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Article

Isolation of Flavonoid Compounds and Anti-Cholesterol Test of Dutch Teak Leaf Extract (*Guazuma ulmifolia* Lamk.)

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Abstract. Dutch teak (*Guazuma ulmifolia* Lamk.) is a tree originating from Latin America (Brazil and Mexico). The results of the phytochemical test showed that Dutch teak leaves positively contained flavonoids, alkaloids (Dragendorff reagent), steroids, and saponins. The purpose of this study was to characterize the flavonoid compounds and test the anti-cholesterol properties of Dutch teak leaf extract. Flavonoid characterization methods used Mg-HCl (yellow), H_2SO_4 (reddish orange), NaOH (yellow), KKt-2A, UV-Vis, and FT-IR reagents. Anti-cholesterol test using the Lieberman-Burchard method. The flavonoid crystals obtained were in the form of amorphous crystals of 0.1047 grams. In KKt-2A there is a change in color from yellow to greenish yellow when viewed with a UV lamp. The UV-Vis spectrophotometer has an absorption wavelength of 326 nm. The results of FT-IR analysis showed the presence of functional groups: OH, CH, C=O, C=C, and COC. Based on these data it can be concluded that the isolated flavonoids are in the form of amorphous crystals which belong to the flavone class with the name 5,7,4' - Trihydroxyflavone-6-Prenyl. The results of the cholesterol reduction test stated that Dutch teak leaves could be anti-cholesterol as seen from the absorbance value being smaller and the percentage of anti-cholesterol activity being greater.

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1. Introduction

Dutch teak plant with the Latin name Guazuma ulmifolia Lamk. is a tree that comes from Latin America (Brazil and Mexico) which has a tropical climate. This plant is known as Mutamba and Guácimo. Dutch teak plants have parts, namely leaves, fruit, flowers, bark, and roots. Dutch teak belongs to the Sterculiaceae family which grows in Ecuador, Panama and other Latin American countries. Dutch teak is found in shrubs and trees in the forest. This plant is often the most needed for respiratory tract infections because it can function in relieving coughs, reducing fever, and can provide antiviral and antibacterial action [1-2].

Dutch teak leaves have been used as a traditional medicine to maintain a healthy body, lose weight as a slimming herb, and lower cholesterol in the body. Medicinal use of this plant is usually consumed as a herbal concoction. Dutch teak leaves are used as a traditional medicine because of the many properties they contain, such as cough medicine, watery swollen feet, cholesterol lowering, and diarrhea medicine [1],[3]. Dutch teak leaves are used by the community as anti-obesity and anti-hyperlipidemic, the use of these leaves is by boiling a few leaves of Dutch teak and then drinking it after it's cold.

The use of Dutch teak leaves as medicine is due to the presence of secondary metabolites which have the potential to promote physiological and pharmacological effects known as active compounds. [4-5]. Secondary metabolites are compounds with biological activity that have the potential to protect plants from pests and diseases for plants and their environment. Phytochemical screening is a simple test that can be used to determine the secondary metabolite content of a plant. The results of the phytochemical screening can be used for the initial analysis of the potential of these plants to become medicinal compounds [6-8] .

Dutch teak leaves have secondary metabolites, such as flavonoids, tannins, sterols, saponins, alkaloids, glucose, calcium oxalate, and phenolic acids. The content of secondary metabolites in Dutch teak leaves such as tannins, saponins, alkaloids, flavonoids, and sterols are known to bind to cholesterol and fat molecules found in food, resulting in a decrease in blood cholesterol levels [9].

Flavonoid compounds are one of the compounds that can lower cholesterol. Flavonoids are compounds that have a C15 atom that is mostly found in plants, which are included in flavonoid compounds, namely flavones, aurons, chalcones, isoflavones, anthocyanins, and leukocyanins. Flavonoids have the potential to reduce cholesterol in the blood by reducing the absorption of cholesterol and bile acids in the small intestine [10-11].

Based on the description that has been explained above, it can be concluded that Dutch teak leaves contain various secondary metabolite compounds that are useful as drugs, one of which is flavonoids as cholesterol lowering. However, until now the secondary metabolites in Dutch teak leaves have only been identified as a group, so it cannot be ascertained which active compound has a role in determining cholesterol lowering.

2. Experimental Section

2.1. Materials

The sample used in this study was a powder of Dutch teak leaves (*Guazuma ulmifolia* Lamk.) taken in the Magelang area, Central Java, Indonesia.

2.2. Sample Preparation

Fresh Dutch teak leaves are selected and separated (sorted) from rotten leaves. Then washed thoroughly with running water, finely chopped to facilitate the drying process, then dried at room temperature. Then crushed and sieved, then stored in plastic as raw material for research.

2.3. Phytochemical Test

2.3.1. Alkaloid Test

Dutch teak leaf powder is taken as much as 4 grams then added a little chloroform, then crushed to form a paste. Next, add 10 mL of 0.05 N chloroform-ammonia, grind again, and take the liquid extract using a dropper pipette which is filtered by placing a cotton swab over the mixture, then placing it in a test tube. Add 5 mL of 2 N sulfuric acid, shake vigorously, and let stand to form two layers. The sulfuric acid layer is taken with a dropper pipette and put into a small size test tube, and save part of the chloroform layer for the terpenoid test. Test the filtrate with Mayer's, Wagner's, and Dragendorff's reagents. Positive alkaloids are indicated by the appearance of a white precipitate on Mayer, a brown precipitate on Wagner, and an orange precipitate on the Dragendorf reagent.

2.3.2. Flavonoid Test

Dutch teak powder as much as 1 gram in a test tube, add methanol to extract then heat for about 5 minutes. Enter the methanol extract in another test tube, then add a few drops of concentrated H_2SO_4. Shake and observe the color change that occurs, if the color changes to brownish red then the positive sample contains flavonoid compounds.

2.3.3. Test Steroids and Terpenoids

Take layer chloroform on test alkaloids previously with a dropper pipette, then pipette a few drops on the drip plate. Then add 5 drops of acetic anhydride and allow it to dry. If Already dry, add 3 drops of sour sulfate concentrated. If formed change color to reddish orange so sample positive terpenoids, whereas the color blue signifies positive steroids.

2.3.4. Saponin Test

Put the Simplicia powder into the test tube, add it aquadest. Then heat 2-3 minutes and let until cold. If, it is already cold, shake with strong. If there is foam stable so samplepositive saponins.

2.4. Flavonoid Isolation

2.4.1. Extraction

1000 grams of Dutch teak leaf powder was extracted by maceration method with methanol. The maceration process was carried out for 2 x 72 hours. The extract from this maceration was then concentrated with a rotary evaporator and a Shinoda test or a flavonoid content test was carried out on the thick extract results.

2.4.2. Fractionation

The thick extract obtained from the extraction was fractionated using a separating funnel successively with n-hexane and ethyl acetate. The fractionation was carried out several times until the n-hexane layer became clear and the final result was a negative Shinoda test to obtain two fractions, namely the aqueous fraction and the n-hexane fraction. The n-hexane fraction was tested for its flavonoid content with the Shinoda test which showed negative results, while the ethyl acetate fraction gave positive results containing flavonoids. The ethyl acetate fraction was then evaporated using a rotary evaporator so that ethyl acetate viscous flavonoids would be obtained.

2.4.3. Separation and Purification

2.4.3.1. Thin Layer Chromatography and Column Chromatography

Thin layer chromatography is used to determine the eluent suitable for the separation of components by column chromatography. The eluent types used were ethyl acetate and methanol with graded polarity. Column chromatography was performed to separate the mixture from the viscous extract using silica gel as the stationary phase, with the mobile phase being ethyl acetate: methanol.

The condensed extract is put into the column above the silica packaging and then eluted with eluent. Collect the resulting eluate in small vials which are numbered. Each vial was monitored by TLC, and the eluate with the same Rf was combined into one vial. Then allow the solvent to evaporate to form an amorphous solid.

2.5. Purity Test

2.5.1. Thin Layer Chromatography

The isolated compound was tested for its purity by Thin Layer Chromatography. The stains produced on the chromatogram can be observed directly if they are visible or with a UV lamp, if the stains are not visible use a color-generating spray reagent with NH_3 steam. If the spot is single, then the compound is pure, but if the spot is not single, then the compound is not pure.

2.5.2. Melting Point Determination

The melting point was determined with the Gallenkamp melting point apparatus. The amorphous solid to be tested for purity is put into a capillary tube which is closed at one end as high as approximately 1 mm, then put into the Gallenkamp melting point apparatus, increasing the temperature of 5°C per minute. Observations were made when the crystals began to melt completely. A substance is said to be pure if its melting point range is less than 2°C.

2.6. Characterization of the Resulting Compounds

2.6.2. Color Test

In the color test the isolated flavonoids with methanol solution, were then divided into three parts in a test tube. Then the first test tube was added 10% NaOH solution, the second test tube was added concentrated H_2SO_4, and the third test tube was added Mg-HCl. Observe the color change that occurs in each tube.

2.6.3. The Two Way Paper Chromatography (KKT-2A)

Using 3 mm Whatman paper size 23x24 cm. Then the sample was spotted on the paper using a capillary tube at 8 cm from the edge of the paper and 1.5 cm from the fold. The paper is put into a vessel containing BAA developer (butanol, acetic acid, and distilled water) with a ratio of 4:1:5. Elude until the developer moves to the upper limit. Then remove the paper and dry it in a fume cupboard. The formed stains were detected using a 365 nm UV lamp.

The paper is then rotated 90° from its initial position and dipped in another vessel containing 15% acetic acid developer solution. Until the elution reaches the upper limit then removed and dried in a fume cupboard. Then it is evaporated with ammonia vapor. Mark the resulting color with a pencil and then calculate its Rf value.

2.6.4. UV-Vis Spectroscopy

Some of the isolated flavonoids were dissolved in methanol pa, then tested by UV-Vis spectroscopy which had been standardized with methanol pa then the sample spectra were recorded using shear reagents (NaOH, $AlCl_3$, $AlCl_3$/ HCl, NaOAc, and NaOAc/H_3BO_3). The spectrum is measured with a wavelength of 200-500 nm.

2.6.5. Infrared Spectroscopy

Measurements using an infrared spectrophotometer were carried out on compounds with a solid state. This measurement is used to determine the functional groups present in the compound. Plates containing isolates in the form of crystals or amorphous with KBr are homogenized and then pressed

to form thin plates. Furthermore, measurements are taken on the plate and the resulting measurements are in the form of peaks that can be analyzed.

2.7. Anti-Cholesterol Test

Siamese pumpkin flavonoid extract with various concentrations was put into a test tube, then 5 ml of standard cholesterol solution was added to it and shaken. After that, add 2 ml of anhydrous acetic acid, then add 0.1 ml of concentrated sulfuric acid. Then leave it in a dark place for 15 minutes. There will be a color change to green. After 30 minutes, do the test with UV-Vis spectrophotometry.

$$A = \frac{C - B}{C} \times 100\%$$

A = % reduction in cholesterol
B = Absorbance of the sample after treatment (methanol extract + standard)
C = Absorbance of initial cholesterol standard

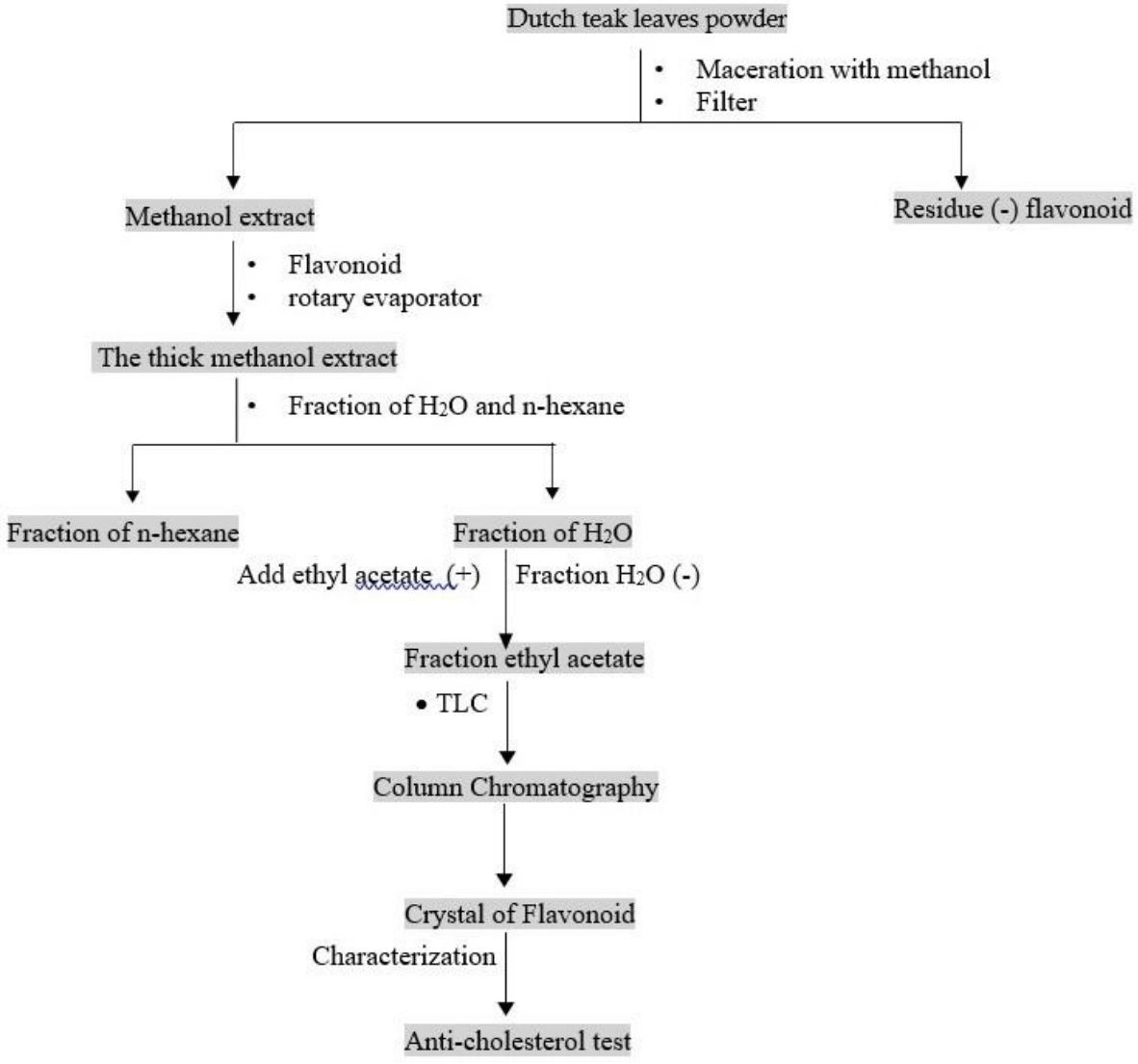

Figure 1. The experiment procedures

3. Results and Discussion

3.1. Isolation and Characterizations

The preliminary test is an initial test to detect what types of secondary metabolites may be contained in natural products which will be studied further. Preliminary test results for secondary metabolites on Dutch teak leaves (*Guazuma ulmifolia*) are shown in Table 1.

Table 1. Preliminary test results

No.	Secondary Metabolites	Reactor	Observation	Results*
1.	Alkaloids	Mayer	No white precipitate formed	(-)
		Wagner	No brown precipitate formed	(-)
		Dragendorff	An orange precipitate formed	(+)
2.	Flavonoids	H_2SO_4 _ _ _	Brownish red solution	(+)
3.	Terpenoids	Lieberman-burchad	No formation of reddish orange color	(-)
4.	Steroids	Lieberman-burchad	A blue solution is formed	(+)
5.	Saponins	H_2O + HCl	Foam appears	(+)

* (+) Identified
(-) Not Identified

The content of secondary metabolites that were positive in this sample were flavonoids, dragendorff in alkaloids, steroids, and saponins. The identification of flavonoid compounds with H_2SO_4 reagent resulted in a color change due to the occurrence of an oxidation-reduction reaction of H_2SO_4 and flavonoids which caused the formation of complex compounds which gave rise to a brownish red color in the sample [12-14].

After phytochemical screening had been done, the sample was subjected to the extraction process, i.e. maceration. The maceration method was carried out with methanol for 2x72 hours. This extraction time causes longer contact between the sample solids and the solvent which will increase the number of broken cells and dissolved active compounds. The solvent used in this maceration process was methanol [15]. After the evaporation process, 70 grams of concentrated methanol extract was obtained. Furthermore, the concentrated methanol extract was added to 200 mL of hot water The aim is to accelerate the solubility of concentrated extracts in water. Furthermore, the fractionation process aims to separate the extract into several fractions according to their polarity, such as non-polar, semi-polar, and polar [6]. The fraction that showed positive results was the ethyl acetate fraction. The concentrated ethyl acetate extract obtained was 2.564 grams.

The separation of flavonoids was carried out using column chromatography. Based on the results of the TLC, a good eluent for separation was the ratio of ethyl acetate: methanol (7:3). Column chromatography was carried out using the Step Gradient Polarity (SGP) method with the eluent ratio of ethyl acetate: methanol which can be seen in Table 2. This SGP method was carried out to obtain good separation between polar, semi-polar, and non-polar compounds present in samples of the eluent used in stages based on their polarity [9].

Table 2. Comparison of ethyl acetate and methanol eluent by Step Gradient Polarity (SGP)

No	Ethyl Acetate	Methanol	Volume Total (mL)	Mark Rf
1	10	0	100	0.8
2	7	3	200	Node a = 0.6Stain b = 0.8
3	5	5	100	Node a = 0.3Stain b = 0.7
4	3	7	100	0.55
5	0	10	100	0.72

Concentrated ethyl acetate extract of 2.564 grams was impregnated first using 5.028 grams of silica gel 60 with a ratio of 1:2. Silica gel was dissolved together with concentrated ethyl acetate extract using acetone in a pestle mortar and stirred until the acetone solvent evaporated to dryness. The results of the impregmentation are entered at the top of the column chromatography. The purpose of this impregnation process is so that the samples to be collected in the column can be dispersed homogeneously and it is hoped that the results of the separation will be good.

Chromatography was carried out until the last band or spot pattern produced a total of 101 vials. The vials with the same Rf values were then combined to obtain 8 large groups which can be seen in Table 3. The groups of vials were allowed to evaporate until the solvent dried at room temperature.

Table 3. The groups obtained from column chromatography

Group	Vials	Rf	Color	Form
1	1-3	0	Clear	Finished Evaporate
2	4-16	0.76	Green	Powder
3	17-20	0.66	Green faded	Wad
4	21-29	0.8	Yellow-brown	Crystal amorphous
5	30-49	0.7	Green faded	A little oil
6	50-67	0.78	Yellow faded	Wad
7	68-93	0.68	Yellow faded	A little oil
8	94-101	0	Clear	Finished evaporate

Based on the phytochemical test, group 4 showed positive results for flavonoids. The amorphous crystals were weighed so that the weight of flavonoid crystals was obtained with a weight of 0.1047 grams. These flavonoids were then tested for purity using TLC with the eluent ratio of ethyl acetate to methanol (7:3) and (5:5). The chromatogram obtained is in the form of a single spot where the Rf values are 0.8 and 0.66. The purity results from this TLC have a single stain indicating that the flavonoid crystals are pure.

The first adding of the shear reagent was with NaOH, the shift that occurred can be seen in Figure 1. When the sample was added with NaOH it was seen that there was a bathochromic shift of 56 nm in band I. Initially, the flavonoid samples with methanol solvent had absorption at a wavelength of 326 nm with an absorbance of 0.9738 Å. The addition of NaOH gives a bathochromic shift to 382 nm with an absorbance of 0.9620 Å. The existence of this bathochromic shift indicates the presence of a hydroxy group at position C number 4' in ring B of the flavonoid. After 5 minutes, there was a slight shift in the wavelength in the I band.

The next shear reagent is $AlCl_3$. Based on the UV-Vis spectrophotometer graph shown in Figure 2, the flavonoid sample with methanol solvent has an absorption of 326 nm at an absorbance of 0.9738 Å. After the addition of $AlCl_3$, there was only a shift in Absorbance to 0.9508 Å, but there was no change in the wavelength of the I band. This indicates that it is possible to have an OH group at C number 5 with a prenyl group at C number 6 [16-17].

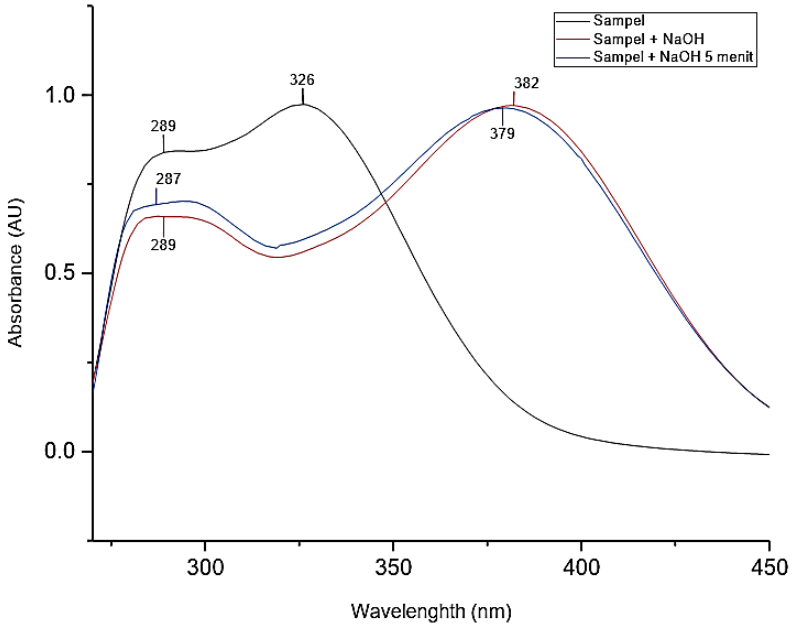

Figure 2. UV-Vis spectrum of flavonoids isolated with NaOH shear reagent

The third sliding reagent uses NaOAc reagent, which can be seen in Figure 3. There was a hypsochromic shift in band II of 5 nm from a sample wavelength of 291 with an absorbance of 0.8484 Å, to 286 nm with an absorbance of 0.8607 Å. This allows the presence of the OH group at C number 7. When boric acid is added, a band I causes a hypsochromic shift of 3 nm from 326 nm with an absorbance of 0.9738 Å to 323 nm with an absorbance of 0.9140 Å [16],[18].

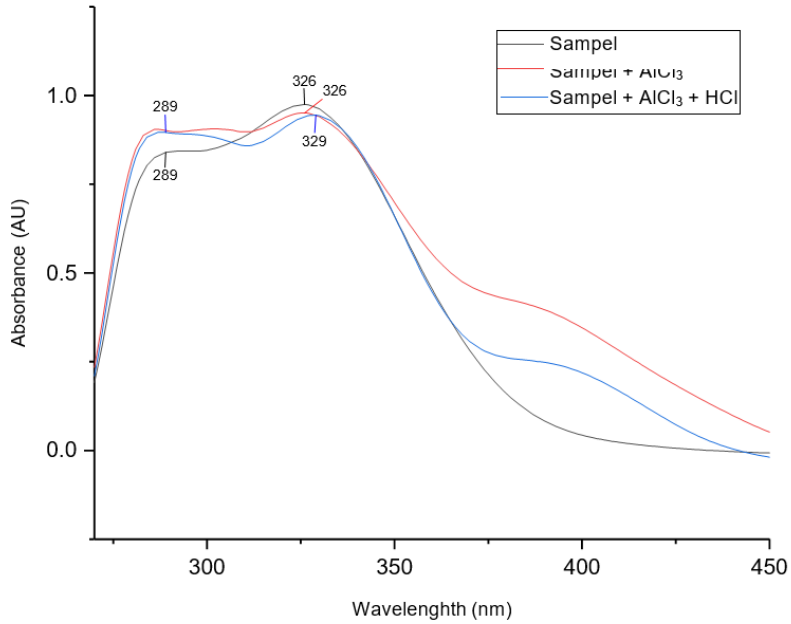

Figure 3. UV-Vis spectrum of flavonoids isolated with $AlCl_3$ /HCl shear reagent

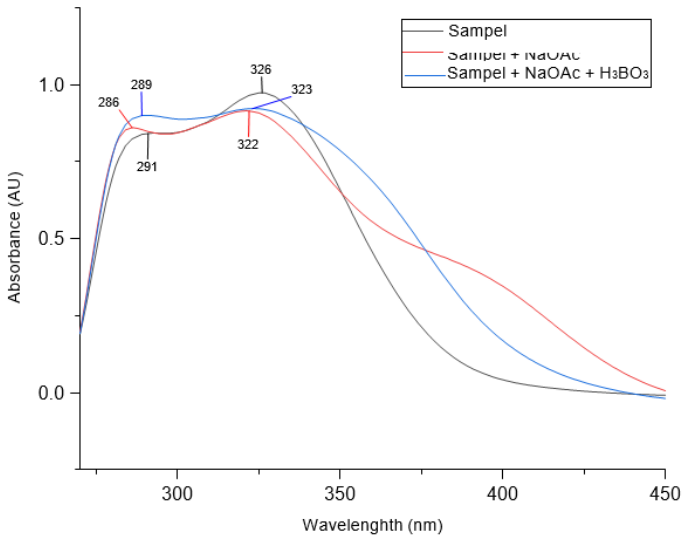

Figure 4. UV-Vis spectrum of flavonoids isolated with NaOAc/H_3BO_3 shear reagent

Characterization using FT-IR serves to determine the functional groups contained in a sample [19]. Based on the characterization of the isolated flavonoid crystal samples, the FT-IR spectrum was obtained which gave a specific absorption at 3361 cm^{-1} as the absorption of the -OH group. This confirms that the isolated flavonoids have a hydroxyl (OH) functional group. Vibration at wave number 2933 cm^{-1} indicates the presence of aliphatic -CH. Other characteristics that support the presence of aromatic rings are shown by absorption in the wave number region 1511 cm^{-1} and 1601 cm^{-1} which is the uptake of the aromatic C=C ring. Vibration at 1651 cm^{-1} and 1700 cm^{-1} with a sharp absorption band indicates the presence of a C=O group. Vibration at wave number 1362 cm^{-1} indicates the presence of aromatic -CH groups as well as at absorption 1450 cm^{-1}. Furthermore, there are COC (ether) strain vibrations at absorptions of 1028 cm^{-1}, 1165 cm^{-1}, 1227 cm^{-1}, and 1265 cm^{-1} [16], [20-21].

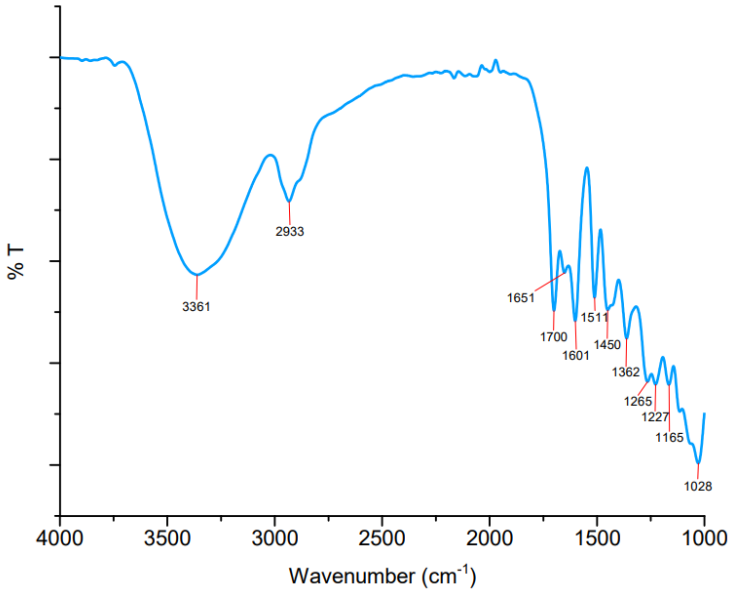

Figure 5. FT-IR spectrum of flavonoid isolation results

Table 4. Results measurement of crystal flavonoids results in isolation in FT-IR

Number Wave (cm^{-1}) Sample	Group Function	Wave Number(cm^{-1}) According to Theory
3361	OH	3000-3750
2933	CH Aliphatic	2700-3000
1651, 1700	C=O	1650-1900
1511, 1601	C=C Aromatic	1500-1676
1362, 1450	CH Aromatic	1300-1475
1028, 1165, 1227, 1265	COC	1000-1300

Based on other research related to the functional groups of flavonoids from the FTIR test, it was found that the functional groups were OH, CH aliphatic, C=O, C=C aromatic, CO, and aromatic CH. The presence of these functional groups, especially the carbonyl group (C=O) is a general characteristic of the flavonoid group compounds and states that the isolate is positive for pure flavonoid compounds [22-23]. Based on data from color reagents, KKt-2A, UV-Vis, and FT-IR, the flavonoids isolated from Dutch teak leaves are thought to contain 5,7,4'-trihydroxyflavone-6-prenil.

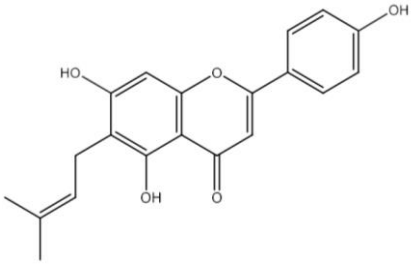

Figure 6. Alleged flavonoid compounds isolated results

3.2. Anti-Cholesterol Test

Testing the sample as a cholesterol-lowering was carried out by comparing the absorbance of the Dutch teak leaf extract resulting from the reaction between free cholesterol plus anhydrous acetic acid and concentrated sulfuric acid (Libermann-Burchard method), compared to the resulting solution from the reaction of free cholesterol plus anhydrous acetic acid and concentrated sulfuric acid [24][25]. The cholesterol concentration used in this study was 140 ppm.

Dutch teak leaf extract was made with 3 concentrations, namely 500 ppm, 600 ppm, and 700 ppm. Each of the sample concentrations was added to a standard cholesterol solution of 140 ppm and reacted with anhydrous acetic acid and concentrated sulfuric acid. The purpose of adding anhydrous acetic acid is to extract cholesterol and remove the water content so that it cannot react with cholesterol, while the addition of concentrated sulfuric acid produces a green color [26].

Next, the solution was left in a dark place to protect it from light for 15 minutes. This is done because the cholesterol solution is photodegradable, unstable to light, and will turn into cholestenone. At this stand, the standard cholesterol solution will form a green-colored complex which is then read with a UV-Vis spectrophotometer. The results of measuring the absorbance value and cholesterol reduction can be seen in Table 5 below.

Table 5. Data on decreased cholesterol levels in dutch teak leaf extract

Sample	Concentration (ppm)	Absorbance	% Decline Cholesterol
Standard raw Cholesterol	140	0.7987	-
Extract Flavonoids I	500	0.5301	50.66%
Extract Flavonoids II	600	0.4960	61.02%
Extract Flavonoids III	700	0.4584	74.23%

The absorbance value shown by each concentration is different. The higher the sample concentration, the lower the absorbance value. When added with the Lieberman-Burchard reagent, the color produced from each test solution fades due to its low absorbance. The high concentration of the sample resulted in a good decrease in cholesterol levels so that the absorbance value became smaller and the percentage of anti-cholesterol activity was greater, reaching 74.23 % of cholesterol into cholestadients and H_2SO_4 into HSO_4, H_3O, and SO_3, each of which will bind to cholestadients.

The result will form a new compound in the form of cholestadient sulfonic acid which will bind to flavonoids so that it can play a role in lowering blood cholesterol levels by replacing the hydroxyl group in cholestadien sulfonic acid with substituent groups from flavonoids. The more flavonoids bind to free cholesterol so that the free cholesterol that remains is more and the color that appears will fade [27-28].

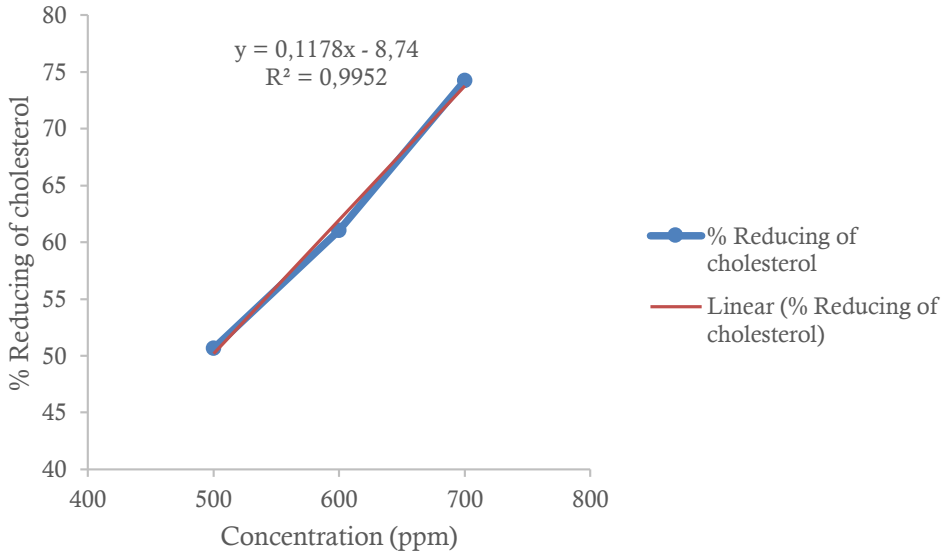

Figure 7. Activity curve of dutch teak leaves on decreased cholesterol levels

The value of EC_{50} is a value to describe the concentration of Dutch teak leaf extract which can reduce total cholesterol levels by 50%. EC $_{50}$ is the concentration needed to reduce cholesterol levels by 50%. Therefore, to calculate The value of EC_{50}, the Y value is replaced with 50 and X is The value of EC_{50}. Based on the curve in Figure 10, the calculation of The value of EC_{50} using the linear line equation y = 0.1178x - 8.74 obtained The value of EC_{50} of 498 ppm which states that at this concentration Dutch teak leaf extract can reduce 50% of initial cholesterol [29][30].

4. Conclusion

The results of the characterization of the flavonoid compounds obtained from this study indicate that the isolated flavonoids belong to the class of flavone compounds that have -OH groups at C-(5,7,4') and prenyl groups at C-6. The results of the FTIR test indicated the presence of functional groups in the form of OH, CH, C=O, C=C, COC. The high concentration of the sample resulted in a decrease in cholesterol levels properly, so that the absorbance value became smaller and the percentage of anti-cholesterol activity was greater. Therefore, it can be concluded that the Dutch teak leaf extract has the potential as an anti-cholesterol agent.

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