katimaha

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Antioxidant activity leaves katimaha (*Kleinhovia hospita* L.)

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Abstract

The free radicals that people face in their daily activities require humans to get additional antioxidants from the outside. To overcome this problem, it is necessary to look for many sources of natural antioxidants considering the need for antioxidants that are increasingly needed Katimaha (K. hospita L.) is believed to have many benefits in the field of medicine. Therefore, katimaha has the potential to be used as a source of natural antioxidants. The research methods carried out were refining katimaha leaf simplicia, extraction, fractionation, antioxidant activity test with TLC plate, purification, determination of compound class, and antioxidant activity test using the DPPH method. The results showed that the active fraction of Katimaha leaf was the n-hexane fraction and the ethyl acetate fraction. Column chromatography on the active fraction found six pure eluates that had antioxidant activity, namely N1, N3, N5, E1, E2, and E4. Groups of compounds N1, N3, N5, E1 are terpenoids, group E2 eluates are tannins, while E3 eluates are flavonoid compounds. The pure eluate antioxidant activity test using the DPPH method obtained IC50 values of N1 are 40,142 ppm, N3 80,057 ppm, N5 271,643 ppm and E1 89,16 ppm, E2 124.91 ppm and E4 52.51 ppm.

Keywords : Katimaha; Antioxidant; DPPH

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1. Introduction

Wild plants have the potential as alternative herbal medicines that are often used by local people as traditional medicines that can help the healing process of diseases such as degenerative diseases. Drugs derived from herbal medicine have small and economical side effects, so people still use wild plants that have secondary metabolic activity that can inhibit several degenerative diseases. *K. hospita* L. is a wild plant that is often used as medicine and food for local people. According to [1] that the *K. hospita* L. plant is one of the tropical plant species and is widely distributed in the Indonesian archipelago, which is believed to be able to produce secondary metabolic compounds that have traditionally attractive and potent bioactivity, such as the use of traditional medicine by the people of South Sulawesi as a cure for hypertension, liver, and diabetes. Degenerative diseases such as cancer, aging caused too often exposed to free radicals.

Antioxidants are substances or secondary metabolic compounds of plants that can capture free radicals. Antioxidants play a very important role for humans as disease prevention, because antioxidants can inhibit cell damage due to free radical oxidation processes. According to [2] states that antioxidants are substances or compounds that have the ability to inhibit, delay or prevent the oxidation process of other easily oxidized materials. Antioxidant activity is based on the scavenging power of free radical compounds using the DPPH method.

Oxidative stress plays an important role in the pathophysiology of the aging process and various degenerative diseases, such as cancer, diabetes, and its complications, as well as atherosclerosis that underlies heart disease, blood vessels and stroke. Antioxidants are needed by the body to overcome and prevent oxidative stress. The use of natural ingredients native to Indonesia contains many antioxidants with various active ingredients.

The group of flavonoid compounds has the ability as an antioxidant, where flavonoids have the ability to reduce free radicals. Secondary metabolic compounds of plants that have antibacterial activity can also generally function as an-

tioxidants, namely groups of phenolic compounds, flavonoids, terpenoids, saponins, and alkaloids. Lack of intake of antioxidants from outside the body can trigger tissue damage due to excessive production of free radicals from the metabolism of fats and proteins in the body, an imbalance between free radicals and antioxidants will cause oxidative stress.

By looking at so many potential plants that have various kinds of secondary metabolic compounds, one of which is wild plants which are rarely used because of the lack of knowledge and not knowing the ingredients in these plants. One of the wild plants that are very abundant in my area, especially in wood, is the Katimaha plant (*K. hospita* L.) which is only used by the local community for food for cattle or goats. Therefore, the researcher wants to knowregarding the antioxidant and antibacterial activity of katimaha leaves (*K. hospita* L.) which later is expected to become additional knowledge for the community and care more about the environment and plants. That even wild plants have many benefits and contain chemical compounds that can be used as traditional medicines, not just food for livestock.

2. Materials and Methods

The research method used in researching the antioxidant activity of katimaha leaves uses experimental exploration methods, where the search or discovery of antioxidant compounds of katimaha plants must be carried out in stages, namely extraction, fractionation, antioxidant activity test, compound purification, compound classification, antioxidant activity test of pure isolates by the method DPPH as well as testing for each stage to reveal the causal relationship to the research variables. According to [3], the experimental research method is a research method to reveal the causal relationship of two or more variables through careful experimental stages. Experimental exploration method aims to clarify and sharpen problems and hypotheses.

The tools used in this study were maceration tools, stationery, blenders, vials, funnels, separating funnels, erlenmeyer, beakers, measuring cups, scissors, hot plates, watch glasses, filter paper, paper labels, micro pipettes or capillary pipettes, dropper pipette, TLC plate, rotary evaporator, UV-Visible spectrometer, test tube and digital scale. The materials used in this study included distilled water, ascorbic acid, sulfuric acid and katimaha (*K. hospita* L.) leaves, Diphenylpicryl Hydrazil Hydrate (DPPH), ethyl acetate, methanol, n-hexane, silica gel TLC plate.

2.1 Making Simplicia and Extracting Katimaha Leaves

Simplicia is made from katimaha leaves which is done by separating the leaves from the stems. Then the katimaha leaves that have been separated from the stems are dried by drying in direct sunlight covered with a thin black cloth to dry. Furthermore, the dried leaves were weighed as much as 500 grams which were then ground using a blender to become a simplicia powder of Katimaha leaves. The extraction process for simplicia katimaha leaves was carried out by inserting 500 grams of simplicia into a glass extractor and extracted by maceration method using 1.5 liters of methanol solvent for 2x24 hours for 5 times and then filtered using filter paper so that the filtrate was separated. Furthermore, the evaporation process is carried out on the katimaha leaf filtrate using a rotary evaporator. The evaporation process is carried out with the aim of separating the active compound from the methanol solvent so that a thick extract will be obtained from katimaha leaves. The results of the maceration extraction process can be calculated the percentage yield with the following formula [4].

$$\%\ \text{of rendemen} = \frac{\text{Thick extract weight}}{\text{Dry Simplicia initial weight}} \times 100\%$$

2.2 Fractionation

The process of fractionation of the thick methanolic extract of Katimaha leaves was carried out by partitioning with the liquid-liquid fractionation method. Fractionation was carried out successively using n-hexane, ethyl acetate, and aquadest solvents which had different polarity levels. The solution in the separating funnel was then shaken until homogeneous, after that it was allowed to stand until two separate layers were formed between the two solvents that were visible directly on the separating funnel. After forming two layers, the bottom layer is an extract with methanol and aquadest solvent, while the top layer is an extract with n-hexane solvent, the aquadest fraction and n-hexane fraction are flowed out from the separating funnel to different places. Then the rest of the distilled water fraction was put back into the separating funnel and then fractionated again using ethyl solvent which was also added to the separating funnel. The solution in the separating funnel is then shaken until homogeneous and allowed to stand until two separate layers are formed, where the bottom layer is an extract with methanol and aquadest solvents while the top layer is an extract with ethyl solvent, the aquadest fraction and ethyl fraction are flowed out from the separating funnel to different places. The three extracts resulting from this fractionation were then evaporated using a rotary evaporator to obtain a thick extract from the n-hexane fraction, ethyl fraction, and aquadest methanol fraction. These extracts were then stored for further testing [5].

The solution in the separating funnel is then shaken until homogeneous and allowed to stand until two separate layers are formed, where the bottom layer is an extract with methanol and aquadest solvents while the top layer is an extract with ethyl solvent, the aquadest fraction and ethyl fraction are flowed out from the separating funnel to different places. The three extracts resulting from this fractionation

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The aquadest fraction and ethyl fraction are drained out of the separatory funnel to different places. The three extracts resulting from this fractionation were then evaporated using a rotary evaporator to obtain a thick extract from the n-hexane fraction, ethyl fraction, and aquadest methanol fraction. These extracts were then stored for further testing [5]. The aquadest fraction and ethyl fraction are drained out of the separatory funnel to different places. The three extracts resulting from this fractionation were then evaporated using a rotary evaporator to obtain a thick extract from the n-hexane fraction, ethyl fraction, and aquadest methanol fraction. These extracts were then stored for further testing [5].

2.3 Activity Analysis of Fractionated Antioxidant Compounds by Thin Layer Chromatography (TLC) Method

This methodusing a stationary phase in the form of a silica gel plate G60F254 which was cut 1 cm × 6 cm and given a lower limit of 0.5 cm and an upper limit of 0.5 cm. The thick extract of each fraction was then spotted using a capillary pipette on the plate and then eluted in the chamber with the appropriate solvent ratio (mobile phase) until the upper limit of the plate had been determined so as to obtain good separation results and color stains. The location of the color spots can be seen with the help of UV light and the Rf values recorded [6].

The eluted TLC plate was then sprayed with DPPH with a concentration of 0.008% then aerated and observed for a color change in the spots to yellow or orange or white on a purple background which means that there is antioxidant activity resulting in the reduction of free radicals by antioxidants [7].

2.4 Purification of Antioxidant Compounds by Gravity Column Chromatography Method

The n-hexane and ethyl acetate fraction extracts were purified by gravity column chromatography, respectively. The stationary phase in the form of silica gel 60 F245 was dissolved with n-hexane solvent and then put into a column that had been plugged with cotton first. The n-hexane fraction extract and ethyl acetate fraction were then put into the column and eluted with n-hexane, ethyl acetate, and methanol as solvents using a stepwise comparison method. The results from the column drops are then collected into vials.

2.5 Identification of Antioxidant Compounds Using the TLC Plate Method

The isolates from the ethyl acetate fraction and the n-hexane fraction resulting from column chromatography purification were tested for purity by thin layer chromatography using several kinds of eluents with good ratios. If the isolate still shows a single stain pattern, it can be said that the isolate is relatively pure [8]. This method uses a stationary phase in the form of a silica gel plate G60F254 which is cut 1 cm × 6 cm and is given a lower limit of 0.5 cm and an upper limit of 0.5 cm. The thick extract of each fraction was then spotted using a capillary pipette on the plate and then eluted in the chamber with the appropriate solvent ratio (mobile phase) until the upper limit of the plate had been determined so as to obtain good separation results and color stains.

The eluted TLC plate was then sprayed with DPPH with a concentration of 0.008% then aerated and observed for a color change in the spots to yellow or orange or white on a purple background which means that there is antioxidant activity resulting in the reduction of free radicals by antioxidants [7]. The pure isolate from the n-hexane and ethyl acetate fractions and has been tested for TLC with a good eluent ratio after drying and then sprayed with sulfuric acid (H_2SO_4) which is then heated on a hot plate until color spots appear on the TLC plate. These spots or stains are calculated how much their Rf value will determine one of the factors in the identification of antioxidant compounds [9].

2.5 DPPH Solution Preparation

DPPH in the form of solid powder as much as 10 mg was dissolved in 25 mL of absolute methanol solvent which then obtained 1 mM DPPH reagent solution. The pure isolates were weighed in an amount of 0.002 g each and then dissolved with absolute methanol solvent up to a volume of 4 mL in order to obtain a mother liquor of 2000 ppm which would be diluted to obtain 5 variations in the concentration of the test solution sample in each fraction, namely 62.5 ppm, 125 ppm, 250 ppm, 500 ppm, 1000 ppm. Samples of the test solution totaling 5 vials were then pipetted as much as 1 mL each and reacted with 1 mL of DPPH solution in a test tube then covered with aluminum foil then vortexed and incubated at room temperature in a dark place. While the comparison solution was made by weighing 0 ascorbic acid. 002 g dissolved in absolute methanol to reach a volume of 2 mL in a volumetric flask. The ascorbic acid solution was also diluted to obtain 5 variations in the concentration of the comparison solution sample, namely 62.5 ppm, 125 ppm,

250 ppm, 500 ppm, 1000 ppm. The comparison solution was then pipetted as much as 1 mL each and reacted with 1 mL of DPPH solution in a test tube then closed and then vortexed and incubated at room temperature in a dark place. Blanks were made by pipetting 4 mL of 1 mM DPPH solution, then covered and coated with aluminum foil and vortexed and then incubated at room temperature of 37oC in a dark room. After completion of incubation, all samples of the test solution and the reference solution and blank were measured for absorbance values using a UV-Vis spectrophotometer at a wavelength of 517 nm [10].

According to [11] the antioxidant activity of the sample by the amount of free radical uptake, in this case DPPH, can be determined by calculating the percentage of inhibition. The percentage of inhibition or antioxidant capacity in inhibiting free radicals is determined by the following equation:

$$\% \text{ inhibition} = \frac{\text{abs control} - \text{abs sampel}}{\text{abs control}} \text{X } 100\%$$

The results of the percent inhibition were made a curve between the percent of free radical scavengers and the concentration of the test solution. The IC50 value is determined from the linear regression equation on the created curve. The linear regression equation formula is as follows:

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$$Y = aX + b$$

Y = Percent Attenuation
a = Gradient
X = Concentration (μg/ml)
B = Constanta

The antioxidant activity of katimaha leaves can be determined by the IC50 value obtained by calculating the X value from the linear regression equation calculated by the following formula [12]:

IC50 = Antilog X

3. Results and Discussion

3.1 Extraction leaves katimaha (*K. hospita* L.)

Based on the simplicia extraction process on katimaha leaves (*K. hospita* L.) by maceration method using methanol as solvent, the results are as shown in Table 3.1. Table 3.1. showed that the methanol solvent used in the extraction was able to extract compounds from katimaha leaves by producing a thick extract weight of 83.7 g from a simplicia weight of 500 g and yielding 16.67% yield. Extraction results are used to determine the amount of extract obtained to perform the method of extracting compounds in simplicia. While the yield is related to the active compounds contained in the extract so that if the percentage value of the yield is high, the number of active compounds contained in the extract increases. Sample extraction in this study was carried out using the maceration method which is a process of immersing the sample using an organic solvent at room temperature.

Table 3.1. Weight of thick extract and percentage of yield of katimaha leaf methanol extract (*K. hospita*L.)

No	Weight of simplicia (grams)	Thick extract weight (grams)	Extract yield weight (%)
1	500 grams	83.72 g	16.67%

3.2 Fractionation of liquid – liquid (FCC) leaves katimaha (*K. hospita* L.)

The results of the fractionation using the liquid-liquid fractionation (FCC) method that has been carried out in this study using n-hexane, ethyl acetate and water methanol solvents, the following results are obtained:

Table 3.2. Fraction weight and percentage yield of katimaha leaf fraction (*K. hospita* L.)

No	Solvent Type	Fraction Weight (g)	Fraction (%)	Yield
1	N-hexane	20.52 g	27.98 %	
2	Ethyl Acetate	17.9 g	24.41%	
3	Methanol Water	34.9 g	47.54%	

Based on Table 3.2. the fractionation process using the liquid-liquid fractionation method obtained the highest weight fractions were methanol water fraction 34.9 g, n-hexane fraction 20.52 g, and ethyl acetate fraction 17.9 g. while the yield value obtained was for methanol water of 47.54%, n-hexane of 27.98%, and ethyl acetate of 24.41%. The yield value obtained from each fraction is different because it depends on the solvent ability of each fraction. According to [13], the yield percentage produced is different because each solvent has its own ability to attract the compounds contained in the leaf extract.

3.3 Antioxidant activity leaves test faction katimaha (Keinhovia hospita L.)

The viscous fraction from liquid-liquid fractionation was tested for antioxidant activity using thin layer chromatography (TLC) method. The results of the antioxidant

activity test of the fractions can be seen in table 3.3.

Table 3.3. Rf value and antioxidant activity of katimaha leaf fraction (*K. hospita* L.)

No	Fraction	Rf value	Antioxidant Activity	Infor-mation
1	N-hexane	0.9	+++	Strong
		0.7	++	Currently
2	Ethyl Acetate	0.8	+++	strong
3	Methanol Water	0.5	+	Weak

Description of [14]:
+++ : Strong antioxidant activity (Intensity of deep yellow color)
++ : Medium antioxidant activity (yellow color intensity)
+ : Weak antioxidant activity (Pale yellow color intensity)
- : No yellow color (No yellow color)

Antioxidant compounds in the n-hexane, ethyl acetate, and methanol fractions in katimahi leaves (*K. hospita* L.) have different retention factor (Rf) values. The n-hexane fraction in katimaha (*K. hospita* L.) leaves had Rf values of 0.9 and 0.7, and the ethyl acetate fraction in katimaha (*K. hospita* L.) leaves had Rf values of 0.8, while the water-methanol fraction in leaves katimaha (*K. hospita* L.) has an Rf value of 0.7. The retention factor (Rf) value is a measure that is obtained based on the position of the stain of each solute on a thin layer chromatography (TLC) plate [15].

The Rf value can be useful for identifying compounds, if the Rf value of an antioxidant compound or other compounds is similar, it can be indicated that both have the same compound. The lower the Rf value of a compound, the more polar the compound. According to [16], the polarity of a compound can be known from the low Rf value obtained. According to [17], a compound that has low polarity has a large Rf value, if it is juxtaposed with high polarity, it produces a low Rf value. So this can be due to the TLC plate (stationary phase) being polar, so the more polar compounds will be retained in the stationary phase and the Rf value is low. When juxtaposed with high polarity, it produces a low Rf value. So this can be due to the TLC plate (stationary phase) being polar, so the more polar compounds will be retained in the stationary phase and the Rf value is low. When juxtaposed with high polarity, it produces a low Rf value. So this can be due to the TLC plate (stationary phase) being polar, so the more polar compounds will be retained in the stationary phase and the Rf value is low.

The n-hexane fraction can attract secondary metabolites of katimaha leaves (*K. hospita* L.) which are non-polar. Non-polar secondary metabolites that have antioxidant activity are derived from the terpenoid group. According to [18], terpenoids are classified as secondary metabolite compounds that are non-polar which will dissolve in non-polar solvents. Research conducted by [19], showed that steroids and terpenoids in several studies could also be attracted to semi-polar solvents.

The ethyl acetate fraction can attract secondary metabolites in katimaha leaves (*K. hospita* L.) which can be dissolved in semi-polar solvents. Secondary metabolites that have antioxidant activity are soluble in semi-polar solvents derived from flavonoids and tannins. The class of antioxidant compounds that are soluble in semi-polar ethyl acetate solvent comes from the flavonoid and tannin groups [19].

The methanol-water fraction can attract polar compounds that have a heavy molecular weight. Secondary metabolites that have polar antioxidant activity mostly come from the alkaloid group. Methanol solvents can dissolve polar and non-polar compounds, the group of compounds thought to be present in methanol solvents mostly comes from the alkaloid, phenolic, steroid and glycoside groups [15].

3.4 Purification and Isolation of Active Compounds with Vacuum Liquid Chromatography

Testing the antioxidant activity of the n-hexane subfraction of Katimaha leaves (*K. hospita* L.) using thin layer chromatography with a size of 6 cm x 1 cm which was eluted with an eluent ratio of n-hexane: ethyl acetate (9:1). The Rf values of the subfraction and the results of the antioxidant activity of the n-hexane subfraction are as follows:

Table 3.4. Rf value and antioxidant activity of the n-hexane subfraction of Katimaha (*K. hospita* L.) leaves with DPPH using TLC

Fraction	Subfraction	Rf value	Activity Antioxidant	Information
	N1	0.64	+++	Strong
	N2	0.62	+++	strong
N-hexane	N3	0.44	++	Currently
	N4	0.4	++	Currently
	N5	0.18	++	Currently

Description of [14]:
+++ : Strong antioxidant activity (Intensity of deep yellow color)
++ : Medium antioxidant activity (yellow color intensity)
+ : Weak antioxidant activity (Pale yellow color intensity)
- : No yellow color (No yellow color)

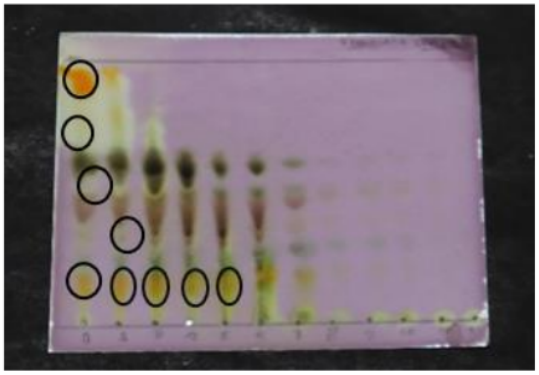

Figure. 3.1.TLC profile of n-hexane subfraction with 9:1 eluent sprayed with DPPH

Results table 3.4. showed that the N1 subfraction had strong antioxidant activity because it had very dense yellow spots and had an Rf N1 value of 0.64. The N2 subfraction has an Rf value of 0.62 with a dark yellow color intensity and it means that the N2 subfraction has strong antioxidant activity similar to that of the N1 subfraction. The N3 and N4 subfractions have Rf values that are not much different, namely 0.44 (N3) and 0.4 (N4), thus the N3 and N4 subfractions have moderate oxidant activity with a yellow color intensity. While the N5 subfraction has an Rf value of 0.18 which means it has moderate antioxidant activity with a yellow color intensity.

Table 3.4. Rf value and antioxidant activity of the ethyl acetate subfraction of Katimaha (*K. hospita* L.) leaves with DPPH using TLC

Fraction	Subfraction	Rf value	Activity Antioxidant	Information
Ethyl Acetate	E1	0.72	+++	Strong
	E2	0.54	+++	strong
	E3	0.54	+++	Strong
	E4	0.22	++	Currently
	E5	0.20	++	Currently

Description of [14]:
+++ : Strong antioxidant activity (Intensity of deep yellow color)
++ : Medium antioxidant activity (yellow color intensity)
+ : Weak antioxidant activity (Pale yellow color intensity)
- : No yellow color (No yellow color)

The n-hexane subfraction in katimaha (*K. hospita* L.) leaves N1 and N2 had relatively the same Rf values, thus it was assumed that these compounds belonged to the same group of compounds with the same characteristics. The n-hexane subfractions N3 and N4 in katimaha leaves (*K. hospita* L.) also have the same Rf value, so it can be assumed that they belong to the same compound group. Compounds with the same Rf value can be combined for compound isolation. According to [16], if the compound has a similar Rf value, it means that the compound has the same compound characteristics and can be combined for the compound isolation process.

Table 3.4. shows the results of the antioxidant activity of the ethyl acetate subfraction and its Rf value, the E1 subfraction has strong antioxidant activity because it has a thick yellow spot and has an Rf value of 0.72. Subfractions E2 and E3 have strong antioxidant activity because they have dark yellow spots and an Rf value of 0.54. Subfractions E4 and E5 have moderate antioxidant activity with Rf values of 0.22 (E4) and 0.20 (E5).

Figure. 3.2.TLC profile of n-hexane subfraction with 9:1 eluent sprayed with DPPH

The final results of the isolation and purification of the ethyl acetate subfraction on katimaha leaves (*K. hospita* L.) produced 3 pure isolates, namely pure isolate E1, pure isolate E2, and pure isolate E4. The antioxidant activity test of four pure isolates were spotted on TLC plates and sprayed with 0.008% DPPH. Based on the results of the activity with TLC, pure isolate E1, pure isolate E2, and pure isolate E4 showed strong antioxidant activity because they caused dark yellow and light yellow spots.

3.4 Identification of Active Isolated Antioxidant Compounds of Katimaha Leaves (*K. hospita* L.)

The results of the identification of active antioxidant compounds from katimaha leaves (*K. hospita* L.) were found in N1, N3, and N5 for the n-hexane fraction, while in the ethyl acetate fraction E1, E2, and E4. For the n-hexane fraction, the compounds contained in the N1 and N2 vials have the same compounds.

In the results table 3.5. It can be seen that the results of the isolation and purification of the n-hexane and ethyl acetate subfraction of Katimaha (*K. hospita* L.) leaf found six pure isolates with different Rf values. Isolates N1, N3, and N5 were active isolates that had been purified from the n-hexane subfraction of Katimaha leaves. Isolates N1, N3, and N5 were classified as terpenoid compounds because blue and purple spots were found after being sprayed with

5% H2SO4 with different values. N1 has an Rf value of 0.64, N3 has an Rf value of 0.18, and N5 has an Rf value of 0.44.

Table 3.5. Active isolate, Rf value, color and class of antioxidant compounds in Katimaha leaves

Isolate	Rf value	Color	Compound Group
N1	0.64	Blue	Terpenoids
N3	0.18	Purple	Terpenoids
N5	0.44	Blue	Terpenoids
E1	0.72	Blue	Terpenoids
E2	0.20	Chocolate	Tannins
E4	0.22	Brick Red	Flavonoids

Isolates E1, E2, and E4 were isolates that had been purified from the ethyl acetate subfraction of Katimaha (*K. hospita* L.) leaves. Isolate E1 was suspected to be a terpenoid compound because blue spots were found after being sprayed with 5% H2SO4 with an Rf value of 0.72. Isolate E2 was suspected to be a tannin compound because brown spots were found after being sprayed with 5% H2SO4 with an Rf value of 0.20. Isolate E5 was suspected to be a flavonoid compound because brick red spots were found after being sprayed with 5% H2SO4 with an Rf value of 0.22.

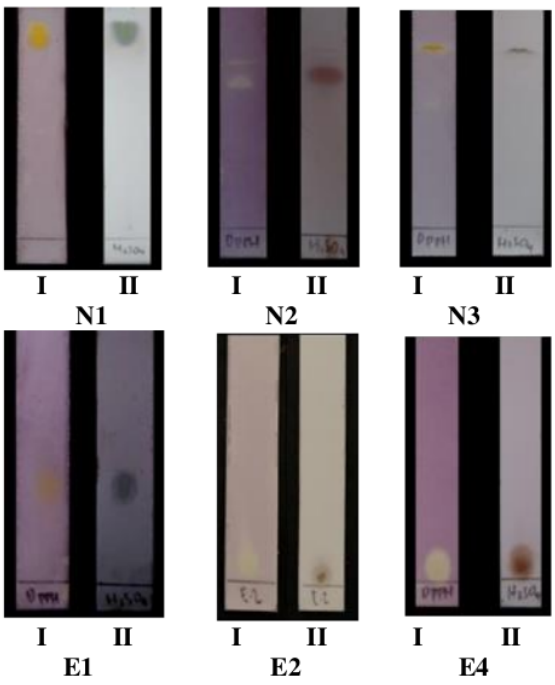

Figure 3.3. Chromatogram Profile of Pure Isolates Antioxidant Compounds Katimaha Leaves
Notes: I. Sprayed with 0.008% DPPH II. Sprayed 0.5% H2SO4

Based on Figure 3.3. above part I shows that the pure isolate has antioxidant activity after being sprayed with 0.008% DPPH solution. Figure II shows pure isolates whose compounds have been identified after being sprayed with 5% H2SO4. Figure I and Figure II in Figure 3.3. showed that the isolate was pure, because there was one spot after testing its purity by thin layer chromatography (TLC) as shown in the *Figure*. According to [20], isolates that have been purified are characterized by the formation of single spots or spots when tested for purity by thin layer chromatography using the appropriate eluent.

The group of terpenoid compounds in Katimaha leaves (*K. hospita* L.) is thought to be derived from the triterpenoid and steroid groups. Triterpenoids and steroids are a group of terpenoids that have antioxidant activity. The mechanism of terpenoids as antioxidants can prevent fat peroxidation, can bind Cu^{2+} and Fe^{2+} metals and bind superoxide free radicals in the body. According to [21], terpenoids such as triterpenoids and steroids can bind free radical compounds, as Cu^{2+} metal chelators in the body and inhibit lipid peroxidation.

Terpenoid antioxidant compounds can prevent fat peroxidation. Fat peroxidation is the result of lipid damage due to oxidation reactions that form lipid peroxides. Lipid peroxide in the body will cause an increase in free radical compounds in the body that have an impact on cell death, DNA damage, so that it can trigger cancer. According to [22], oils containing lipid peroxides if consumed will cause an increase in free radicals in the body, triggering skin cancer, lung cancer and esophageal cancer, as well as DNA damage and even cell death. Therefore, antioxidant compounds are needed to prevent lipid peroxidation.

The antioxidant activity of terpenoid compounds is able to bind Cu^{2+} and Fe^{2+} metals in the body. The transition metals Cu^{2+} and Fe^{2+} are reactive and can increase the formation of Reactive Oxygen Species (ROS) in the body. Terpenoid antioxidant compounds are able to become metal binding agents because they have phenolic groups and carboxyl groups that form stable complexes with metal ions Cu^{2+} and Fe^{2+} so that they can inhibit the formation of free radical compounds.

Group of compoundsTannins are compounds synthesized by plants which are usually combined with antioxidants. Tannins also have active compounds in addition to antioxidants that can be efficacious in antidiarrheal, antibacterial. Tannins have two types, the first, condensed tannins and hydrolyzed tannins, both types of tannins are found in plants. Condensed tannins have the most compounds in plants compared to hydrolyzed tannins [23].

The group of flavonoid compounds is characterized by a change in the spot to yellow-orange because it is reacted with sulfuric acid. Based on research according to [24], the presence of flavonoid compounds was indicated by a change in the color of the pure isolate to a yellow-orange color. This

happened due to the formation of flavilium salts with sulfuric acid which was marked by the formation of yellow-orange spots on the isolates. According to [25], there are two mechanisms by which flavonoid antioxidants scavenge free radicals, namely by transferring an electron to free radicals and forming complexes with metals.

The mechanism of the flavonoid compound group is able to increase the work of cellular antioxidants by protecting and increasing the work of lipophilic antioxidants. Lipophilic antioxidants are cellular antioxidants that are able to bind to ROS, causing damage to hydrophobic systems such as cell membranes. Damage to cell membranes can reduce the ability of cells which in time will reduce the permeability of cell membranes and lead to cell damage. Cell damage by free radicals causes degenerative diseases such as: cardiovascular disease, tumors and liver. According to According to [21], flavonoids can prevent tumor, cardiovascular and liver diseases because flavonoids are antioxidants that can increase the work of lipophilic antioxidants.

The group of flavonoid compounds is an antioxidant, the mechanism of flavonoid as an antioxidant can directly prevent the regeneration of ROS in the body and can indirectly improve the performance of cellular antioxidants in the body. Flavonoids can prevent the regeneration of ROS by releasing their hydrogen atoms directly to free radicals, besides that flavonoids prevent the formation of ROS by inhibiting the activity of xanthine oxidase enzymes and nicotinamide adenine dinucleotide phosphate enzymes which can increase free radicals in the body, and can bind metals.

3.5 Antioxidant Activity of Purified Compounds of Katimaha Leaves (*K. hospita* L.)

The table of antioxidant activity test results for pure isolates of katimaha leaves (*K. hospita* L.) can be seen in the following 3.6. Based on table 3.6. The antioxidant activity of Katimaha leaves was obtained using the DPPH method, which shows the value of Inhibition Concentration (IC50) N1 40,142 ppm, N2 80.057 ppm, N5 271 ppm, 643 ppm, E1 89.16 ppm, E2 124.91 ppm, and E5 52.51 ppm. Isolate N1 is an antioxidant that has very strong activity because its IC50 value is below 50 ppm. Isolates N3, E1, and E4 are antioxidants that have strong activity because their IC50 values are in the range of 50-100 ppm. Isolate N5 is a weak antioxidant because its IC50 is above 200 ppm. Meanwhile, isolate E2 is a moderate antioxidant because its IC50 value is above 100 ppm. According to Molyneux (2004), an antioxidant compound is said to be very strong if IC50 is less than 50 ppm, strong if IC50 is 50-100 ppm, moderate if IC50 is 100-150 ppm and weak if IC50 is 150-200 ppm.

Table 3.6. The results of the antioxidant activity test of the pure isolate of katimaha leaf (*K. hospita* L.) using the DPPH method

No	Isolate	Concentration (ppm)	Free radical inhibition (%)	IC50 (ppm)	Antioxidant activity
1	Ascorbic Acid	1000	69.51	16,115	Very strong
		500	68.73		
		250	65.63		
		125	63.68		
		62.5	60		
2	N1	1000	59.58	40,142	Very strong
		500	58.82		
		250	57.87		
		125	56.73		
		62.5	55.4		
3	N3	1000	63.35	80.057	Strong
		500	59.93		
		250	56.33		
		125	54.62		
		62.5	52.39		
4	N5	1000	62.23	271,643	Weak
		500	61.34		
		250	59.57		
		125	56.73		
		62.5	51.95		
5	E1	1000	61.1	89.16	Strong
		500	57.88		
		250	57.65		
		125	56.05		
		62.5	53.92		
6	E2	1000	60.98	124.91	Currently
		500	59.55		
		250	57.65		
		125	56.05		
		62.5	53.92		
7	E4	1000	58,47	52.51	Strong
		500	53.59		
		250	53.22		
		125	52.71		
		62.5	51.52		

The IC50 value is the amount of concentration required to inhibit free radicals by 50%. According to Molyneux (2004), the IC50 value of antioxidant compounds is the concentration of antioxidant compounds that are able to ward off free radicals, therefore it is concluded that the smaller the IC50 value of antioxidant compounds, the stronger the antioxidant activity, because with just a small concentration these compounds have been able to inhibit as much free radicals as possible 50%. To see the comparison of pure isolates and the positive control of ascorbic acid can be seen clearly through Figure 3.4. below this:

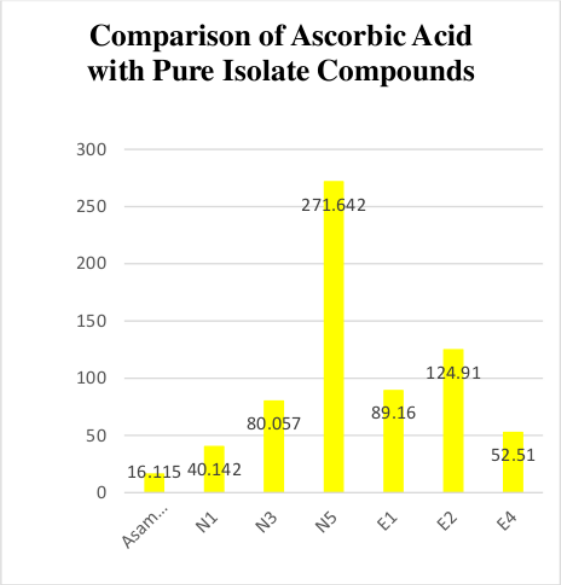

Figure 3.4. Comparison graph of IC50 value of ascorbic acid and pure compound of katimaha leaves. Description of antioxidant activity by [26]:

1. Very strong : IC50 value less than 50 ppm
2. Strong : IC50 value 50 – 100 ppm
3. Currently : IC50 value 100-200 ppm
4. Weak : IC50 value is greater than 200 pp

Based on table 3.6. and figure 3.4. The above shows that the IC50 value of pure isolates of antioxidant compounds is still below the IC50 of ascorbic acid which has antioxidant activity with an IC50 value of 16.115 ppm and is classified as a very strong antioxidant. Ascorbic acid or vitamin C is often used as a comparison in testing antioxidant activity because vitamin C includes natural antioxidants such as secondary metabolites that have antioxidant activity. According to [27], ascorbic acid has very strong antioxidant activity because it has many polyhydroxyl groups to increase antioxidant activity.

Based on table 3.6 ascorbic acid has a very strong antioxidant activity which is indicated by an IC50 value of less than 50 ppm, in several studies on antioxidant activity using the DPPH method, ascorbic acid shows a very strong activity.

Based on the results table 3.6. it can be seen that the greater the concentration value of pure isolates, the greater the percent inhibition or percent inhibition of antioxidant compounds in inhibiting DPPH free radical compounds. The difference in the percentage of inhibition means that the antioxidant levels are higher if the concentration of pure isolate compounds is higher. Testing the IC50 value of antioxidant compounds shows the in vitro mechanism of antioxidant compounds in reducing free radicals. According to [28], the highest antioxidant activity was indicated by the large percentage value of the reduction or inhibition of DPPH free radicals.

4. Conclusion

The active fraction as an antioxidant from the methanol extract of Katimaha leaves (*K. hospita* L.) was the n-hexane fraction and the ethyl acetate fraction, while the methanol fraction was inactive. Pure compounds that have been isolated from katimaha leaves are from the n-hexane fraction, namely compounds N1, N3, and N5 terpenoid groups, while from the ethyl acetate fraction, compounds E1 are terpenoid groups, E2 compounds are tannins and E4 compounds are flavonoids. Pure compound N1 has a very strong antioxidant activity with an IC50 value of 40,142. Pure compounds N3, E1, and E4 have strong antioxidant activity with IC50 values of 80.057 ppm, 89.16 ppm, and 52.52 ppm. Compound E2 has antioxidant activity that falls into the medium category with an IC50 value of 124.91 ppm.

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