 0.05, it is considered negative for the presence of the test compound [43].

Table 3. Rf value of extraction results

No.	Sample	Replication (R)	The distance traveled by the compound from the point of origin	The distance traveled by the solvent from the point of origin	Rf
1	Quercetin in 96% ethanol solvent		1.6	6.5	0.25
2	96% ethanol extract	R1	2.2	6.5	0.34
		R2	2.15	6.5	0.33
3	Quercetin in methanol solvent		1.3	6.5	0.2
4	Methanol extract	R1	1.7	6.5	0.26
		R2	1.75	6.5	0.27

The results of the extraction of *Cosmos caudatus* leaves using 96% ethanol and methanol solvents showed no significant differences in yield values. However, neither extract showed any flavonoid compounds. This is likely due to the use of a different eluent in the TLC test than usual, namely a combination of chloroform and ethyl acetate instead of ethyl acetate and n-hexane. Therefore, the selection of eluent combinations in the TLC test is crucial for proper separation of compounds on the TLC plate.

4. CONCLUSION

This study concluded that solvent selection is crucial for the extraction process, as well as for the eluent or mobile phase in the TLC test. The results showed that 96% ethanol and methanol had nearly equal extraction efficiency, with no significant differences in their yields. However, the use of chloroform and ethyl acetate as eluent in a 7:3 ratio in the TLC test failed to detect the presence of flavonoids. The results of this study can inspire further research in the extraction process from other plants. Furthermore, similar studies should be complemented with an assessment of the chemical profile of the extraction results, such as using LC-HRMS (Liquid Chromatography - High Resolution Mass Spectrometry). This is done

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to determine whether the active compounds extracted from both solvents are the same or not.

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AUTHOR CONTRIBUTIONS

The first author drafted the article and calculated the research data. The second and third authors proofread the draft and directed the discussion. The second author was also responsible for submitting the article and its content, reflecting the research conducted.

CONFLICT OF INTEREST

All authors in this article have no conflict of interest.

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