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PHYTOCHEMICAL ANALYSIS AND TOTAL FLAVONOID CONTENT ON ETHANOL AND ETHYL ACETATE EXTRACT FROM NEEM (*Azadirachta indica* juss.) LEAVES USING UV–VIS SPECTROPHOTOMETRIC

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ABSTRACT
The plants are rich sources of secondary metabolites, a bioactive compound that has various activities. Flavonoids, as a type of secondary metabolite, have been reported to possess anticancer, antioxidant, antibacterial, anti-inflammatory, and antiviral activities. Flavonoid has been found abundantly in Neem (*Azadirachta indica* Juss.) leaves. The difference in total flavonoid content might be an occurrence of the different solvent types and concentrations. The present study was conducted to analyze the phytochemical and determine the total flavonoid content of neem leaves extract using two different solvents (namely 70% ethanol and ethyl acetate) using UV-Vis spectrophotometric. The extraction from neem leaves was performed by maceration method. Phytochemical analysis of neem leaves reveals several secondary metabolites: flavonoids, steroids/triterpenoids, tannins, and saponins. Total flavonoid content from both extracts was determined by utilizing the UV-Vis spectrophotometric method at a maximum wavelength of 428.2 nm with three repetitions, and also quercetin was used as a standard. Total flavonoid content from neem leaves extracts in solvents of 70% ethanol and ethyl acetate had a value of 118.57 ± 0.08 mg/g QE and 74.17 ± 0.20 mg/g QE, respectively. Neem leaves extract in solvents of 70% ethanol and ethyl acetate had identical phytochemical content. Total flavonoid content of neem leaves from 70% ethanol extract was higher than ethyl acetate extract.

Keywords: Neem Leaves; Phytochemical analysis; Total flavonoid content; UV-Vis Spectrophotometer

1. INTRODUCTION

Indonesia is rich in biodiversity, especially medicinal plants. Neem (*Azadirachta indica* Juss.) is one of the medicinal plants mostly found in Madura, Java, Bali, and NTB (Javandira et al., 2016; Supriyanto et al., 2017). Utilization neem as a medicinal plant is due to its secondary metabolite content. The secondary metabolite content of neem is abundantly found in most parts of plants, including stems, leaves, and seeds. The flavonoids compound is a type of secondary metabolite that plays a role in medication (Aryal et al., 2019).

The flavonoids have been reported to possess various pharmacological activities, including anticancer, antioxidant, antibacterial, anti-inflammatory, and antiviral (Panche et al., 2016; Ekin et al., 2017; Xu et al., 2017). Flavonoids are important in the health sector and needed in various pharmaceutical, cosmetic, and nutraceutical applications. Flavonoids function as inhibitors of several enzymes, such as: xanthine oxidase (XO), cyclooxygenase (COX), lipooxygenase (LOX) and phosphoinositide-3-kinase (PI3K) (Panche et al., 2016). Flavonoids from neem leaves extract have been studied for their strong antioxidant activity in methanol solvent with a concentration of 80% and an IC_{50} value of 83.28 ppm (IC_{50} < 100 ppm = strong) compared to water and ethanol

solvents (Supriyanto et al., 2017). Furthermore, Supriyanto and colleagues compared the antioxidant content of the methanol and ethyl acetate fractions. The ethyl acetate fraction has higher antioxidant activity than methanol with an IC_{50} value of 101.54 ppm (Supriyanto et al., 2018).

Therefore, this study was conducted to measure of total flavonoid content with a variety of solvents, namely ethanol and ethyl acetate. Flavonoids can be extracted by a maceration procedure. Maceration has several advantages, i.e., it is simply to perform, cheap and stable without heating. The maceration method's ability to extract the total flavonoid content of neem leaves is depend on the polarity of the solvent (Mukhriani et al., 2015; Supriyanto et al., 2018).

The UV-Vis spectrophotometry method utilized for the calculation of total flavonoid content is based on the measurement radiation from the flavonoid compound at its maximum wavelength. The presence of conjugated aromatic compounds provides strong absorption at a wavelength of 200–700 nm (Mukhriani et al., 2015). This investigation aims to determine the phytochemical and total flavonoid content using the UV-Vis spectrophotometry method on neem leaves extract in solvents of 70% ethanol and ethyl acetate. The flavonoids content from both extracts might be different due to the difference in solvent polarity.

2. METHODS

2.1. Materials

The chemicals and reagents utilized in the present investigation were all of analytical quality.

2.2. Preparation of neem leaves extract

The neem leaves powder simplicia was weighed (25 g) and placed in maseration vessel (jars). Afterward, 250 mL of 70% ethanol was added to the vessel and macerated for 3×24 hours. Then, the vessel was sealed and kept out us from direct light. To obtain a crude extract of neem leaves, the solvent was removed after maceration using a rotary evaporator. The technique was repeated for the ethyl acetate solvent.

2.3. Phytochemical analysis

The phytochemical analysis in the extract was conducted on neem leaves using the standard method previously described. Flavonoids, steroids/triterpenoids, tannins, and saponins were among the secondary metabolites examined for phytochemical analysis (Syafitri et al., 2014; Ilmiyah, 2019).

2.3.1. Flavonoid's content

The extract (4 mL) was combined with 1.5 mL of 50% ethanol before being heated (500°C). The filtrate was then transferred to a spotted plate, and five droplets of concentrated sulfuric acid were added. The presence of flavonoid compounds is indicated by the formation of a red solution during the reaction.

2.3.2. Steroids/triterpenoids content

Salkowaski test: The extract (2 mL) was combined with concentrated sulfuric acid (5 droplets). Afterward, the solution was shaken and set aside for a moment. The presence of triterpenoid compounds is indicated by the formation of a greenish-blue solution during the reaction.

Libermann Burchard test: The extract (2 mL) was placed in the reaction tube, and 10 droplets of acetic anhydride were added to the inside tube. Afterward, sulfuric acid was added to the extract through the inside wall of the reaction tube. The presence of triterpenoid compounds is indicated by the formation of a greenish-blue solution during the reaction.

a. Tannin's content

   The extract (4 mL) was dissolved in water, and 3–4 droplets of $FeCl_3$ were added. The presence of gallic tannins and catechol tannins is indicated by the formation of blue and green solutions, respectively.

b. Saponins content

The extract (2 mL) was poured in the reaction tube, and added with diethyl ether. The extract residue (which is not soluble in diethyl ether) was then separated and mixed with water (5 mL). Afterward, the solutions were shaken. The presence of saponin compounds is indicated by the formation of a stable foam during the reaction.

2.4. Total flavonoid content analysis

2.4.1. Preparation of quercetin standard stock solution (100 mg/L)

The quercetin standard powder was weighed at 10 mg and then poured into a volumetric flask (100.0 mL). After that, ethanol was added to a volumetric flask and dissolved using sonication assistance.

2.4.2. Determination of the maximum wavelength of quercetin

The maximum wavelength was measured from a twenty (20) mg/L standard quercetin solution. The quercetin solution was pipetted (1.0 mL) and placed into a reaction tube. Afterward, the reaction tube was filled with 2% $AlCl_3$, 5% acetic acid, and distilled water of 1.0 mL, 1.0 mL, and 2.0 mL, respectively. Thereafter, the solution was shaken and kept away from light for 30 minutes. Absorbance was measured in the wavelength range of 200–400 nm with a UV-Vis spectrophotometer. The blank solution was made up of a mixture of 2% $AlCl_3$ (1.0 mL), 5% acetic acid (1.0 mL), and distilled water (2.0 mL).

2.4.3. Determination of quercetin calibration curve

In brief, the standard solution of quercetin was created with various concentrations (10, 20, 30, 40, and 50 mg/L). The solution from each concentration was pipetted (1.0 mL) and then placed into a reaction tube. Afterward, the reaction tube was filled with 2% $AlCl_3$, 5% acetic acid, and distilled water of 1.0 mL, 1.0 mL, and 2.0 mL, respectively. Thereafter, the solution was shaken and kept away from light for 30 minutes. The absorbance value was determined using a UV-Vis spectrophotometer at a maximum wavelength of 428.2 nm.

2.4.4. Determination of total flavonoid content

The extract was weighed (50.0 mg) and then mixed with ethanol to obtain dissolution. Afterward, the solution was poured into a volumetric flask (10.0 mL) and then was added with ethanol up to the mark-line to obtain an extract content of 5000 mg/L. Thereafter, the extract solution of 5000 mg/L was pipetted (1.0 mL) and then placed into a volumetric flask (10.0 mL). Next, the solvent was added up to the mark-line to obtain an extract content of 500 mg/L. Afterward, the extract solution of 500 mg/L was pipetted (1.0 mL) and then transferred into a reaction tube. Thereafter, 2% $AlCl_3$ solution, 0.1 M sodium acetate solution, and distilled water were added into the reaction tube in amounts of 1.0 mL, 1.0 mL, and 2.0 mL, respectively. After that, the reaction tube was shaken and incubated for 30 minutes in a dark place. After incubation, the absorbance was measured at a maximum wavelength of 428.2 nm. The data was collected from triplicate experiments. The total flavonoid content was calculated using the following formula (1):

$$\text{Total flavonoid content} = (Y \times F \times V) / W \quad (1)$$

Where Y represents the flavonoids concentration from the standard curve equation (mg/L), F represents the dilution factor, V represents the volume of extract used (L), and W represents the weight of the sample utilized (g).

3. RESULTS AND DISCUSSION

The maceration method was used to extract neem leaves, which uses two different solvents: 70% ethanol and ethyl acetate. The extract percentage was calculated by dividing the mass of the extract by the dry simplicia and multiplying by 100%. The yield percentage of neem leaves extract

in a solvent of 70% ethanol is 10.12 percent, which is higher than the yield percentage of neem leaves extract in an ethyl acetate solvent (8.64 percent).

Based on **Table 1**, the phytochemical analysis of neem leaves revealed that the extract of 70% ethanol and ethyl acetate contained flavonoids, steroids, tannins and saponins. Phytochemical analysis revealed that neem leaves extract in both solvents (70% ethanol and ethyl acetate) contained flavonoids, steroids, tannins, and saponins. Different types and concentrations of solvent did not affect the types of secondary metabolites contained in neem leaves extract (Ilmiyah, 2019).

Table 1. Phytochemical Analysis of Neem Leaves Utilizes Two Different Solvents

Secondary Metabolites	Solvents	
	70% Ethanol	Ethyl Acetate
Flavonoids	+	+
Steroids (Salkowaski)	+	+
Steroids (Libermann Burchard)	+	+
Tannins	+	+
Saponins	+	+

(+) : contain the compound
(−) : not contain the compound

The total flavonoid content was determined quantitatively using the colorimetric method with aluminum chloride (Pekal et al., 2014; Ekin et al., 2017). The maximum wavelength of quercetin was obtained at 428.2 nm and then was used to assess the absorbance of the calibration curve and the absorbance of samples (**Figure 1**). The calibration curve data in **Table 2**. The calculation of the quercetin calibration curve (from a concentration of 10–50 mg/L), i.e., $y = 0.0153x + 0.0013$; $R^2 = 0.998$. Total flavonoid content was calculated using quercetin equivalent (QE) per weight of extract (g), as shown in **Table 3**. Total flavonoids in neem leaves extract were 118.57 ± 0.08 mg/g QE in 70% ethanol and 74.17 ± 0.20 mg/g QE in ethyl acetate.

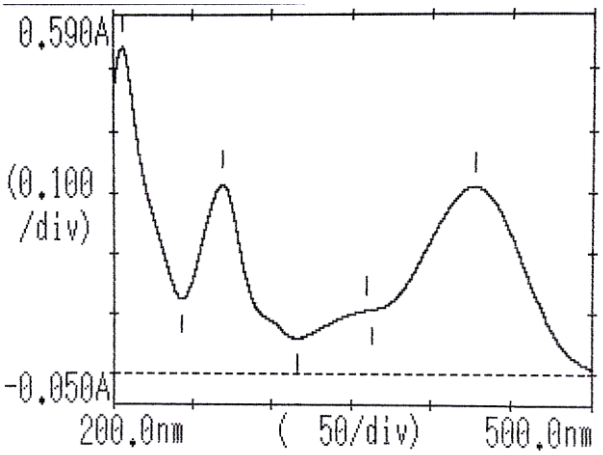

Figure 1. The Maximum Wavelength of Quercetin

Table 2. Absorbance of the Quercetin Calibration Curve

Concentration (mg/L)	Absorbance
10	0.152
20	0.306
30	0.469
40	0.589
50	0.778

Table 3. Total Flavonoid Content of Neem Leaves Extract

Extract	Concentration (mg/L)	Total Flavonoid (mg/g QE)
70% ethanol	59.28 ± 0.04	118.57 ± 0.08
Ethyl acetate	37.08 ± 0.10	74.17 ± 0.20

Aluminum chloride will be complex and stable in binding with the carbonyl group on carbon number 4 (C_4), the hydroxyl group on carbon number 3 (C_3), and the carbon number 5 (C_5) of flavonols and flavonoids in an analysis of total flavonoid content (Pekal et al., 2014; Fadillah et al., 2017; Sembiring et al., 2018). This binding could also result in the formation of a complex of an acid non-stable with a hydroxyl in the ortho position of the flavonoids' B-ring (Kamtekar et al., 2014; Fadillah et al., 2017; Senet et al., 2017; Sembiring et al., 2018; Niah et al., 2019). Because quercetin is a type of flavonoid (flavonol), the total flavonoid content was determined using standard quercetin (Sembiring et al., 2018; Wirasati, 2019). The value of R^2 closely reached 1, indicating a linear relationship between concentration and absorbance of standard quercetin from the calibration curve.

The addition of sodium acetate solution in the analysis of total flavonoids aims to detect the 7-hydroxyl group, followed by an incubation aims to obtain an impeccable reaction with maximum color intensity. Total flavonoids from neem leaves extract in solvents of 70% ethanol and ethyl acetate had a value of 118.57 ± 0.08 mg/g QE and 74.17 ± 0.20 mg/g QE, respectively. These results revealed that total flavonoid content from the solvent of 70% ethanol was more dissolved compared to ethyl acetate. These findings indicate that solvents with high polarity will provide higher total flavonoids.

4. CONCLUSION

Neem leaves extract in solvents of 70% ethanol and ethyl acetate had identical phytochemical content. Total flavonoids from neem leaves extract in solvents of 70% ethanol and ethyl acetate had a value of 118.57 ± 0.08 mg/g QE and 74.17 ± 0.20 mg/g QE. The total flavonoid content of neem leaves from 70% ethanol extract was higher than ethyl acetate extract. This finding might emphasize the potency of neem leaves as a source of natural antioxidants according to the analysis of their total flavonoid content. However, total phenolic content as well as antioxidant activity studies are required for the further research of this plant.

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6. CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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