MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Sutrisno *et al.* | Malaysian Journal of Fundamental and Applied Sciences, Vol. 20 (2024) 1493-1505

RESEARCH ARTICLE

# Neem Leave (*Azadirachta Indica*): Extraction, Fractionation, Phytochemical Screening, Antioxidant and Food Antifungal Activities

**Sutrisno[a]*, Elfira Devi Yuniarti[a], Husni Wahyu Wijaya[a], Megawati[b]**

[a]Department of Chemistry, Universitas Negeri Malang, Jl. Semarang 5 Malang 65145, Indonesia; [b]Pusat Riset Bahan Baku Obat dan Obat Tradisional, Badan Riset dan Inovasi Nasional, Tangerang Selatan, Banten, Indonesia

Abstract Neem (*Azadirachta indica*), a widely recognized medicinal plant, is known for its numerous therapeutic properties. People use neem leaves to increase their appetite to treat dysentery, malarial ulcers, and bacterial infections. Research on neem leaves that has been done is still focused on extracts, but not their fractions. This study aimed to extract neem leaves that separate the components based on their polarity (fractionation), analyze and identify their components, and test antioxidant and antifungal activities. The research stages caried out include (1) extraction and fractionation, (2) analysis (thin layer chromatography, bromine, visible and IR spectrophotometry), (3) phytochemical identification, and (4) assay of antioxidant and antifungal activities. The yield of extraction with methanol is 8.08%. Fractionation of the methanol extract using *n*-hexane, ethyl acetate, 1-butanol, and methanol-water produced yields of 13.86, 6.84, 16.58, and 48.68%, respectively. The methanol extract contains alkaloids, flavonoids, tannins, terpenoids, and saponins, while each fraction contains flavonoids, phenolics, tannins, terpenoids, and saponins. Flavonoid and phenolic compounds is dominant in methanol extract, ethyl acetate fraction, and 1-butanol fraction. Both the two groups of compounds are most content in the ethyl acetate fraction, namely total flavonoid content 17.32 and total phenolic content 12.80 mg GAE/g sample. Ethyl acetate fraction shows the best antioxidant and antifungal activity. The antioxidant activity of this fraction is in the strong category with $IC_{50}$ 19.06 ppm, but weaker than ascorbic acid. The ethyl acetate fraction was able to withstand the growth of mold on white bread for 96 hours at 500 ppm and 120 hours at 2000 ppm. The content of flavonoid and phenolic compounds indicates that it mainly contributes to antioxidant and antifungal activity, and there is a significant relationship between antioxidant and antifungal activity. Neem leaf extract has the potential to be an antioxidant and antifungal for food, especially methanol extract, ethyl acetate, and 1-butanol fractions. Therefore, ethyl acetate and 1-butanol fractions could be an alternative to natural additives in white bread formulations.

**Keywords**: neem leave extract, total phenolic, total flavonoid, antioxidant, food antifungal.

***For correspondence:**
sutrisno.kimia@um.ac.id

**Received:** 01 July 2024
**Accepted:** 17 Nov. 2024



## Introduction

Abundant biodiversity is characteristic of the tropics, and Indonesia is no exception. A wide variety of plants can grow well on this tropical earth. Plants are used as food, energy, and some of them are also used as medicines, especially traditional medicines. More than 1000 species of plants in Indonesia are medicinal plants [1]. One important aspect of the plant's medicinal usefulness is the pharmacological properties of its antioxidant activity. Research on antioxidant activity continues to grow, both from natural and synthetic ingredients, pure and blended. Neem (*Azadirachta indica*) is a tropical plant and a popular medicinal plant. Neem is a tall plant that can grow up to 20 m [2]. Traditionally, neem is grown on the side of the main road and at the same time used as shade and leaves for animal feed [3]. Neem leaves are used to increase appetite, treat dysentery, malaria, ulcers, and bacterial infections [4, 5, 6]. In addition, it is also used to treat itching by punching and then attaching it to the itchy part [7], [8]. Some Indonesians (Sampang Regency, Madura), use neem leaf decoction water to treat colds, diarrhea, and

diabetes. In addition, neem leaf decoction is also used to treat eczema (Indonesia: *gudig*) [2, 9]. The benefits of neem leaves are undeniable because of the content of compound components contained in them.

Neem is used as antifungal, antimalarial, hypoglycemic, immunomodulatory, antipyretic, anti-inflammatory, antibacterial, antigastric ulcer, antiarthritic, spermicidal diuretic and antitumor [10]. Neem is exhibit antibacterial, antiviral, immune modulatory, anti-inflammatory, anti-hyperglycemic, antiulcer, antimalarial, antimutagenic, antidiabetic and anticarcinogenic properties [11], and the potential to be a prostate cancer therapy [12, 13]. The phytochemical study of neem's antibacterial activity was conducted by Mudenda *et al*. [14], highlighting the effectiveness of ethanol extracts as a growth inhibitor for *Streptococcus* sp. [15] and demonstrating the resistance properties of neem leaf extracts against *Staphylococcus aureus* and *Bacillus subtilis* [16].

Research reports that ethanol 96% extract of neem leaves contains alkaloids, tannins, flavonoids, saponins, and steroids/terpenoids [17]. Other research suggests that the methanol extract of neem leaves contains flavonoids, saponins, tannins, alkaloids and steroids [18]. Screening of these compounds is carried out through extraction. One extraction method that is often used is maceration. This method has the advantage that the process is simple, easy to carry out, and does not require heating so it can minimize the possibility of the substance becoming damaged [19, 20, 21]. Neem leaf extract is a colored extract. Chemically, colored substances have long conjugated C=C double bonds so they are rich in electrons. Compounds that are rich in electrons can act as electron donors, so they have the potential to act as antioxidants. Antioxidants are preservative additives that ward off, fight or prevent oxidative damage to both food and non-food. As additives, antioxidants are added during processing or storage. Food antioxidants help maintain nutrient levels, texture, color, taste, freshness, functionality, aroma, and attractiveness [22]. In general, the characteristics of antioxidant compounds are that they have groups that are easily oxidized, such as primary hydroxyl groups ($CH_2$-OH), secondary hydroxyl groups (CH-OH), phenolics, aldehyde groups (CHO), and double bonds (C=C). Assessment of antioxidant capacity can be done based on its capacity to scavenge free radicals or measure the ability of antioxidants to reduce ferric ions [23]. The free radical scavenge capacity uses DPPH (2,2-diphenyl-1-picrylhydrazyl) through its absorption of visible light radiation.

Several studies have suggested that neem leaf extracts that have antioxidant activity are ethanol-water extract ($IC_{50}$ 226.118 ppm) [21], 80% methanol extract ($IC_{50}$ 80.28 ppm [24]. Research on the antioxidant activity of neem leaves that has been carried out is still focused in the extract, while there has been no report on the fraction of the extract. The content of phenolic and terpenoid compounds in neem leaves is thought to also have the potential to provide antifungal activity because these compounds can disrupt the permeability of fungal cell membranes [26]. Previous research stated that ethanol extract of neem leaves inhibits the growth of *Microsporum gypseum* [26]. In other research, ethanol extract of neem leaves showed inhibition of the growth of *Aspergillus sp.* isolated from rice leaves [27].

This study aims to extract and fractionate neem leaves, then identify and evaluate their antioxidant and antifungal activities. Methanol was used for extraction, while fractionation was performed by partitioning with n-hexane, ethyl acetate, and 1-butanol solvents. The extracts and partitioned fractions were subsequently analysed to determine their phytochemical content and tested for antioxidant and antifungal activities. The antioxidant activity test employed the DPPH radical scavenging method, while antifungal activity in food was assessed using a simple method on white bread as the medium.

# Materials and Methods

## Materials

The neem leaves used as research samples were collected from the *Puspiptek* (Science and Technology Research Center) Garden, Serpong, Banten Province, Indonesia. White bread is obtained from bread sellers in the market in Malang City – East Java Province – Indonesia. The chemicals used are technical methanol (technical grade and p.a.), ethanol p.a. (*Merck*), n-hexane (technical grade), ethyl acetate (technical grade), 1-butanol (technical grade), chloroform p.a. (*Merck*), TLC plate on silica gel 60 $F_{254}$ (*Merck*), quercetin (*Sigma Aldrich*), gallic acid (*Merck*), ascorbic acid (*Merck*), DPPH powder (*Sigma Aldrich*), Mayer reagent, Bouchardat reagent, Drangendorf reagent, magnesium powder (*Merck*), hydrochloric acid, HCl (*Merck*), sulfuric acid, $H_2SO_4$(*Merck*), iron(III) chloride, $FeCl_3$ powder (*Merck*), sodium nitrite, $NaNO_2$ (*Merck*), sodium hydroxide, NaOH (*Merck*), Folin Ciocalteu reagent (*Merck*), sodium carbonate, $Na_2CO_3$ (*Merck*), and aluminum chloride, $AlCl_3$ (*Merck*).

## Equipment

The equipment used in the study are a set of glassware, a set of maceration tools, analytical balance (*Kern ABJ 320-4NM,* with an accuracy of 0.0001 grams), analytical balance (Durascale DAB200 with an accuracy of 0.001 grams), *rotary vacuum evaporator* (*Buchi R-124*), *chamber*, tweezers, UV lamps (254 and 366 nm), micropipette, *spray* bottle (20 mL), plastic clip (7x10 cm), Visible (722 AP), UV-Vis (Jena Specord 200 plus), IR (Shimadzu IR Prestige 21) spectrophotometers. Analysis of UV-Vis and IR spectrophotometrically was carried out at the Minerals and Advanced Materials Laboratory, Universitas Negeri Malang.

## Sample Preparation

Fresh neem leaves (2 kg) are dried in the oven for 48 hours at a temperature of ±50°C. Drying is stopped when a constant weight has been obtained. Next, the leaves are squeezed to reduce their size, then ground to obtain a powder with a size of 50 mesh. This powder is then stored and prepared to be used as a research sample or stock material to be extracted.

## Neem Leaves Extraction

The amount of 800 g of dried neem leaf powder was macerated using 3 liters of methanol (technical grade), and after 24 hours it is filtered. The filtrate obtained is evaporated with the solvent using a rotavapor (rotary vacuum evaporator) at ~50°C. This process is repeated 3 times. The results of this experiment are called neem leaf extract, and then partitioning is carried out.

## Fractionation of Neem Leaf Extract

***Fractionation using n-hexane.*** A total of 50 g of neem leaf extract is put into 10 mL of methanol (technical grade) then added with 500 mL of water, then stirred until homogeneous. 500 mL of n-hexane (technical grade) was added and stirred again. The mixture is put into a separate funnel and then shaken. Leave for 11-12 hours until two layers are formed, and then separated. The upper layer is the n-hexane phase (n-hexane fraction), the lower layer is the methanol-water phase (methanol-water fraction). The top layer (n-hexane fraction) is evaporated with its solvent (n-hexane) with rotavapor. The residue of this evaporation is an n-hexane fraction (colorless). This process is repeated several times until enough is obtained for the next experiment. Then the methanol-water fraction which is the residue from this process is further partitioned using ethyl acetate.

***Fractionation using ethyl acetate.*** A total of 500 mL of ethyl acetate (technical grade) is incorporated into the methanol-water fraction (residue), then stirred. The mixture is put into a separate funnel and shaken, left for 11-12 hours until two layers are formed, then separated. The upper layer is the ethyl acetate phase, while the lower layer is the methanol-water phase (methanol-water fraction, as residue). The top layer is evaporated with its solvent (ethyl acetate) using a rotavapor. The resulting extract is referred to as the ethyl acetate fraction (colorless). This process is repeated until a sufficient layer of ethyl acetate is obtained. The methanol-water fraction which is the residue of this process is further partitioned using 1-butanol.

***Fractionation using 1-butanol.*** To the methanol-water fraction (residue) 500 mL of 1-butanol (technical grade) was added, then stirred. Next, it is placed into a separate funnel and shaken. Leave for 11-12 hours until two layers are formed, then separated. The upper layer is the 1-butanol phase, while the lower layer is the methanol-water phase (methanol-water fraction). The top layer is evaporated with its solvent (1-butanol) using a rotary vacuum evaporator. The evaporation residue obtained is referred to as the fraction of 1-butanol (colorless). This process is repeated until a sufficient fraction of 1-butanol is obtained. The bottom layer which is the residue of this process is evaporated with the solvent (methanol-water) and the extract obtained is here in after referred to as the methanol-water fraction.

## Analysis of Neem Leaf Extract and Fractions

***Analysis with thin layer chromatography.*** The extract and fraction of neem leaves are dissolved in their respective solvents. It is taken in small amounts using capillary pipes and then tapped on top of the TLC plate. The distance between the dots is 0.5 cm. The TLC plates that have been given the extract and fractions are inserted into a chamber containing eluent, then lysed. By the time the eluent had reached the upper limit, the elution was stopped. Spots stains are observed with UV lamps at 254 and 366 nm.

***Analysis of unsaturation with bromine water***. The extract and neem leaf fractions (10 mg each) are dissolved into 10 mL of solvent so that a solution with a concentration of 1000 ppm is obtained. A total of 1 mL of the solution is put into a test tube, then 2 drops of bromine water are added, beaten vigorously. Furthermore, a change in the color of bromine water was observed and noted.

***Visible spectrophotometry analysis***. The extract and neem leaf fractions (10 mg each) are dissolved into 10 mL of solvent so that a solution with a concentration of 1000 ppm is obtained. It was analyzed using the visible spectrophotometer at 380-800 nm with a cuvette thickness of 1 cm.

***Infra-red (IR) spectrophotometric analysis***. The extract and fraction of neem leaves (50 mg each) are dissolved into 5 mL of solvent each so that a concentration of 10000 ppm or 1% is obtained. Analyzed using the FT-IR (*Fourier Transform Infrared*) spectrophotometer at 4000-500 $cm^{-1}$.

## Phytochemical Identification of Neem Leaf Extract and Fractions

Fresh neem leaves (2 kg) are dried in the oven for 48 hours at a temperature of ±50°C. Drying is stopped when a constant weight has been obtained. Next, the leaves are squeezed to reduce their size, then ground to obtain a powder with a size of 50 mesh. This powder is then stored and prepared to be used.

***Alkaloid test.*** Neem leaf extract and fractions (250 mg each) were added with 0.5 mL of 0.2 M HCl solution heated for 2 minutes, then cooled and filtered. The filtrate obtained is treated as follows [28]:

1) *test with Mayer's reagent.* A total of 0.5 mL of filtrate was added with 2 drops of Mayer's reagent. Positive test results are indicated by the formation of a white or yellow precipitate.
2) *test with Bouchardat's reagent.* A total of 0.5 mL of filtrate was added with 2 drops of Bouchardat's reagent. Positive test results are indicated by the formation of a brown to black precipitate.
3) *test with Dragendorf's reagent.* A total of 0.5 mL of filtrate was added with 2 drops of Bouchardat's reagent. Positive test results are indicated by the formation of an orange precipitate.

***Flavonoid test.*** The extract and fractions of neem leaves (10 mg each) were dissolved in 10 mL of each solvent, then 2 mL of each solution was added with 2 mg of magnesium powder and 5 drops of concentrated HCl solution. Positive test results are indicated by the formation of an orange solution [29].

***Phenolic or tannin test.*** The extract and fractions of neem leaves (10 mg each) were dissolved in 10 mL of each solvent. A total of 2 mL of each solution was added with 5 drops of 5% $FeCl_3$ solution. Positive test results are indicated by the formation of a dark green, blue, or brown solution [30, 31].

***Terpenoid test.*** The extract and fractions of neem leaves (10 mg each) were dissolved in 1 mL of chloroform, then 0.5 mL of concentrated $H_2SO_4$ solution was added. Positive test results are indicated by the formation of an orange to brown ring [32].

***Determination of Total Flavonoid Content (TFC).*** The TFC (total flavonoids content) was determined by spectrophotometry using $AlCl_3$ reagents and quercetin as standards. 5 mg of quercetin is dissolved into 5 mL of methanol, resulting in a quercetin 1000 ppm solution stock solution. Amount of 25, 50, 100, 150, and 200 µL of quercetin stock solution were placed into the test tube and then distillate water was added until the volume became 2.7 mL. This solution is referred to as the quercetin standard solution series. The preparation of the sample solution is carried out in the following way: as much as 5 mg of the sample is dissolved into 5 mL of methanol so that the sample master solution with a concentration of 1,000 ppm is obtained. A total of 500 µL of sample master solution was added distillate water until the volume became 2.7 mL. To a series of standard quercetin solutions and sample solutions, 150 µL of $NaNO_2$ 5% solution was added, shaken until homogeneous and left for 5 minutes. Then, 150 µL of 10% $AlCl_3$ was added, left for 6 minutes, then 2 mL of 1M NaOH was added, homogenized and left for 5 minutes. Next, the absorbance was measured at 510 nm. The TFC were calculated through the calibration curve regression equation of quercetin standard solutions. The results are expressed in milli grams (mg) of quercetin equivalent per gram of sample (mg QE/g sample) [29, 34, 37]. The test was carried out three times (triplo).

***Determination of Total Phenolics Content (TPC).*** The preparation of the sample solution was carried out as follows: 5 mg of the sample was dissolved into 5 mL of methanol (p.a) so that a stock solution with a concentration of 1000 ppm was obtained. 500 µL of stock solution is added distillate water until the volume becomes 4 mL. To the standard solution series of gallic acid and sample solution 250 µL of Folin Ciocalteu reagent is added, then homogenized, and left for 8 minutes. Then, add 750 µL of $Na_2CO_3$ 20% solution and incubate for 2 hours in a dark room at room temperature. Furthermore, its absorption was measured at 765 nm [29, 34]. The total phenolic content is calculated through the regression equation of the standard calibration curve of gallic acid. The result is expressed in milligram gallic acid equivalent per gram of sample (mg GAE/g sample) [29, 34]. The test was carried out three times (triplo).

## Test of Antioxidant Activity of Neem Leaf Extract and Fractions

Antioxidant activity was determined through radical capture using DPPH by visible spectrophotometry. The procedures followed the method by Idris *et al*. [23]. The steps carried out were: (1) preparation of

sample solution series (extract and neem leaf fractions), (2) preparation of ascorbic acid solution (as a positive control), and (3) preparation of DPPH solution series (as a radical source) and negative control. *First stage*, sample solution sequence preparation: a series of solution samples of extracts and fractions with concentrations of 100, 200, 300, 400, and 500 ppm. For ethyl acetate fractions, series solutions with concentrations of 50, 75, 100, 125, and 150 ppm. *Second stage*, the preparation of ascorbic acid solution with concentrations of 1, 2, 3, 4, and 5 ppm. *Third stage,* DPPH solution preparation with a concentration of 20 ppm, and negative control is each solvent (without samples).

The test was carried out with the following experiment: 7.2 mL of DPPH 20 ppm solution was added to 0.8 mL of each sample solution (extract and fractions) and ascorbic acid solution at each concentration. It was incubated for 30 minutes, then its absorption was measured at wavelengths 497, 517, and 537 nm using a Vis 722 AP spectrophotometer. The calculation of absorbance to assess antioxidant activity is performed using the following equation:

$$A_{calc} = A_{517} - \frac{A_{497} + A_{537}}{2}$$

with $A_{497}$ is absorbance at wavelength of 497 nm, $A_{517}$ is absorbance at a wavelength of 517 nm, $A_{537}$ is absorbance at a wavelength of 537 nm, and $A_{calc}$ is the calculated absorbance. Determination of radical scavenging inhibition of DPPH with the following equation:

$$Inhibition = \frac{A_o - A_t}{A_o} \times 100\%$$

where $A_0$ is the absorbance of the control and $A_t$ is the absorbance of the sample. The absorbance obtained is made a calibration curve in linear regression equation:

$$y = ax + b$$

where $x$ is the concentration of the sample (in ppm) and $y$ is the inhibition of DPPH radical scavenging (in %). Furthermore, the $IC_{50}$ value is determined by substituting 50 to the $y$ value so that the value $x$ (sample concentration) is obtained when the DPPH inhibition is 50%.

### Test of Antifungal Activity of Neem Leaf Extract and Fraction

The test of food antifungal activity uses a simple technique, namely with the medium of white bread. This medium is cut to a size of (4 x 4 x 1) cm. A total of 20 mg of extract and neem leaf fractions, each dissolved into 10 mL of 70% ethanol, was obtained in the sample master solution with a concentration of 2000 ppm. These solutions were diluted into 1000 and 500 ppm, and 10 mL each was taken as a test sample. The samples of the 2000, 1000, and 500 ppm test solution were put into spray bottles, respectively. 3 slices of prepared white bread are sprayed with each test sample solution. The preparation of this white bread is then put into a plastic clip and the appearance of mold stains is observed every 12 hours for 192 hours (16 x 12 hours), that is, the appearance of mold stains is permanent. The experimental procedure followed the method by Torrijos *et al*. [37] with major modifications.

## Results and Discussion

### Extraction of Substances in Neem Leaves

Drying in an oven at 50°C for 48 hours for 2 kg of fresh neem leaves resulted in dried neem leaves or simplicial (constant weight) of 768.9 g (yield 38.45%). So, the moisture content in neem leaves is 61.55%. Simplicial preparations aim to produce samples that are resistant to fungi and other microbes. Simplicial is squeezed and milled so that a simplicial powder with a maximum size of 50 mesh is produced. Next, this simplicial powder was extracted by maceration with methanol 3 x 24 hours. Methanol is a universal solvent and extractant [38]. This solvent can dissolve or extract non-polar to polar compounds, because it has a methyl molecule ($-CH_3$) which is non-polar and a hydroxyl group (-OH) which is polar. From this maceration, 62.1605 g of methanol extract was obtained (yield 8.08%). This yield is higher than another previous research, namely 7.9% [39]. Differences in yield values can be caused by several factors, including the plant growing environment, age, drying method, and extraction time and method. The methanol extract of neem leaves is then partitioned using the liquid-liquid extraction method. The methanol extract of neem leaves is then fractionated using liquid-liquid extraction with various solvents, namely n-hexane, ethyl acetate, 1-butanol, and (methanol + water = 1 + 5).

## Fractionation to Neem Leaf Extract

Fractionations are carried out to isolate compounds based on their polarity differences. This step or procedure is a standard procedure in the isolation technique of natural products. The fractionation was carried out by liquid-liquid extraction using a separate funnel, with a solvent whose polarity was increasing, starting with n-hexane then ethyl acetate and ending with 1-butanol. These solvents have a lower polarity than methanol and water. The appropriate components are distributed into each solvent with the corresponding polarity. Each solvent used does not mix with each other with the solvent of the leaf extract, thus forming two layers. The upper layer is a solvent layer that is empathetic with the extracted neem leaf components, while the lower layer is an extract residue (in methanol-water solvent) that still contains relatively polar components. From this fractionation process, fractions are obtained according to the solvent, and are referred to as n-hexane fraction, ethyl acetate fraction, 1-butanol fraction, and methanol-water fraction (as residue), the results are summarized in Table 1. Referring to this table, the content of secondary metabolites of neem leaf methanol extract is dominated by relatively polar compounds.

**Table 1.** Fractionation yields of 50 g methanol extract of neem leave

| Fraction | Weight (gram) | Colour | Yield (%) |
|---|---|---|---|
| n-Hexane | 6.93 | dark green | 13.86 |
| Ethyl acetate | 3.42 | dark green | 6.84 |
| 1-Butanol | 8.29 | blackish brown | 16.58 |
| Methanol-water | 24.34 | blackish brown | 48.68 |

The analysis of the components contained in methanol extract and fractions was carried out by thin layer chromatograph (TLC) with mixed eluent (n-hexane + ethyl acetate = 3 + 1). Visible stains with UV lamps at 254 and 366 nm, the results are shown in Figure 1. The symbols on the TLC plates are as follows, E is methanol extract, ***Hx*** is n-hexane fraction, ***EA*** is ethyl acetate fraction, ***Bu*** is 1-butanol fraction, and ***Air*** is methanol-water fraction (extract residue). Based on the results of this TLC analysis, it appears that the n-hexane fraction contains the most components (12 stains). The complete results of the component content are methanol extract, n-hexane fraction, ethyl acetate fraction is 6, 12, and 4 respectively. The 1-butanol fraction and the methanol-water fraction are not delusional with this eluent.

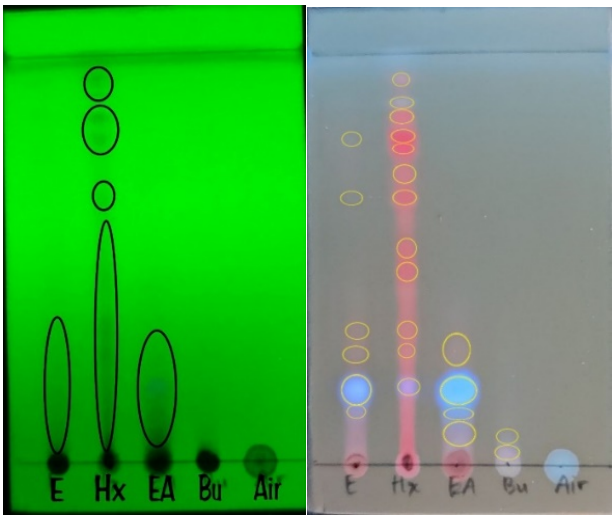


**Figure 1**. Results of TLC analysis by UV lamp at 254 nm (left) and 366 nm (right)

Referring to the chromatogram (**Figure 1**), the n-hexane fraction not only cares for non-polar components, but also polar components. This is suspected to be due to the presence of methanol in this fraction. Methanol with its methyl group and hydroxyl group is capable of being dissolved in non-polar and polar solvents. During partitioning, methanol can dissolve with water or n-hexane, so its non-polar components are dissolved in n-hexane. This condition similar to what occurs in the partitioning process with ethyl acetate and 1-butanol.

## Analysis by Bromine

This analysis is intended to identify the presence of non-aromatic C=C double bonds in neem leaf extracts and fractions. The reaction that occurs is the addition of bromine molecules to the C=C bond which is characterized by a decrease in intensity (fading) of the color of bromine water. Thus, a positive test of non-aromatic C=C double bonds is characterized by a decrease in the intensity of the color of the bromine. This appearance of reduced intensity is observed qualitatively-visually, the results are listed in Table 2. The results of the test with bromine water indicate that all fractions have a double bond C=C, with different degrees of unsaturation. Based on this test, the n-hexane fraction and the ethyl acetate fraction have a lower degree of unsaturation than other fractions.

This analysis is intended to identify the presence of non-aromatic C=C double bonds in neem leaf extracts and fractions. The reaction that occurs is the addition of bromine molecules to the C=C bond which is characterized by a decrease in intensity (fading) of the color of bromine water. Thus, a positive test of non-aromatic C=C double bonds is characterized by a decrease in the intensity of the color of bromine water. This appearance of reduced intensity is observed qualitatively-visually, the results are listed in Table 2. The results of the test with bromine water indicate that all fractions have a double bond C=C, with different degrees of unsaturation. Based on this test, the n-hexane fraction and the ethyl acetate fraction have a lower degree of unsaturation than other fractions.

**Table 2.** Results of analysis by bromine

| Samples | Results of | |
|---|---|---|
| | observation | detection |
| Bromine (in water) | reddish solution | |
| Methanol extract | yellow solution | ++ |
| n-Hexane fraction | two layers:<br>top (brownish yellow) and bottom (yellow) | + |
| Ethyl acetate fraction | brownish yellow solution | + |
| 1-Butanol fraction | yellow solution | ++ |
| Methanol-water fraction (residue) | yellow solution | ++ |

*Note: +: weak detected and ++: moderate detected*

Methanol extract, n-hexane and ethyl acetate fractions have maximum uptake at λ greater than 380 nm as shown in Figure 2 and Table 3. The absorption pattern of these four fractions is relatively the same, each with three $\lambda_{max}$, except for the n-hexane fraction with four three $\lambda_{max}$. Since the analysis was carried out at the same concentration, namely 10 mg in 10 mL (1 mg. $mL^{-1}$) at path length of visible light is 1 cm, the value of absorptivity (a) is equal to its absorption, in $cm^2.mg^{-1}$. This represents the existence of a long-conjugated C=C and/or C=O bond (≥ 4 C=C double bond). Possible electron transitions are $\pi \rightarrow \pi^*$ and/or $n \rightarrow \pi^*$ transitions. The 1-butanol fraction and methanol-water fraction with concentration is 1000 ppm are yellow substances. Theoretically, colored substances should have $\lambda_{max}$ character in the range of 400-800 nm. However, in this study neither showed any identifiable $\lambda_{max}$.

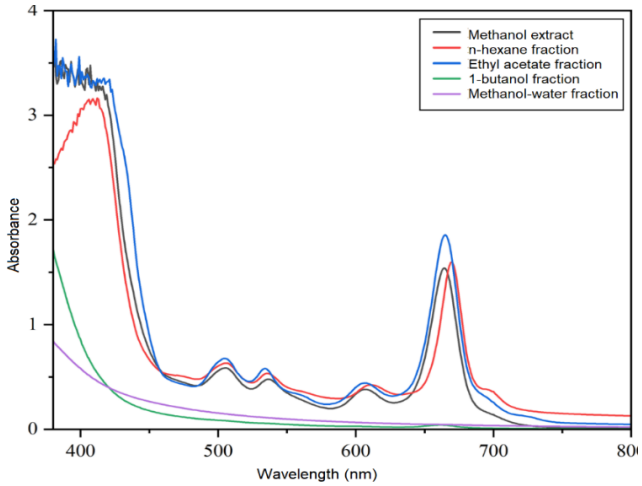


**Figure 2.** Visible spectra of neem leaf extract and fractions

**Table 3.** Analysis results of $\lambda_{max.}$ and absorbance

| Sample | $\lambda_{max}$ (nm) | A | a ($cm^2$. $mg^{-1}$) |
|---|---|---|---|
| Methanol extract | 664 | 1.5385 | 1.5385 |
| n-Hexane fraction | 409 | 3.1576 | 3.1576 |
| | 670 | 1.5972 | 1.5972 |
| Ethyl acetate fraction | 665 | 1.8570 | 1.8570 |
| 1-Butanol fraction | *NA* | *NA* | *NA* |
| Methanol-water fraction | *NA* | *NA* | *NA* |

*Note:* **a** is absorptivity, and *NA is not available data*

## Analysis of Infra-Red Spectrophotometry

The IR spectrum (infra-red) results of the analysis of the extract and neem leaf fractions at the wave number of 4000–500 $cm^{-1}$ are shown in **Figure 3**. Based on this spectrum, it shows that the extract and fractions of neem leaves have bands including vibrating, stretching, and bending bands at a certain number of waves indicating the presence of typical groups of compounds contained in them.

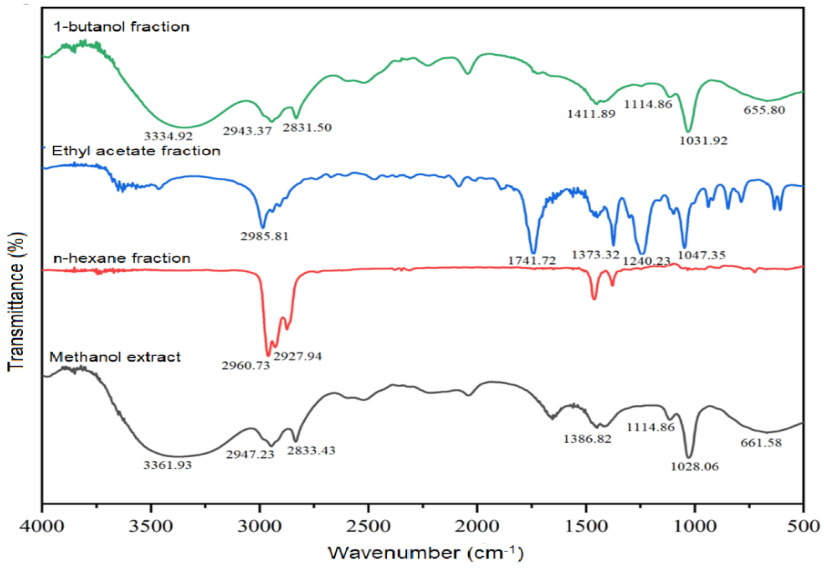


**Figure 3.** Infra-red spectra of neem leaf extract and fractions

Neem leaf methanol extract showed a distinctive band with strong and broad intensity at a wave number 3361.93 $cm^{-1}$ indicating the stretching vibration of the O-H bond and supported by the appearance of a band of 661.58 $cm^{-1}$ as of O-H bending vibration. In addition, bands at 1114.86 and 1028.06 $cm^{-1}$ indicated C-O bending vibrations. Based on the data, it is suspected that methanol extract contains alcoholic compounds. In addition, a distinctive band was also observed at the wave number of 1386.82 $cm^{-1}$ which indicates aromatic C=C linear vibration. This is supported by the appearance of bands at wave numbers 2947.23 and 2833.43 $cm^{-1}$ which are the C-H stretching vibration of the alkyl chain, as well as bands at 582.5 $cm^{-1}$ which are aromatic C-H bending vibrations.

The n-hexane fraction does not have a characteristic band of vibrational functional groups (4000 to 1500 $cm^{-1}$), and this is consistent with the results of the test analysis with bromine water. However, the visible spectrum shows that there are four maximum wavelengths indicating conjugation not of the C=C double bond. The ethyl acetate fraction has a distinctive band with strong and sharp intensity at wave numbers 1741.72 $cm^{-1}$ which represents the stretching vibration of the C=O group. Other bands also appear at 2985.81 $cm^{-1}$ indicating C-H stretching vibration and at 1373 $cm^{-1}$ indicating C-H bending vibration. In addition, a weak and broad band appears around 3500 $cm^{-1}$ which indicates O-H stretching vibration.

The IR spectrum of the 1-butanol fraction has an identical pattern to that of methanol extracts. The characteristic band at 3334.92 $cm^{-1}$ which is the O-H stretching vibration. There is a band at 655.8 $cm^{-1}$ indicating as a O-H stretching vibration. Other bands were observed at 1114.86 and 1031.92 $cm^{-1}$ which

are C-O bending vibrations. Based on the data, it is suspected that the 1-butanol fraction contains alcoholic compounds. In addition, a distinctive band also appears at a wave number of 1411.89 $cm^{-1}$ indicating aromatic C=C tensive vibrations. This is supported by the appearance of bands at wave numbers 2943.37 and 2831.5 $cm^{-1}$ which are C-H stretching vibrations.

## Quantitatively Antioxidant Activity, TPC, and TFC Relationship of Neem Leaf Extract and Fractions

Methanol extracts and neem leaf fractions show the total phenolic content (TPC) and total flavonoids content (TFC). Quantitatively analyzed, the ethyl acetate fraction has the largest total phenolic content, while the n-hexane fraction contains the largest total flavonoid content. TPC and TFC indicate an important contribution to antioxidant activity (Table 4). The methanol extract of neem leaves has an $IC_{50}$ value of 70.29 ppm and is classified as very weak. The antioxidant activity of the methanol extract of neem leaves in this study was slightly stronger than previous research with an $IC_{50}$ value of 80.28 ppm [25]. Quantitatively, the antioxidant activity of neem leaf extracts and fractions was determined using the DPPH method. The $IC_{50}$ value represents the concentration when the sample can capture 50% of DPPH radicals. Good antioxidant activity is indicated by the small $IC_{50}$ value. The $IC_{50}$ values of neem leaf extracts and fractions, as well as ascorbic acid. There is a quantitative relationship between the total phenolic content and total flavonoid content in each extract or fraction and its antioxidant activity (Figure 4). The ethyl acetate fraction with the highest content of these two groups of compounds has the strongest antioxidant activity, followed by the 1-butanol fraction.

**Table 4.** TPC, TFC, and $IC_{50}$ of neem leaf extract, fractions, and ascorbic acid

| Sample | TPC (mg GAE/g sample) | TFC (mg QE/g sample) | $IC_{50}$ (ppm) |
|---|---|---|---|
| Methanol extract | 5.53 | 11.15 | 70.29 |
| n-Hexane fraction | 3.09 | 18.82 | 82.38 |
| Ethyl acetate fraction | 12.08 | 17.32 | 19.06 |
| 1-butanol fraction | 9.2 | 7.2 | 43.68 |
| Methanol-water fraction | 6.2 | 1.5 | 78.15 |
| Ascorbic acid (positive control) | -- | -- | 16.64 |

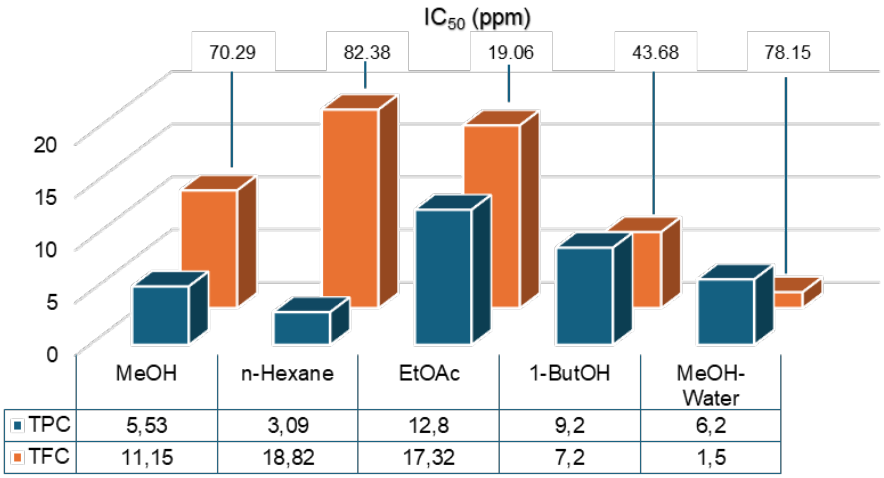


**Figure 4.** Relationship of phenolic and flavonoid contents and antioxidant activity

The highest antioxidant activity is the ethyl acetate fraction, although it is a very strong category, although it is weaker than ascorbic acid. The ethyl acetate fraction with the highest total phenolic content and total flavonoid content provides a dominant contribution in determining antioxidant activity. DPPH captures H radicals from the -OH group of phenolic compounds and flavonoids (for example, symbolized ROH) to form DPPHH, while ROH phenolic compounds form RO radicals. The change in DPPH to DPPHH (reduced DPPH) is characterized by a decrease in the absorbance of the DPPH solution compared to before capturing hydrogen radicals from phenolic compounds [22]. Flavonoids and phenolics act as

antioxidants because they have hydroxyl groups (-OH) which can stabilize free radicals by donating hydrogen atom [41, 42]. Antioxidants (phenolic and flavonoid compounds) play an important role in maintaining cellular health by protecting the body's cells from oxidative damage. Their main mechanisms include free radical capture, electron or hydrogen donation, and enzymatic activation, which collectively form an effective antioxidant defense in the human body.

The appearance of mold on white bread growing media pada jam ke 90
Mold begins to appear at the 90th hour

## Antifungal Activity of Neem Leaf Extract and Fractions

The antifungal activity of neem leaf extracts and fractions was determined qualitatively using a simple but very practical method, namely white bread media. The appearance of mold on the white bread media was observed every 12 hours starting from 0 (null) hour to the 192 hours (8 days). During the period up to the 60 hours (2.5 days), mold had not grown in all test samples. On the 72 hours (3 days) mold began to grow on the bread without treatment. The experimental results showed that in the 72$^{nd}$ hour of untreated bread, mold began to grow and continued to grow throughout the observation. White bread treated with 70% ethanol solution (as a comparison) and methanol extract, n-hexane fraction, and methanol-water fraction; mold grew at the 96$^{th}$ hour. Meanwhile, treatment with the ethyl acetate fraction or 1-butanol fraction, the mold began to grow at the 108$^{th}$ hour, and so on, briefly presented in Table 5. The 2000 ppm ethyl acetate fraction showed the best antifungal activity, because it could inhibit fungal growth for up to 120 hours and survived until the end of the experiment (192 hours). So, it has the potential to act as food antifungal. This can be attributed to the high total phenolic content in the ethyl acetate fraction. Phenolic compounds can interact with the hydrophilic part of the fungal cell membrane so that its permeability is disturbed and can cause cell death [25, 43]. Therefore, ethyl acetate and 1-butanol fractions could be an alternative to natural additives in bread formulations since they satisfy consumer requirements.

**Table 5.** Data on fungal growth on white bread with treatment extract and fractions of neem leave

| Threat white bread by | Concentration (ppm) | Observed at hour: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 60 | 72 | 84 | 96 | 108 | 120 | 132 | 144 | 156 | 168 | 180 | 192 |
| Without threat (blank) | | - | + | ++ | ++ | +++ | +++ | +++ | +++ | ++++ | ++++ | ++++ | ++++ |
| Ethanol 70% | | - | - | - | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ |
| MeOH extract | 500 | - | - | - | - | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | 1,000 | - | - | - | - | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | 2,000 | - | - | - | - | - | + | +++ | +++ | +++ | +++ | +++ | +++ |
| *n*-Hexane fraction | 500 | - | - | - | + | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 1,000 | - | - | - | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | 2,000 | - | - | - | - | - | + | ++ | ++ | ++ | ++ | ++ | ++ |
| EtOAc fraction | 500 | - | - | - | - | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | 1,000 | - | - | - | - | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | 2,000 | - | - | - | - | - | - | + | ++ | ++ | ++ | ++ | ++ |
| 1-BuOH fraction | 500 | - | - | - | - | + | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 1,000 | - | - | - | - | + | ++ | ++ | ++ | ++ | ++ | +++ | +++ |
| | 2,000 | - | - | - | - | - | + | ++ | ++ | ++ | ++ | ++ | ++ |
| M-W fraction | 500 | - | - | - | + | +++ | +++ | +++ | +++ | ++++ | ++++ | ++++ | ++++ |
| | 1,000 | - | - | - | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ |
| | 2,000 | - | - | - | + | ++ | ++ | ++ | ++ | ++ | +++ | +++ | +++ |

*Note:* mold growth indicated by (-): not yet growth, (+): start growth, (++): little, (+++): medium, and (++++): a lot

Antifungal mechanisms involve a variety of different strategies to disrupt the function and integrity of fungal cells. This approach is important for the development of effective antifungal agents, which can be used in agriculture to control plant diseases, and in medicine to treat fungal infections in humans. Phenolic compounds and flavonoids have different antifungal mechanisms, depending on their compound structure and interactions with target fungal organisms [36, 44, 45, 46]. This class of compounds shows interesting potential as antifungal agents based on their various mechanisms of action and could be an important research area in agriculture and biotechnology, as well as in the development of more effective antifungal drugs.

## Conclusions

Extraction of neem leaves (*Azadirachta indica*) using methanol resulted in a yield of 8.08%. Fractionation of methanol extract with solvents produced n-hexane, ethyl acetate, 1-butanol, and methanol-water fractions with a yield of 13.86; 6.84; 16.58; and 48.68% respectively. Methanol extract contains alkaloids, flavonoids, phenolics, tannins, terpenoids and saponins. Each fraction contains flavonoids, phenolics, tannins, terpenoids and saponins. The content of phenolics and flavonoids compounds is dominant in methanol extract, ethyl acetate fraction, and 1-butanol fraction. The ethyl acetate fraction contained the most total flavonoid content 17.32 mg QE/g and total phenolic content 12.80 mg GAE/g. The best antioxidant and antifungal activity were demonstrated by the ethyl acetate fraction. The antioxidant activity of this fraction is in the strong category ($IC_{50}$ 19.06 ppm). The ethyl acetate fraction was able to prevent mold growth on white bread for 96 hours and 120 hours at concentrations of 500 and 2000 ppm respectively. The content of flavonoid and phenolic compounds indicated that they contributed mainly to antioxidant and antifungal activities, and there was a significant relationship. Neem leaf extract has potential as a food antioxidant and antifungal, especially methanol extract, ethyl acetate fraction, and 1-butanol fraction. Therefore, ethyl acetate and 1-butanol fractions could be an alternative to natural additives in bread formulations.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

## Acknowledgement

This work is supported by the Research Grant by Non-Budgeting (*Non-APBN)* Institute of Research and Community Engagement (*Lembaga Penelitian dan Pengabdian kepada Masyarakat*), Universitas Negeri Malang.

## References

[1] Nugraha, S. P., & Agustiningsih, W. R. (2015). Pelatihan penanaman tanaman obat keluarga (Toga). *Jurnal Inovasi dan Kewirausahaan, 4*(1), 58–62.

[2] Latif, M. J., Hassan, S. M., Mughal, S. S., Aslam, A., Munir, M., Shabbir, N., Mushtaq, M., & Perveis, S. (2020). Therapeutic potential of *Azadirachta indica* (Neem) and their active phytoconstituents against diseases prevention. *Journal of Chemistry and Chemical Sciences, 10*(3), 98–110.

[3] Sodirun, F., Hayati, A., & Zayadi, H. (2016). Persepsi masyarakat tradisional Pulau Mandangin Kabupaten Sampang terhadap tanaman mimba (*Azadirachta indica* Juss). *e-Jurnal Ilmiah Biosaintropis (Bioscience-Tropis), 2*(1), 11–18.

[4] Mihra, M., Jura, M. R., & Ningsih, P. (2018). Analisis kadar tanin dalam ekstrak daun mimba (*Azadirachta indica* A. Juss) dengan pelarut air dan etanol. *Jurnal Akademika Kimia, 7*(4), 179–183.

[5] Uzzaman, S. (2020). Pharmacological activities of Neem (*Azadirachta indica*): A review. *International Journal of Pharmacognosy and Life Science, 1*(1), 38–41.

[6] Gibreel, H. H., Rabie, R., & Salih, M. (2019). The antibacterial effect of some Sudanese plants (Neem, Garad and Sidr). *Agriculture and Forestry Journal, 3*(2), 89–94.

[7] Witria, W., & Zainuri, M. (2021). Pengaruh ekstrak daun mimba (*Azadirachta indica*) terhadap histamin daging ikan tongkol abu. *Jurnal Pengolahan Hasil Perikanan Indonesia, 24*(2), 209–217.

[8] Andhiarto, Y., Andayani, R., & Ilmiyah, N. H. (2019). Uji aktivitas antibakteri ekstrak etanol 96% daun mimba (*Azadirachta indica* A. Juss.) dengan metode ekstraksi perkolasi terhadap pertumbuhan bakteri *Staphylococcus aureus*. *Journal of Pharmacy Science and Technology, 2*(1), 102–111.

[9] Reddy, I. V. S., & Neelima, P. (2022). Neem (*Azadirachta indica*): A review on medicinal kalpavriksha. *International Journal of Economic Plants, 9*(1), 059–063.

[10] Eid, A., Jaradat, N., & Elmarzugi, N. (2017). A review of chemical constituents and traditional usage of Neem plant (*Azadirachta indica*). *Palestinian Medical and Pharmaceutical Journal, 2*(2), article 3.

[11] Kothawade, M., Jangle, H., Bhadane, H., Sufiyan, M., & Daphal, G. (2022). Active constituent in Neem (*Azadirachta indica*) and their therapeutic role. *International Journal of Pharmaceutical Research and Applications, 7*(5).

[12] Batra, N., Kumar, V. E., Nambiar, R., de Souza, C., Yuen, A., Uyen Le, Verma, R., Ghosh, P. M., & Vinall, R. L. (2022). Exploring the therapeutic potential of Neem (*Azadirachta indica*) for the treatment of prostate cancer: A literature review. *Annals of Translational Medicine, 10*(13), 754.

[13] Damtew, M. (2022). A review on chemical composition, medicinal value, and other applications of *Azadirachta indica*. *AGBIR, 38*(2), 268–272.

[14] Mudenda, S., Banda, M., Mohamed, S., & Chabalenge, B. (2023). Phytochemical composition and antibacterial activities of *Azadirachta indica* (Neem): Significance of traditional medicine in combating infectious diseases

and antimicrobial resistance. *Journal of Pharmacognosy and Phytochemistry, 12*(5), 256–263.
[15] Abdurrahman, M., Lestari, E. S., & Prihatiningsih, T. (2022). The effectiveness of ethanol extract of Neem leaf (*Azadirachta indica*) mouthwash against the growth of *Streptococcus* sp. *Journal of Biomedicine and Translational Research [Online], 8*(2), 76–80.
[16] Oyekanmi, A. A., Kumar, U. S. U., H. P. S., A. K., Olaiya, N. G., Amirul, A. A., Rahman, A. A., Nuryawan, A., Abdullah, C. K., & Rizal, S. (2021). Functional properties of antimicrobial Neem leaves extract-based macroalgae biofilms for potential use as active dry packaging applications. *Polymers, 13*, 1664.
[17] Ruwandha, D., Yani, D. F., & Iskandar, D. (2021). Uji aktivitas tanin daun mimba (*Azadirachta indica*) terhadap bakteri *Salmonella typhi*. *Jurnal Kimia Riset, 6*(1), 77–85.
[18] Rahmawati, I., Fachri, B. A., Nurtsulutsiyah, N., Manurung, Y. H., Reza, M., Palupi, B., Rizkiana, M. F., & Amini, H. W. (2022). Penerapan response surface methodology dalam optimasi kondisi proses ekstraksi antosianin pada limbah kulit kakao dengan metode maserasi menggunakan pelarut etanol. *JC-T (Journal Cis-Trans): Jurnal Kimia dan Terapannya, 6*(1), 24–31.
[19] Susanty, S., & Bachmid, F. (2016). Perbandingan metode ekstraksi maserasi dan refluks terhadap kadar fenolik dari ekstrak tongkol jagung (*Zea mays* L.). *Jurnal Konversi, 5*(2), 87–93.
[20] Farahani, Z. K. (2021). The effect of extraction method (ultrasonic, maceration, and Soxhlet) and solvent type on the extraction rate of phenolic compounds and extraction efficiency of *Arctium lappa* L. roots and *Polygonum aviculare* L. grass. *Food & Health, 4*(2), 28–34.
[21] Septiyani, R., & Wibowo, C. (2019). Identification of active compounds and testing the antioxidant properties of Neem leaf extract. *AIP Conference Proceedings, 2094*, 020034.
[22] Wilson, D. W., Nash, P., Buttar, H. S., Griffiths, K., Singh, R., De Meester, F., Rie Horiuchi, R., & Takahashi, T. (2017). The role of food antioxidants, benefits of functional foods, and influence of feeding habits on the health of the older person: An overview. *Antioxidants, 6*(81).
[23] Idris, L., Adli, M. A., Yaacop, N. N., & Zohdi, R. M. (2023). Phytochemical screening and antioxidant activities of *Geniotrigona thoracica* propolis extracts derived from different locations in Malaysia. *Malaysian Journal of Fundamental and Applied Sciences, 19*, 1023–1032.
[24] Supriyanto, S., Wijanarko, S. B., Rifa'i, M., & Yunianta, Y. (2020). Chemical characteristic and antioxidant activity of methanol extract Neem leaves (*Azadirachta indica* Juss). *EurAsian Journal of BioSciences, 14*, 7881–7887.
[25] Blanco, S. R., Fernández, J., Ibáñez, S. L., Miguélez, E. M., Villar, C. J., & Lombó, F. (2020). Plant phytochemicals in food preservation: Antifungal bioactivity: A review. *Journal of Food Protection, 83*(1), 163–171.
[26] Sudan, P., Goswami, M., & Singh, J. (2020). Exploration of antifungal potential of *Azadirachta indica* against *Microsporum gypseum*. *Biomedical and Pharmacology Journal, 13*(2), 921–925.
[27] Keta, J. N., Suberu, H. A., Shehu, K., Yahayya, U., Mohammad, N. K., & Gudu, G. B. (2019). Effect of Neem (*Azadirachta indica* A. Juss) leaf extract on the growth of *Aspergillus* species isolated from foliar disease of rice (*Oryza sativa*). *Science World Journal, 14*(1), 98–102.
[28] Syahadat, A., & Siregar, N. (2020). Skrining fitokimia daun katuk (*Sauropus androgynus*) sebagai pelancar ASI. *Jurnal Kesehatan Ilmiah Indonesia, 5*(1), 85–89.
[29] Marsoul, A., Ijjaali, M., Elhajjaji, F., Taleb, M., Salaim, R., & Boukir, A. (2020). Phytochemical screening, total phenolic and flavonoid methanolic extract of pomegranate bark (*Punica granatum* L): Evaluation of the inhibitory effect in acidic medium 1 M HCl. *Materials Today: Proceedings, 27*(4), 3193–3198.
[30] Tamasi, A. A., Shoge, M. O., Adegboyega, T. T., & Chukwuma, E. C. (2021). Phytochemical analysis and in-vitro antimicrobial screening of the leaf extract of *Senna occidentalis* (Fabaceae). *Asian Journal of Natural Product Biochemistry, 19*(2), 58–65.
[31] Kumar, N., & More, D. R. (2019). Phytochemical analysis and bioactivity of selected medicinal plant of butterfly-pea (*Clitoria ternatea* L.) used by Kolam tribe adjoining region of Telangana and Maharashtra states. *The Pharma Innovation Journal, 8*(1), 417–421.
[32] Ushie, O. A., Neji, P. A., Abeng, F. E., Okpashi, V. E., Baba, N. H., & Azuaga, T. I. (2019). Phytochemical screening and antimicrobial activities of chloroform and ethyl acetate extracts of *Physalis angulata*. *Journal of Chemical Society of Nigeria, 44*(6), 1062–1069.
[33] Sundowo, A., Artanti, N., Hanafi, M., Minarti, & Primahana, G. (2017). Phytochemical screening, total phenolic, total flavonoids contents and antioxidant activity of *Cinchona ledgeriana* leaves ethanol extract. *AIP Conference Proceedings, 1904*, 020067.
[34] Prifiharni, S., Mashnafie, G., Priyotomo, G., Royani, A., Ridhova, A., Elya, B., & Soedarsono, J. N. (2022). Extract sarampa wood (*Xylocarpus moluccensis*) as an eco-friendly corrosion inhibitor for mild steel in HCl 1M. *Journal of the Indian Chemical Society, 99*(7).
[35] Selahuddeena, M. L., Bouguerrae, M., Hashima, S. E., Wahaba, R. A., Samad, A. F. A., & Abdullah, F. (2024). Investigation on optimized flavonoid extraction from *Leucas zeylanica* and its anthelmintic activity. *Malaysian Journal of Fundamental and Applied Sciences, 20*(2), 497–513.
[36] Torrijos, R., Nazareth, T. d. M., Quiles, J. M., Mañes, J., & Meca, G. (2021). Application of white mustard bran and flour on bread as natural preservative agents. *Foods, 10*(431).
[37] Nisa, F. Z., Astuti, M., Haryana, S. M., & Murdiati, A. (2016). Antioxidant activity and total flavonoid of *Carica papaya* L. leaves with different varieties, maturity and solvent. *Agritech, 39*(1), 54–59.
[38] Apra, M., Prasetyaningsih, A., & Madyaningrana, K. (2021). Potensi biosakarida ekstrak daun mimba (*Azadirachta indica*) dan bunga cengkeh (*Syzygium aromaticum*) terhadap tungau penyebab penyakit krepes pada jamur kuping. *Edumatsains, 5*(2), 225–238.
[39] Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Pandey, A. G. R. P., & Chang, C. M. (2022). Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules, 27*, 1326.
[40] Munteanu, I. G., & Apetrei, C. (2021). Analytical methods used in determining antioxidant activity: A review.

*International Journal of Molecular Sciences, 22*, 3380.

[41] Phuyal, N., Jha, P. K., Raturi, P. P., & Rajbhandary, S. (2020). Total phenolic, flavonoid contents, and antioxidant activities of fruit, seed, and bark extracts of *Zanthoxylum armatum* DC. *The Scientific World Journal, 2020*, Article ID 8780704.

[42] Chen, J., Yang, J., Ma, L., Li, J., Shahzad, N., & Kim, C. K. (2020). Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Scientific Reports, 10*(1).

[43] Liu, Y., Benohoud, M., Yamdeu, J. H. G., Gong, Y. Y., & Orfila, C. (2021). Green extraction of polyphenols from citrus peel by-products and their antifungal activity against *Aspergillus flavus*. *Food Chemistry X, 12*.

[44] Neves, A. M., Fontenelle, R. O. dos S., Lopes, F. S., Mendes, J. de F. S., Rodrigues, A. L. M., Marinho, M. M., Marinho, E. M., & de Morais, S. M. (2021). Phenolic profile, antioxidant and antifungal activity of extracts from four medicinal plants of the *Anacardiaceae* family. *Research, Society and Development, 10*(8), e44510817421.

[45] Ramos, A. J. J., Palestina, C. U. L., Espinoza, J. M. P., Romo, S. E. A., Saenz, Y. O. S., Mancilla, C. L. A., & Tlahque, J. G. (2020). Phenolic compounds, antioxidant properties and antifungal activity of jarilla (*Barkleyanthus salicifolius* [Kunth] H. Rob & Brettell). *Chilean Journal of Agricultural Research, 80*(3), 352–360.

[46] Simonetti, G., Brasili, E., & Pasqua, G. (2020). Antifungal activity of phenolic and polyphenolic compounds from different matrices of *Vitis vinifera* L. against human pathogens. *Molecules, 25*, 3748.