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CHEMICAL CHARACTERISTIC AND ANTIOXIDANT ACTIVITY OF METHANOL EXTRACT NEEM LEAVES (Azadirachta indica JUSS)

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Abstract
The neem plant (Azadirachta indica) is a herb with the potential as a source of antioxidants. This study aimed to identify compounds that exist in the neem leaf and determine their antioxidant activity. Neem leaf extract was collected by using 80% methanol. Furthermore, methanol extracts from neem leaves with the highest antioxidant activity were partitioned with n-hexane, ethyl acetate, and water. This research were analyzed by partition for antioxidant activity, and DPPH (1,1-diphenyl-2-pycrilhydrazil) and the half-maximal inhibitory concentration (IC50) values were determined. The results showed that the ethyl acetate partition had the highest antioxidant activity, with IC50 values of 1.004 µg/ml. Neem leaf extract has the potential for a use as an herbal medicine in the treatment of various diseases.

Keyword
Antioxidant; DPPH; fractionation; IC50; neem leaf
INTRODUCTION

Neem is a plant in the family Meliaceae (Dash, Dixit, & Sahoo 2017) that can be found in tropical countries such as India and Indonesia (Igwenyi et al. 2017). Neem plants in Indonesia were first encountered as shade plants. Neem tree trunks can grow to 20 m tall and have a slightly rough stem and oval-shaped leaves with jagged edges, with pointy stone fruit measuring 1 cm in length. Neem plants have the potential for use as an herbal remedy and are known in India as the God of Plants because it can cure various diseases. Originally, neem plants were used as herbal remedies for their antibacterial and anti-inflammatory properties (Revankar, 2014). Neem contains secondary metabolites that can inhibit oxidation by competitively binding oxygen and hindering the initiation phase, blocking the propagation stage, and inhibiting or stabilizing the catalyst hydrogen peroxide. This is indicated when the solution becomes a greenish brown color. The color change occurs because of the reduction reaction. Tannins are polyphenols which are soluble in polar solvents like water; while ethanol and methanol are semi-polar solvents. Saponins are soluble in polar solvents like water; while ethanol and methanol are semi-polar solvents, saponins in the neem leaf still be

This study aimed to identify compounds that exist in the neem leaf and determine their antioxidant activity. The research was conducted through a multilevel extraction process using the maceration method. The results provide an overview of the compounds that make up the extracts from neem leaves and the role they play in capturing the power of free radicals.

METHODE

Collection of plant material

Neem leaves (Azadirachta indica Juss) were obtained from the Kamal Bangkalan Madura from plants that were approximately five years old. The leaves were collected, cleaned, washed under running tap water, and sun-dried.

Extraction of neem leaves

The neem leaves used were dark green and were collected about two months after the shoot developed. Before using neem leaves, they were dried for one day used sun drying, then pulverized into a powder using a blender. As much as 100 g of neem leaf powder was added to solvent extraction that contained water, 60% ethanol, 80% methanol, or 80% methanol, using the maceration method to extract. The ratio between the substance and the solvent is 1: 3. Maceration was done in a closed space for 48 hours with shaking.

Phytochemical measurement

Phytochemical analysis using the (Khanam et al., 2015) method with several modifications

Tannins

When testing neem leaf extract samples for tannin using a 0.1% FeCl₃ reagent, a positive test is indicated when the solution becomes a greenish-brown color. The color change occurs because of the reduction reaction. Tannins are polyphenols classes of compounds, and polyphenols can reduce iron (III) to iron (II) (Delimont et al. 2017). This is also a traditional way to detect phenol compounds, namely by adding a solution of 1% iron (III) chloride to water or ethanol in a solution wherein green, red, purple, blue or black (Ares et al. 2018). According to previous studies, tannins are soluble in water and ethanol.

Saponin

Saponins are soluble in polar solvents like water; while ethanol and methanol are semi-polar solvents, saponins in the neem leaf still be
extracted. In this test, 10 ml of sample extract plus 5 ml of distilled water were mixed, then shaken until frothy. On top of the foam, three drops of olive oil were added, then it is shaken again. This forms an emulsion of the two samples, indicating a positive test for saponins. The addition of olive oil is a source of cholesterol so as to purify the saponins and add a large amount of cholesterol, which causes the formation of adduct complex compounds that were not soluble in water (Bajad et al. 2019).

**Flavonoid test**

For the flavonoid test, 0.5 ml of sample extract plus 5 ml of aqueous ammonia were mixed. Visible changes in solution color are seen, and the samples become slightly yellow. This occurs because the flavonoids contain phenolic compounds. When phenol reacts with a sample, a base color will form because of a conjugated system of aromatic groups (Delimont et al., 2017).

**Terpenoids**

Measuring terpenoids concentration can be performed by adding ethanol, and chloroform, and sulfuric acid to the filtrate. A reddish-brown color formed at the interface, which indicates that the sample contains terpenoids (Tyagi and Agarwal 2017). The formation of a reddish-brown color at the interface area of the added reactant or reactants chlorosulfonic acid and Briner Brieskorn were often used to distinguish the typical triterpenoids, which are red and brown steroid compounds (Delimont et al. 2017) so that the resulting color looks reddish-brown.

**Antioxidant activity measurement**

After 48 hours, the macerated samples (from neem leaves extraction process) were filtered and collected in Erlenmeyer flasks. The filtrate was then concentrated using a rotary evaporator at 40°C until the solution is concentrated. Concentrated extracts were analyzed for antioxidant activity using DPPH and for IC50 values. Those extracts with the highest antioxidant activity were further fractionated using n-Hexane, ethyl acetate, and water.

**Fractionation of neem extract**

Concentrated extracts that had the highest antioxidant activity in previous studies were further fractionated to get a purer compound. As many as 100 g of extract was added to 400 ml of distilled water and homogenized, then inserted into a separating funnel. Next, 200 ml of n-Hexane was added, shaken, and allowed to stand so that the solution separated into two parts. The top layer was the n-Hexane solution, and the bottom layer was a water solution. The layers were separated by opening the tap on separating funnel to let the water layer run through and collect in a separate tube, after which the n-Hexane solution was evaporated using a rotary evaporator in order to obtain a thick extract. For the filtrate or water solution that was separated, 200 ml of ethyl acetate was added, then shaken, and allowed to stand until separation occurred. The top layer of solution was ethyl acetate, while the bottom was water. The two solutions were separated by opening the tap on the bottom of the separating funnel. Each solution was evaporated using a rotary evaporator until concentrated extract was obtained. Concentrated extract from each fraction was then analyzed using DPPH (1,1-diphenyl-2-picrylhydrazyl) for antioxidant activity with IC50 values. Fractions that had the highest activity were also analyzed by Fourier Transform Infrared (FTIR) and Liquid Chromatography-Mass Spectrometry (LCMS).

**Analysis of antioxidants**

Analysis of antioxidants was determined according to the modified method of (Filbert et al. 2014). Samples of each fraction of neem extract were prepared. The first step was to make liquor of each sample at 100 ppm by dissolving 10 mg of extract in 100 ml of methanol. Each sample was further diluted in methanol to 5, 6, 7, 8, and 9 ppm. Prepared stock solution DPPH at 50 ppm was made by dissolving 5 mg of solids into a 100 ml methanol DPPH. Then, the control solution was prepared for comparison to the test samples by mixing 2 ml of methanol and 1 ml of 50 ppm DPPH. For the test samples, 2 ml of each sample solution was mixed with 2 ml of 50 ppm DPPH. The samples were then incubated for 30 minutes at a temperature of 27°C until color changed (from DPPH activity) was apparent. All samples were performed in triplicate. A UV-Vis spectrophotometer was used to test absorbance values at a wavelength of 517 nm, and the antioxidant activity was expressed by calculating the half-maximal inhibitory concentration (IC50) values obtained from the linear regression equation of the data absorbance.

**FTIR (Fourier Transform Infrared) analysis**

A certain amount of dried extract was mixed uniformly with potassium bromide (KBr) to form pellets using manual press equipment (Shimadzu, Tokyo, Japan). The FTIR spectrum was made
using a Tensor 37 FTIR spectrophotometer (Bruker Optik GmbH, Karlsruhe, Germany) with a deuterated triglycine sulfate (DTGS) detector in the middle infrared region (400–4000/cm) at 4/cm resolution with a total of 32 OPUS (version 4.2, Bruker Optik GmbH, Karlsruhe, Germany). FTIR spectrum in OPUS format was stored in data point table (DPT) format. When the extract passed into the FTIR region, the functional groups of the components were separated based on their peak ratio (Pakkirisamy et al., 2017).

**LCMS (Liquid Chromatography-Mass Spectrometry) analysis**

LCMS analysis used the model Shimadzu LCMS-8040LC/MS with the column shim pack FO-ODS (2 mm x 150 mm, 8 µm), a capillary voltage of 3.0 kV and a column temperature of 35°C for a sample injection volume of 1 µl and a flow gradient of 0/100 at 0 min, 15/85 at 5 min, 21/79 at 20 min and 90/100 at 24 min, and a flow rate of 0.5 ml/min. The sampling cone was at 28.0 V with the solvent CH3ON (0.1% TFA)/H2O (0.1% TFA), and the MS focused ion mode was [M]+, collision energy 5.0 V and desolvation gas flow 600 L/h with desolvation temperature set for 350 °C. The fragmentation method was low energy OID and ionization ESI for scanning at 0.6 sec/scan (10–100 mhz), with source temperature at 100 °C and a run time of 80 minutes.

**Data Analysis**

One-way analysis of variance (ANOVA) was used for statistical analysis with a significance level of 0.05, then followed by the Duncan test for pairwise comparison using SPSS 21. P values less than 0.05 were considered to be significant.

**RESULT AD DISCUSSION**

**Phytochemical of neem leaf extract**

All samples tested were positive because tannins are soluble in water, alcohol, and acetone (Hanani, 2015). Qualitative analysis results for saponins (Table 1) showed that all samples contained saponin from neem leaves extract. Saponins are soluble in polar solvents like water; while ethanol and methanol are semi-polar solvents, the saponins in the neem leaf can still be extracted. The qualitative test results for flavonoids also revealed that all samples were positive. According to a study, flavonoids are polyphenolic compounds that are soluble in polar solvents, such as chloroform, ethyl acetate, acetone, and methanol (Mariana et al., 2017). Then, test results for terpenoids showed that all samples of neem leaf extract contained terpenoids. Therefore, four types of phytochemicals were present in neem leaf extract by using any treatment used.

**Antioxidant activity of neem leaf extract from DPPH analysis**

Neem leaf extract antioxidant activity is expressed by IC50, which is the effective concentration of extract needed to remove 50% of the total free radical DPPH. IC50 analysis results for neem leaf extracts were shown in Table 2. IC50 values of all samples ranged from 83.28 to 90.39. A smaller IC50 values indicates higher antioxidant activity. According to a study, the activity of antioxidants can be classified into four groups, based on IC50 values. The strength of the antioxidant activity can be seen in Table 3. Based on these parameters, neem leaf extract has potent antioxidant activity (IC50< 100). IC50 was lowest in neem leaf extract with an 80% methanol solvent (Santos et al. 2018).

**Antioxidant activity of methanol neem leaf extract fractions**

Further fractionation processing was done using n-Hexane, which is non-polar. Fractionation is the process of the withdrawal of a compound using two different solvent polarity properties; thus, using n-Hexane aims to extract grease and terpene. Methanol-water residue obtained was fractionated with ethyl acetate to isolate the semi-polar compounds. In the methanol extract, there are different groups of secondary metabolites, so needed to separate the compounds through the fractionation process.

| Solution   | Tannin | Saponin | Flavonoid | Terpenoid |
|------------|--------|---------|-----------|-----------|
| Ethanol 60%| +      | +       | +         | +         |
| Ethanol 80%| +      | +       | +         | +         |
| Methanol 60%| + | + | + | + |
| Methanol 80%| + | + | + | + |
The use of different solvent polarity levels affected the type of compounds extracted. The ethyl acetate fraction contained compounds such as fatty acids and phytosterols, while the water extract contained carbohydrates (glucose and sucrose). As shown in Table 4, the fraction of ethyl acetate has a higher antioxidant activity than the fractions of n-Hexane and water. This is because ethyl acetate is a semi-polar solvent, and as such, it can attract polar and non-polar compounds.

Another peak occurs at the top of wave number 675–995/cm, which may indicate the presence of the C-H alkene group that usually appears in wave numbers 675–995/cm and 3,010–3,095/cm.

**LCMS Results**

Figure 2 showed LCMS peaks appearing at wavelengths of 498, 554, 540, and 596, which are identified as deacetylnimbin, deacetylsalannin, nimbin and salannin, respectively. These results are similar to a previous study (Caboni et al. 2006). Deacetylnimbin (or 6-desacyl nimbin) has the soluble properties of chloroform and methanol that are used in traditional medicine, such as anti-tumor agents (Zhao et al. 2010).

The results showed that Neem leaf extract contained tannins, saponins, flavonoids, and terpenoids. Tannin serves as an antimicrobial, antidiarrheal, and anti-helminthic agent (Igwenyi et al. 2017, Bolade et al., 2018). Saponins are powerful active surface compounds that create a foam when shaken with water. Some saponins work as antimicrobial. Saponin glycosides are complex compounds that are a condensation product of sugar with an organic hydroxy compound, which produces sugars (glucose) and non-sugars (aglycone) when hydrolyzed. This type of saponin is composed of two groups: triterpenoid saponins and steroidal saponins. Both types of saponin are soluble in water and ethanol but insoluble in ether (Bajad et al., 2019). Saponins also comprise a large family of structurally related compounds containing a steroid or triterpenoid aglycone. They are reported to have a wide range of beneficial properties.
pharmacological properties, such as anti-inflammatory and anti-diabetic effects (Unekwu et al. 2014).

Flavonoids are one of the largest natural phenols and are generally found in plants bound to sugars like glycosides or aglycone. Flavonoids are mainly found in plants as a mixture and are rarely found in a single form. These compounds help create pigment in plants, such as flower pigments that attract birds and insects to pollinate flowers. In addition, flavonoids are known to be growth regulators and regulators of photosynthesis, as well as antimicrobial and antiviral. In the body, flavonoids function to inhibit enzymes involved in the biosynthesis of prostaglandin lipoxigenase. The antioxidant mechanism of flavonoids is to capture ROS directly, prevent ROS regeneration, and indirectly increase the antioxidant activity of cellular antioxidant enzymes. Flavonoids belong to the group of polyphenolic compounds and are typically known for health-promoting properties, such as having antioxidant, anti-allergic, anti-inflammatory, antimicrobial and anticancer effects (Mariana et al., 2017). The ability of flavonoids to act as potent antioxidants depends on their molecular structures, the position of the hydroxyl group and other features in its chemical structure (Iqbal et al. 2015).

Terpenes or terpenoids are active against bacteria, fungi, viruses, and protozoa (Khanam et al., 2015). Triterpenoid betulinic acid has been shown to inhibit the effects of HIV (Xiao et al., 2006). The working mechanism of terpenes is not well understood, but it is speculated to be involved in the destruction of cell membranes by lipophilic compounds. Terpenoids found in plant essential oils have been beneficial for the control of Listeria monocytogenes in foods (Penduka et al., 2014). A type of terpenoids in pepper, known as capsaicin, has a number of biological activities in humans that can affect the nervous, cardiovascular and digestive systems.

Figure 1. FTIR results of ethyl acetate fraction of neem leaf extracts
Figure 2. Showed that there are four compounds from the LCMS test, namely diacetyl nimbin, nimbin, deacetyl salannin and salannin. According to Chen et al., (2019), there are four new limonoid types of nortriterpenoids, namely 1-detigloyl-1-O-methacryloylsalannin, 28-deoxo-2,3-dihydrornimboline, 12-acetoxy-3-O-acetyl-7-O-tigloyvilasinin and 12-acetoxy-3-O-acetyl-7-O-methacryloylvilasinin. In that study, results from an in vitro cytotoxic assay showed inhibitory activity against the human breast cancer MDA-MB-231 cell line, as well as inhibiting growth of the human cervical cancer HeLa cell line, the melanoma A375 cell line, and the promyelocytic leukemia HL-60 cell line.

CONCLUSIONS

The results revealed that neem leaf extracts contained bioactive compounds, such as tannins, saponins, flavonoids, and terpenoids, and had strong antioxidant activity. In addition, the LCMC test results of neem leaf extract contain deacetyl nimbin, nimbin, deacetyl salannin, and salannin compounds. Neem leaf extract has the potential for use as an herbal medicine in the treatment of various diseases. Furthermore, the research needs to be done in vitro and in vivo to determine the effect of neem leaf extract to treat various diseases.

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