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Quantification of Total Flavonoids in Morning Glory (*Ipomoea carnea* Jacq.) Leaves Extract

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Abstract

Flavonoids, saponins, xanthoproteins, triterpenoids, and tannins are among the secondary metabolites found in Morning Glory (*Ipomoea carnea* Jacq.) leaves. *I. carnea* has antibacterial, anti-inflammatory, anti-allergic, analgesic, and anti-cancer activities. To obtain optimum results, these secondary metabolites can be extracted using suitable solvents. The method, solvent, temperature, and extraction time are all factors that influence the extraction process, and these all have an impact on the yield amount and extract quality. The objective of this study was to investigate the most effective solvent and soxhletation time for the total flavonoid content of *I. carnea* leaves extract. This was a quantitative study that used a pre-experimental design. Time and soxhletation solvent were examined as independent variables on total flavonoid levels. Methanol, ethyl acetate, and n-hexane were utilized as solvents, and the soxhletation times were 1, 3, and 5 hours. The results of this study could be concluded that the highest total flavonoid level obtained was 5.12 ± 0.06%, using methanol Soxhletation at 5 hours. So it can be one of the best suggestion for getting the optimal total flavonoid level using methanol solvent for soxhletation as long as 5 hours for the extraction proccess.

INTRODUCTION

Morning Glory, also locally known as "Krangkungan" or "Kangkung Hutan", is one of the plants that receives little attention because it is only used as a source of livestock feed. Although it is used as feed for livestock, it should be used with caution because it is toxic to livestock (Ganjari, 2016). The phenolic group is the most prevalent secondary metabolite content in *I. carnea* plants, and it is typically found in flowers and stems. Flavonoids, particularly catechol and quercetin, are secondary metabolite chemicals found in flowers, leaves, and stems. Flavonoids have antibacterial, anti-inflammatory, anti-allergic, and anti-cancer activities (Widyaningrum, et al, 2021)..

To obtain the optimum results, the metabolites should be extracted with an appropriate solvent. The solvent type, method, and extraction time are factors that influence the extraction process, yield, and extract quality (Xiao, et al, 2015). This underlies our research interest in quantifying total flavonoid, particularly in the *I. carnea*. Soxhletation was chosen as the extraction method, with the solvent and extraction time varied. The advantages of this procedure include more comprehensive extraction of secondary metabolites because it is done frequently, saving solvent, time, and the sample quantity. The extraction solvents used might affect the yield and secondary metabolites produced (Pratama, et al, 2017). N-hexane, ethylacetate, and methanol are solvents widely

used for extraction depending on polarity levels ranging from low to high. The solvent n-hexane was chosen because of its low boiling point (65–70 °C), and rapid evaporation, so it is appropriate for soxhletation extraction (Susanti, et al, 2016). Ethylacetate was chosen based on its semi-polarity, low toxicity, volatility, and boiling point (around 77 °C). Methanol was used due to its advantages of being universal and capable of extracting polar, semipolar, and nonpolar secondary metabolites. Besides, methanol has low boiling point of around 64.7 °C, making it excellent for soxhletation extraction without decomposing the metabolites (Lenny, 2016).

The ideal extraction period might yield the optimum secondary metabolites. Long extraction time might affect the decomposition of the bioactive components, but short time might be inefficient (Budiyanto, et al, 2018). Generally, the saturation times used were 1, 3, and 5 hours. The soxhletation time was chosen following Wahyuni's (2021) work, which found that between 1 and 3 hours of soxhletation time increased the metabolites yield. According to Budiyanto and Yulianingsih (2018), the optimum extraction period produces optimum bioactive components from plants. The objective of this research was to investigate the effect of extraction solvent type and time on total flavonoid levels in *I. carnea* leaves extract. The differents of this research from the others are this research focus on solvent and time soxhletation optimation to total flavonoid level on *I. carnea* extract. The optimal solvent and time extraction will be measured by the highest total flavonoid level on *I. carnea* extract.

MATERIALS AND METHODS

The study utilized the equipment as follows: analytical balance (Ohaus), water bath (Memmert Wnb 6), UV-Vis spectrophotometer (Shimadzu, UV-1280), set of soxhlet and glass tools (Pyrex®), chamber, and UV lamps (254 and 366 nm). The materials used in this study are Morning Glory leaves (*Ipomoea carnea* Jacq.), aquadest, methanol, ethanol, ethylacetate, n-hexane, acetic acid, HCl, zinc powder, FeCl3, quercetin standard, which were purchased from Sigma®, and TLC plates (silica gel GF254).

Plant Determination

I. carnea plant determination was carried out in Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT), Tawangmangu to ensure the validity of plant species taken as samples.

Simplicia Standard Parameter Analysis

The macroscopic organoleptic observation of *I. carnea* leaves simplicia powder covered the physical characteristics of colour, texture, smell and flavor. Dry simplicia water levels were determined gravimetrically by drying in oven at of 105℃ for 30 minutes until the weight was stable (Dirjen POM, 2000).

Simplicia Extraction by Soxhlet

I. carnea leaves from Central Java Province, Indonesia, were collected, selected, washed, wet sorted, and drained. The leaves then dried under sunlight indirectly (covered with black clothes) to prevent the active substance decomposition. Dry leaves were dry sorted, refined and extracted by Soxhlet using solvents of different polarity levels, namely methanol, ethylacetate, and n-hexane. The soxhletation time was also varied, namely 1, 3, and 5 hours at 60 0C each. The filtrate was evaporated using a rotary evaporator and aired until each solvent evaporates and a thick extract was obtained. The thick extract was stored in a desiccator for further drying. This process was modified from Widyaningrum et al, (2021) and modified into three solvents for the extraction process using the soxhletation method.

Phytochemical Screening

Phytochemical screening test using the tube method to identify the extracted active compounds, such as flavonoids, alkaloids, steroids, terpenoids, saponins, tannins and polyphenols.

Qualitative Test of Flavonoids by Thin Layer Chromatography (TLC)

TLC analysis of flavonoids was to identify the flavonoid compounds presence in extracts and simplicia. The mobile phase used was butanol: glacial acetic acid: water (4:1:5). Flavonoids were detected using AlCl3 and cytoroborate reagents. Both the elution results and detection with spray reagents were observed for spots produced under 254 and 365 nm UV lights. Positive results containing flavonoids were indicated by a yellow color change after being sprayed with a reagent detector. Quercetin was used as standard due to it is a flavonoid of the flavonol group that has a keto group at C-4 and has a hydroxyl group on neighboring C-3 or C-5 atoms of flavones and flavonols (Islamiyati and Saputri, 2018).

Quantification of Total Flavonoids with a UV-Vis Spectrophotometer (Pratiwi, 2020)

Determination of the maximum wavelength of quercetin standard solution

Three ml of quercetin stock solution (1,000 ppm), 0.2 ml AlCl3, and 0.2 ml acetic acid, respectively, were accurately added into 5 ml

volumetric flask, then aquadest up to 5 ml. The final quercetin concentration was 15 ppm. Blanks were made from the mixture of ethanol, AlCl3, acetic acid and aquadest. The absorbance was measured using visible spectrophotometry at a wavelength of 370-500 nm.

Determination of operating time (OT)

Pipette 0.15 ml (15 ppm) from the stock quercetin solution then add ethanol up to 10 ml. From the resulting solution, pipette 1.5 ml, add 0.2 ml AlCl3, 0.2 ml acetic acid and aquadest up to 5 ml. The absorbance of the solution was measured at the maximum wavelength obtained every 5 minutes with a time interval of 25 minutes.

Determination of the quercetin standard curve

The standard curve solution was made from 15 ppm solution into several series of concentrations and then put into a 5 ml volumetric flask by pipetting 1.5 ml of the solution from each; 2.1 ml; 2.8 ml; 3.5 ml; and 4.1 ml (4.5; 6.5; 8.5; 10.5 and 12.5 ppm) then added with 0.2 ml AlCl3, 0.2 ml acetic acid and distilled water. Each concentration was left for 15 minutes (OT), and absorbance readings were taken at the maximum wavelength (414 nm).

Determination of total flavonoid levels

Each stock solution of 10,000 ppm extract samples was made in ethanol. As much 0.1 ml of the solution, 0.2 ml AlCl3, and 0.2 ml 1M acetic acid, respectively, were accurately added into volumetric flask and distilled water up to 5 ml, left for 15 minutes (OT) at room temperature then measured the absorbance on maximum wavelength. Repetition was carried out 3 times.

RESULTS AND DISCUSSION

Simplicia Standard Parameter Analysis

The organoleptic test results of *I. carnea* leaves simplicia powder were brownish green with a rough texture, had a distinctive tea-like odor and tasteless. Water level determination aimed to provide a minimum limit or range for the amount of water level in simplicia. The Indonesian Ministry of Health (2008) sets a standard for simplicia water level of less than 10%. The results of the analysis of water level in *I. carnea* leaves simplicia had met quality standards with an average value of 6.8%.

***Ipomoea carnea* Jacq. Leaves Extraction**

Ipomoea carnea leaves dry simplicia extraction was performed using the soxhletation method with different solvents, namely n-hexane, ethyl acetate, and methanol. Additionally, variations in soxhletation duration were applied, specifically 1, 3, and 5 hours. Each soxhletation process was conducted at 60°C to prevent the mass transfer coefficient decreasing. Temperatures above 75°C can cause the coefficient decreasing due to the being close to the flavonoids boiling point. This proximity can lead to the evaporation of flavonoid compounds and decreasing their levels (Purwanti, et al, 2016). Each filtrate was collected and evaporated by water bath at 50 °C until the thick extract was achieved.

Table 1. *Ipomoea carnea* leaves extract yield

Extract	Yield (%)		
	1 h	3 h	5 h
Methanol	19.30	22.40	32.10
Ethylacetate	2.50	5.40	6.50
n-Hexan	8.20	8.34	9.30

The most efficient extraction was achieved using methanol as the solvent and a soxhletation time of 5 hours, resulting in a yield of 32.10% (Table 2). Flavonoids are classified as polar chemicals due to their high content of sugar groups (glycosides), which possess polarity. Consequently, they readily dissolve in polar solvents like methanol (Widyaningrum, et al, 2021). Variations in the kind of dissolution will impact the quantity of extract that is obtained. Methanol is a versatile solvent capable of binding all chemical constituents present in natural plant materials, including non-polar, semi-polar, and polar compounds. Methanol is a permeable fluid that readily penetrates cells by crossing the cell membrane, allowing for the complete extraction of secondary metabolites contained in the cytoplasmic solvent (Lenny, 2016). The significant yield of *I. carnea* leaves extract using methanol as a solvent indicated that methanol was more effective in extracting chemicals. This is due to the similarity in polarity between the compounds and the solvent.

Secondary Metabolites Qualitative Analysis

Phytochemical Screening

Qualitative analysis of the presence of secondary metabolites was carried out by phytochemical screening of simplicia powder and thick extract. The identification results of secondary metabolites are shown in Table 2.

The phytochemical analysis of simplicia powder was positive for the presence of flavonoids, saponins, tannins, polyphenols, and alkaloids. The methanol extract contained flavonoids, saponins, tannins, polyphenols, and alkaloids, whereas the ethyl acetate and n-hexane extracts contained flavonoids, tannins, and polyphenols. The findings were in line with Abriyani's (2021) research, which indicates that *I. carnea* leaves contain flavonoids, saponins, polyphenols, and alkaloids. The

compounds extracted in methanol differ from both ethylacetate and n-hexane due to variations in their polarity levels, which cause the presence of unextracted compounds. Polar solvents have a greater capacity to extract polar chemicals compared to semi-polar and non-polar solvents. Polar chemicals exclusively dissolve in polar solvents, including ethanol, methanol, butanol, and water. Non-polar chemicals exclusively dissolve in non-polar solvents, such as ether, chloroform, and n-hexane (Budiyanto, dan Yulianingsih, 2018).

Thin Layer Chromatography (TLC) Analysis

Thin Layer Chromatography (TLC) analysis of the compound using the stationary phase silica gel GF254 and the mobile phase butanol: acetic acid: water (4:1:5), produced spots with RF values shown in Table 3.

Table 3. RF and HRF values of *I. carnea* leaves extracts

Analytes	Length (h)	RF	HRF
Quercetin std.	-	0.875	87.5
Methanol extract	1	0.937	93.7
	3	0.937	93.7
	5	0.937	93.7
Ethylacetate extract	1	0.856	85.6
	3	0.843	84.3
	5	0.875	87.5
n-hexane extract	1	0.937	93.7
	3	1.000	100.0
	5	0.968	96.8

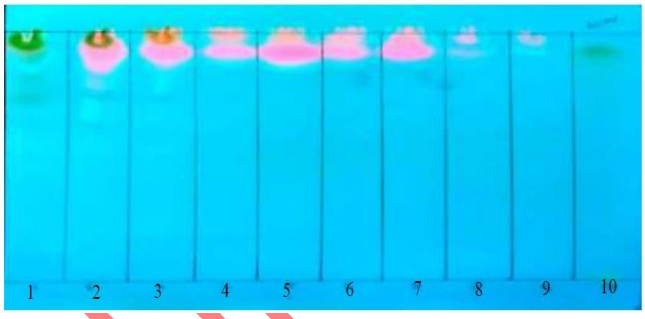

Figure 1. TLC profile of *I. carnea* leaves extract. 1. Methanol extract 1h; 2. Methanol extract 3h; 3. Methanol extract 5h; 4. Ethylacetate extract 1h; 5. Ethylacetate extract 3h; 6. Ethylacetate extract 5h; 7. Hexane extract 1h; 8. Hexane extract 3h; 9. Hexane extract 5h; 10. Quercetin standard.

TLC analysis of flavonoids was using the GF silica gel stationary phase. Before use, the GF silica plate was activated first by heating it at 70˚C for an hour. GF (gypsum flourescence) silica is silica gel with a binder and fluorescence indicator. This type of silica gel usually fluoresces greenish when viewed in short wavelength ultraviolet light. The stationary phase used was silica gel which was polar, while the mobile phase (eluent) used was n-butanol: acetic acid: distilled water (4:1:5) which was very polar because it contained much water. The choice of eluent is based on the polarity of each solvent, where polar active compounds will be more easily eluted by a polar mobile phase than a non-polar mobile phase. On the other hand, non-polar compounds are more easily eluted by non-polar mobile phases (Purwanti, et al, 2016).

Extracts were positive for containing flavonoids if there were yellow spots, and positive for phenolics if there were red, blue or purple spots. The results of the identification of flavonoids in extract samples using citroborate and AlCl3 spray reagents were positive for containing flavonoids with clearer yellow spots after being sprayed. The yellow spots detected in *I. carnea* leaves extract were compared with the yellow color in the standard quercetin solution so that it was suspected to contain flavonoid compounds of the type flavonols and flavones found in *I. carnea* leaves. Flavonoid compounds produce fluorescence in 366 nm UV light and show yellow, green or blue fluorescent colors. TLC identification of flavonoids from *I. carnea* leaves extracts produced RF value that was closely to the standard RF value of quercetin, namely 0.875. Seen in table 9, the RF value of ethylacetate extract is the closest to the standard RF value of quercetin. The RF results with n-hexane solvent with soxhletation times of 3 and 5 hours produced different RF values. This might be caused by the flavonoid compounds contained in both extracts having different structures to quercetin as standard (Susanti, et al, 2016).

Total Flavonoids Content of *I. carnea* Leaves

The determination of maximum wavelength was carried out by using quercetin as standard due to most widely distributed flavonoid compound found in plants. Quercetin and its glycosides are approximately 60-75% of the flavonoids. Quercetin is also a flavonoid compound that can react with AlCl3 to form a complex (Kelly, 2015) which maximum wavelength obtained in this study was 414 nm. This is not much different from the research of Kumalasari (2018) and Alfiansyah (2017) who obtained the maximum wavelength of the quercetin complex, namely 420 nm and 416 nm, respectively. However, all these results are in accordance with the theory which states that the maximum wavelength of quercetin is between 380-560 nm. The results of the operating time measurements showed that the absorbance was stable at 15 minutes. These results are in line with research (Indriyani, 2018) which determined a

stable operating time of 16 minutes. This absorbance stability indicates that the complex formation reaction is optimal.

Table 1. *I. carnea* leaves extracts phytochemical screening

Metabolites	Reagent; Positive Indication	Simplicia	Extract		
			Methanol	Ethylacetate	n-Hexan
Flavonoids	Mg powder + HCl; red color[a]	+	+	+	+
Saponins	Hot aquadest + HCl; stable foam[a]	+	+	-	-
Tannins	$FeCl_3$ 10%; dark blue or greenish black color[b]	+	+	+	+
Polyphenols	Hot aquadest + $FeCl_3$ 10%; dark blue or greenish black color[b]	+	+	+	+
Steroids	Bouchardat; bluish green ring[c]	-	-	-	-
Triterpenoids	Bouchardat; brownish or violet ring[c]	-	-	-	-
Alkaloids	Mayer; orange precipitate[d]	+	+	-	-
	Dragendrof; yellow precipitate[d]	+	+	-	-

Note : (+) = positive ; (-) = negative; a : Depkes RI, 1995; b : Robinson, 1991; c : Ciulei, 1984 ; d : Farnsworth, 1966.

The linear regression equation of quercetin standard curve using visible spectrophotometry ws Y= 0.0708x + 0.0224. The correlation coefficient (r) value shows the linearity relationship between two variables. The standard quercetin solution obtained a relationship between concentration and absorbance with a value of 0.989. An r value that is close to 1 indicates that there is a good linear relationship between these variables (Christian, 2014).

The absorbance readings were taken at the maximum wavelength 414 nm . The addition of AlCl3 in this study aims to detect the presence of 7-hydroxyl groups which can form complex colors, while the incubation treatment for 15 minutes before measurement is intended so that the reaction runs perfectly, thereby providing maximum color intensity. The addition of acetic acid aims to maintain the wavelength in the visible area (Chang, et al, 2013). The results of determining total flavonoid content was shown in Table 4.

The results of the assay showed that the highest flavonoid content was obtained using methanol as solvent and soxhletation time of 5 hours. Methanol had a higher total flavonoid content than ethylacetate and n-hexane because the ability and properties of the solvent in dissolving flavonoid compounds vary, depending on the level of polarity of the extracted compound. Flavonoids are generally polar components because they have sugar attached, therefore flavonoids tend to prefer polar solvents, which shows a tendency that the more polar the solvent, the higher the flavonoid content that is extracted. Flavonoid compounds are divided into several types, each type has different polarity depending on the number and position of hydroxyl groups, which will affect the solubility of flavonoids in solvents. Methanol is able to attract a greater number of secondary metabolites such as phenolic compounds, flavonoids and tannins compared to ethanol. Apart from that, methanol solvent also has a boiling point that is not too high so it can be used to extract flavonoids by soxhletation without destroying the compound components which are susceptible to high heating, which is what produces high levels of flavonoids.

Tabel 4. Total flavonoids content

Extract	Length (h)	Total Flavonoids (%) (Mean±SD; n=3)
Methanol	1	2.80 ± 0.068
	3	4.91 ± 0.058
	5	5.12 ± 0.178
Ethylacetate	1	4.49 ± 0.083
	3	3.77 ± 0.129
	5	3.56 ± 0.061
N-Hexan	1	3.61 ± 0.128
	3	3.75 ± 0.146
	5	4.37 ± 0.171

The highest total flavonoid results were found at an extraction time of 5 hours, this time indicates the best time for the extraction process, because at that time the compounds contained in the *I. carnea* leaves can be extracted optimally. At times 1 and 3 hours the compounds contained in the sample had not been completely extracted, so the total flavonoids produced were lower than the extraction time of 5 hours. According to Irawan (2016), a short extraction time will provide low results because not all components are extracted. The results of total flavonoids in ethyl acetate solvent were higher during extraction, the levels decreased. This occurs because the compounds that are extracted evaporate with prolonged heating. The compounds extracted are thermolabile so they disappear with prolonged heating (Susanti, et al, 2016).

The results of statistical analysis using two-way ANOVA of solvent and soxhletation time provided statistically significant differences in flavonoid levels, where methanol solvent for 5 hours

increased the total flavonoid levels of *I. carnea* leaves extract. The results of solvent analysis show statistical differences, while the results of time analysis also show statistical differences. From these statistical results it can be concluded that there are differences between the types of soxhletation solvents on the total flavonoid content of *I. carnea* leaves extract. Soxhletation times of 1, 3, and 5 hours were statistically different with a P value <0.05. Statistical results showed that there was a difference between the soxhletation time and the total flavonoid content of *I. carnea* leaves extract with the p value <0,05.

CONCLUSION

The results of this study could be concluded that the highest total flavonoid level obtained was 5.12 ± 0.06%, using methanol Soxhletation at 5 hours. The optimum solvent for soxhleting *I. carnea* leaves was methanol. The methanol solvent was statistically different from both ethylacetate and n-hexane with a sig value <0.05. The solvents ethyl acetate and n-hexane were not statistically different where the sig value was > 0.05. The optimum time for soxhleting *I. carnea* leaves was 5 hours. The extraction time was statistically different for *I. carnea* leaves flavonoid content, the test results showed that the differences in time of 1, 3 and 5 hours were all significantly different with a sig value <0.05 .

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