WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Phytochemical Screening and Antioxidant Activity Test of Gambir Leaves Extract (*Uncaria Gambir* (Hunter) Roxb) with n-Hexane, Acetone and Ethanol Solvents

Zikra Azizah, Rosi Wulandari, Rina Desni Yetti, Ulfa Ismirza, Sestry Misfadhila

Departement of Pharmaceutical Chemistry, School of Pharmaceutical Science (STIFARM), Padang, West Sumatera, Indonesia, 25147

A B S T R A C T

A research on the phytochemical screening and testing of antioxidant activity of gambir leaves extract (Uncaria gambir (Hunter) Roxb) n-hexane, acetone and ethanol has been done. this study was to determine the content of secondary metabolites and antioxidant activity of extracts n-hexane, acetone and ethanol from gambir leaves (Uncaria gambir (Hunter) Roxb). Extraction of gambir leaves using the maceration method, then phytochemical screening and antioxidant testing were carried out using the DPPH (1,1-diphenyl-2-pikrilhidrazil) method. The results of phytochemical screening showed that the n-hexane extract contained a class of steroid and terpenoid compounds, in acetone extract contains phenol compounds, flavonoids, alkaloids, tannins and terpenoids. Where as the ethanol extract contains phenol compounds, flavonoids, alkaloids, tannins, terpenoid and saponins. Based on the calculation of IC50 gambir acetone extract has the highest antioxidant activity with IC50 of 56.6772 µg/mL, ethanol extract is a moderate antioxidant with IC50 of 135.2190 µg/mL and n-hexane extract is a weak antioxidant with an IC50 of 1948.6277 µg/mL

Keywords: Phytochenical Screening, Antioxidant, Gambir Leaves (*Uncaria gambir* (Hunter) Roxb)

1. Introduction

The use of antioxidant compounds is rapidly expanding in health and food science, particularly in the fields of drug and nutritional research in the foods we consume (Huang *et al*., 2005). Its use as a medicinal ingredient is growing along with increasing knowledge about the activity of free radicals in several degenerative diseases such as heart disease and cancer (Winarsi, 2007). Gambir plants belong to the Rubiaceae family. This plant has been used for generations for medicinal purposes (Agricultural Research and Development Agency, 2013). Indonesia uses gambir as a raw material for pharmaceuticals, but the development and research of gambir plants as herbal medicines towards standardized herbal medicines and phytopharmaceuticals remains limited (Hernani, 2011).

Gambir leaves are traditionally used as an ingredient in betel chewing, a remedy for wounds, fever, headaches, stomach aches, and fungal and bacterial infections. Water extracts of gambir leaves and young twigs are used for diarrhea and dysentery, and as a mouthwash to treat sore throats (Indonesian Food and Drug Administration, 2006; Taniguchi *et al*., 2007). Catechins are the main component of the gambir plant. Besides catechins, there are several other components such as alkaloids, catechu tannic acid, quercetin, red catechu, gambir fluorescence, fats, and waxes (Heiztman *et al*., 2005; Damanik *et al.*, 2014). Dried leaves extracts of gambir have been identified as containing catechins, the main bioactive compounds for antioxidants (Apea-Bah *et al*., 2009; Anggraini *et al*., 2011). The use of gambir is supported by research showing that it contains the active ingredient catechin, which has nonspecific immunostimulant activity. Tests of the effect of catechin on gastric ulcers in female rats showed a reduction in ulcers, normalization of gastric acidity, and inhibition of Helicobacter pylori in vitro (Indonesian Food and Drug Administration, 2006).

Ethanol extract of gambir (*Uncaria gambir*) leaves was methylated using dimethyl sulfate (DMS) and purified by column chromatography to yield two isolates with different colors and solubilities. The two isolates were characterized using UV-Visible and FT-IR spectrophotometers. The results indicated the presence of phenolic compounds, indicated by the OH and CH aromatic stretches. Isolate 1 exhibited antioxidant activity with an IC50 value at a concentration of 13.41 µg/mL, while isolate 2 exhibited antioxidant activity with an IC50 value at a concentration of 121.81 µg/mL (Kresnawaty *et al*., 2009).

The methanol extract of gambir (*Uncaria gambir*) leaves contains flavonoids, alkaloids, and phenolics. The total phenolic and total flavonoid contents were 18.37% and 5.82%, respectively. The extract showed significant antioxidant activity with an IC50 value at a concentration of 35.95 µg/mL, so it can be used as a potential source of natural antioxidants (Amir *et al*., 2012).

Antioxidant compounds from plants can be obtained by extraction using solvents. Solvents are selected based on different levels of polarity with the aim of obtaining the best solvent, namely a solvent that can extract large amounts and can extract the group of antioxidant compounds that have the highest

activity (Mangela et al., 2016). Therefore, as an effort to optimize the use of natural materials, researchers are interested in conducting phytochemical screening and testing the antioxidant activity of gambir leaves extract (*Uncaria gambir* (Hunter) Roxb) with n-hexane, acetone and ethanol solvents using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method using a UV-Visible spectrophotometer.

2. Research Method

Tools and Materials

Tools

Double beam UV-Vis spectrophotometer (Shimadzu UV-1800), analytical balance (Precisa), blender (Miyako), rotary evaporator (Ika), desiccator, oven (Memmert), moisture balance (Ohaus), carbolite furnace (CWF 1200), parchment paper, silica gel 60 F254, UV lamp (Camag), beaker (Pyrex Iwaki®), filter paper, spatula, suction bulb, funnel, aluminum foil, maceration container (dark bottle), Erlenmeyer flask (Iwaki), volumetric flask (Iwaki), stirring rod, beaker glass (Iwaki), porcelain cup, porcelain crucible, volumetric pipette (Iwaki), test tube, test tube rack, and dropping plate.

Materials

Magnesium powder (Mg) (Merck), lead acetate ($Pb(C_2H_3O_2)_2$) (Merck), sulfuric acid p (H_2SO_4) (Merck), chloroform ($CHCl_3$) (Merck), hydrochloric acid p (HCl) (Merck), ferric (III) chloride ($FeCl_3$) (Merck), potassium iodide (KI) (Merck), sodium hydroxide (NaOH) (Merck), aluminum chloride ($AlCl_3$) (Merck), anhydrous acetic acid ($(CH_3CO)_2O$) (Merck), mercury (II) chloride ($HgCl_2$) (Merck), chloral hydrate, n-hexane (C_6H_{14}) (PT Bratachem), acetone (C_3H_6O) (Merck), distilled water (PT Bratachem), 70% ethanol (C_2H_5OH) (PT Bratachem), methanol p.a. (CH_3OH) (Merck), DPPH (1,1-Diphenyl-2-Picrilhydrazil) p.a (Sigma), vitamin C p.a ($C_6H_8O_6$) (PT Bratachem).

Characterization Test of Simplicia

The characterization test for simplicia included drying loss, total ash content, acid-insoluble ash content, water-soluble extract content, and ethanol-soluble extract. Ministry of Health of the Republic of Indonesia (1985).

Preparation of Gambir Leaves Extract

Five grams of gambir leaves simplicia powder was macerated by immersing the simplicia in 500 mL of each solvent (n-hexane, acetone, and 70% ethanol) (1:10 w/v ratio). The mixture was soaked for the first 6 hours, stirring occasionally, and then allowed to stand for 18 hours. The macerate was separated by filtration. This filtration process was repeated twice, using the same type and amount of solvent. All macerate was collected and evaporated using a rotary evaporator at a temperature below ±50°C to obtain a thick extract. The yield was weighed and recorded.

Extract Characterization Test

Extract characterization tests include determining water content, total ash content, acid-insoluble ash content, water-soluble extract content, and ethanol-soluble extract content. Ministry of Health of the Republic of Indonesia (2000).

Phytochemical Tests

1. Alkaloid Test

a. Mayer's Reagent

To 2 mL of plant extract, two drops of Mayer's reagent are added along the side of the test tube. A creamy-white precipitate indicates the presence of alkaloids (Banu & Cathrine, 2015).

b. Wagner's Reagent

Two drops of Wagner's reagent are added to 2 mL of plant extract along the side of the test tube. A reddish-brown precipitate confirms a positive test (Banu & Cathrine, 2015).

2. Flavonoid Test

Evaporate 1 mL of the test solution to dryness, dissolve the remainder in 1 mL of 95% ethanol, add 0.1 g of magnesium P powder and 10 mL of concentrated HCl. A reddish-orange to red-purple color indicates the presence of flavonoids. A yellow-orange color indicates the presence of flavones, chalcones, and aurones (Hanani, 2015).

3. Phenol Test

Three drops of 5% $FeCl_3$ are added to one mL of the test solution. A sample is positive for phenol if a blue or blackish-green color forms (Banu & Cathrine, 2015).

4. Terpenoid Test

Add 2 drops of anhydrous acetic acid and 1 drop of concentrated sulfuric acid to 1 mL of the extract. A brown or red color change indicates the presence of terpenoids (Banu & Cathrine, 2015).

5. Steroid Test

Add chloroform and observe the resulting layer. Then, add 3 drops of H_2SO_4 P. A blue or green color will form. The blue or green color can be observed at the edge of the dropper plate (Hanani, 2015).

6. Tannin Test

Add 3 drops of 10% $FeCl_3$ to one mL of the extraction solution. A sample is positive for tannin if a blackish-green or blackish-blue color forms (Hanani, 2015).

7. Saponin Test

One mL of the test solution is added to 10 mL of water and shaken vigorously for 10 minutes. If the foam formed remains stable for approximately 10 minutes, reaching a height of 1 cm to 10 cm. If the foam persists after the addition of 1 drop of 2N HCl, the extract is positive for saponin (Tiwari *et al.*, 2011).

Determination of Antioxidant Activity of Gambir Leaves

Preparing a 30 µg/mL DPPH Solution

Approximately 10 mg of DPPH (MW 394.33) is accurately weighed, then dissolved in methanol p.a. to 100 mL, then placed in a volumetric flask lined with aluminum foil. Add the solvent to the mark and shake until homogeneous, resulting in a DPPH solution with a concentration of 100 µg/mL. Next, dilute by pipetting 15 mL of DPPH solution with a concentration of 100 µg/mL into a 50 mL measuring flask, add the solvent to the limit mark, then shake until homogeneous and obtain a DPPH solution with a concentration of 30 µg/mL (Molyneux, 2004).

Preparation of Control Solution and Optimization of Maximum DPPH Wavelength

Pipetted 3.8 mL of DPPH solution (30 µg/mL) into a test tube. Then, 0.2 mL of methanol was added and homogenized. Cover the vial with aluminum foil. Incubate in a dark room for 30 minutes. Determine the absorption spectrum using a UV-Visible spectrophotometer at a wavelength of 400-800 nm and determine the maximum wavelength.

Preparation of Vitamin C Reference Solution

Weighed 25 mg of pure vitamin C into a volumetric flask, then added methanol p.a. to 25 mL (1000 µg/mL). Pipetted 1 mL of stock solution (1000 µg/mL) into a 10 mL volumetric flask (concentration 100 µg/mL). Next, a series of concentrations of 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL and 10 µg/mL were made, by pipetting the stock solution (100µg/mL) as much as 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL and 1 mL then adding methanol p.a to the 10 mL volumetric flask mark. Then pipette 0.2 mL of each concentration into a vial and add 3.8 mL of DPPH solution (30 µg/mL) then the vial is closed with aluminum foil. Incubated in a dark room for 30 minutes. The absorbance of various concentrations was measured using a UV-Visible spectrophotometer at the maximum wavelength of DPPH. **(**Andayani *et al*., 2008)

Antioxidant Activity Testing of Gambir Leaves n-Hexane Extract

250 mg of hexane extract was weighed and dissolved in methanol p.a. in a 50 mL volumetric flask to obtain a concentration of 5000 µg/mL. Next, a series of concentrations was prepared: 1500 µg/mL, 2000 µg/mL, 2500 µg/mL, 3000 µg/mL, and 3500 µg/mL. By pipetting 3 mL, 4 mL, 5 mL, 6 mL, and 7 mL of the stock solution (5000 µg/mL) and then topping up with methanol p.a. to the 10 mL mark. To determine the antioxidant activity of each concentration, 0.2 mL of sample solution was pipetted with a micro pipette and put into a vial, then added 3.8 mL of DPPH solution (30 µg/mL). The mixture was homogenized and left for 30 minutes in a dark place, the absorbance was measured with a UV-Vis spectrophotometer at the maximum wavelength. The antioxidant activity of the sample was determined by the amount of inhibition of DPPH radical absorption through the calculation of the percentage of DPPH absorption inhibition (Andayani *et al*., 2008).

Antioxidant Activity Testing of Gambir Leaves Acetone Extract

5 mg of acetone extract was weighed and dissolved in methanol p.a. in a 10 mL volumetric flask to obtain a concentration of 500 µg/mL. A series of concentrations was then prepared: 30 µg/mL, 40 µg/mL, 50 µg/mL, 60 µg/mL, and 70 µg/mL. The concentrations were then pipetted into 0.6 mL, 0.8 mL, 1 mL, 1.2 mL, and 1.4 mL of the stock solution (500 µg/mL), and then made up to the 5 mL mark with methanol p.a. To determine the antioxidant activity of each concentration, 0.2 mL of the sample solution was pipetted into a vial with a micropipette, then 3.8 mL of DPPH solution (30 µg/mL) was added. The mixture was homogenized and left for 30 minutes in a dark place, the absorbance was measured with a UV-Vis spectrophotometer at the maximum wavelength. The antioxidant activity of the sample was determined by the amount of inhibition of DPPH radical absorption by calculating the percentage of DPPH absorption inhibition (Andayani *et al*., 2008).

Antioxidant Activity Testing of Gambir Leaves Ethanol Extract

Twenty-five mg of the ethanol extract was weighed and dissolved in methanol p.a. in a 25 mL volumetric flask to obtain a concentration of 1000 µg/mL. Next, a series of concentrations was prepared: 100 µg/mL, 120 µg/mL, 140 µg/mL, 160 µg/mL, and 180 µg/mL. Pipetting 1 mL, 1.2 mL, 1.4 mL, 1.6 mL, and 1.8 mL of the stock solution (1000 µg/mL) was done, then making up to the 10 mL mark with methanol p.a. To determine the antioxidant activity of each concentration, 0.2 mL of the sample solution was pipetted into a vial with a micropipette, then 3.8 mL of DPPH solution (30 µg/mL) was added. The mixture was homogenized and left in the dark for 30 minutes. Absorption was measured using a UV-Vis spectrophotometer at the maximum

wavelength. The sample's antioxidant activity was determined by the amount of inhibition of DPPH radical absorption by calculating the percentage inhibition of DPPH absorption (Andayani *et al*., 2008).

Data Analysis

Data analysis used percentage inhibition and IC50 calculations using a regression equation.

The formula for calculating the percentage inhibition is:

% Inhibition $=\frac{Control\ Absorbance-Sample\ Absorbance}{Control\ Absorbance}$ x100%

3. Result and Discussion

In this study, the samples used were deep green gambir leaves. The leaves were collected in the morning at 9:00 a.m. Western Indonesian Time (WIB). Gambir leaves were collected from smallholder plantations in the Ampalu area, Aur Duri Village, Sutera District, Pesisir Selatan Regency. The gambir leaves were cleaned of impurities, then chopped into 1-2 cm pieces. They were left to dry for approximately two weeks. The dried leaves were then ground using a blender to form a powder. This allowed the solvent to penetrate easily, allowing for more effective extraction of the active ingredient.

The gambir leaves medicinal plant was tested for characterization, including determining drying loss using a 100-200 μm sample. This is especially true for extracts that do not contain high essential oil content. The drying loss was 8.8909%. The total ash content aims to provide an overview of the internal and external mineral content from the beginning until the formation of ash, the ash content obtained is 1.4486%.

The acid-insoluble ash content was used to determine the amount of impurities. The acid-insoluble ash content was 0.4149%. The water-soluble compound content was used to determine the amount of compounds that could be extracted with water, resulting in a water-soluble compound content of 37.0641%. The ethanol-soluble compound content was used to determine the amount of compounds that could be extracted with ethanol, resulting in a 71.4496% soluble compound content in ethanol.

The powdered gambir leaves extract was macerated. This maceration method was chosen because it can handle large samples, is simple to implement, requires no special treatment, and avoids the possibility of decomposition of active substances due to temperature influences because there is no heating process. According to Sie (2013), antioxidants are compounds that are not heat-resistant, so the maceration method, which is a cold extraction method, is more optimal in extracting antioxidant compounds.

Sample maceration was carried out using three solvents with different polarities to determine the yield and obtain active compounds from gambir leaves based on their polarity. The yield of n-hexane obtained was 6.696%, the yield of acetone was 16.9294% and the yield of ethanol was 13.5646%. Furthermore, the characterization of the extracts from the three solvents was carried out, namely determining the ash content of the extract, which obtained n-hexane extract was 0.084%, acetone extract was 0.482%, and ethanol extract was 0.254%. For the determination of the ash content insoluble in acid, the n-hexane extract was 0.068%, acetone extract was 0.163% and ethanol extract was 0.131%. Determination of water content aims to provide a minimum limit or range of water content in the extract where the water content of the n-hexane extract was 14.09%, the water content of the acetone extract was 12.11% and the water content of the ethanol extract was 6.55%.

Determination of phytochemical content in n-hexane, acetone and ethanol extracts carried out seven phytochemical tests, namely phenol test, tannin test, flavonoid test, alkaloid test, saponin test, terpenoid test, and steroid test. Positive phytochemical screening test containing flavonoid compounds is indicated by a red-orange reaction, the extract reacts when ethanol, Mg powder and concentrated hydrochloric acid are added. A positive phenol compound test is indicated by a blackish blue or blackish green color when reacted with iron (III) chloride.

Table 1. Phytochemical Screening

No	Phytochemical Test	Hexane Extract of Gambir Leaves	Acetone Extract of Gambir Leaves	Ethanol Extract of Gambir Leaves
1	Alkaloid Mayer Wagner	 - -	 - +	 + +
2	Flavonoid	-	+	+
3	Phenol	-	+	+
4	Saponin	-	-	+
5	Tanin	-	+	+

No	Phytochemical Test	Hexane Extract of Gambir Leaves	Acetone Extract of Gambir Leaves	Ethanol Extract of Gambir Leaves
6	Terpenoid	+	+	+
7	Steroid	+	-	-

Determination of the antioxidant activity of n-hexane, acetone, and ethanol extracts from *Uncaria gambir* (Hunter) Roxb leaves using an antioxidant assay using DPPH reagent, determined by double-beam UV-Vis spectrophotometry, as shown in Fig. 1. Antioxidants are defined as compounds capable of protecting cells from the dangers of reactive oxygen free radicals (Hazimah *et al*., 2013).

DPPH donates a hydrogen atom when reacting with a compound, then becomes reduced, indicated by the loss of the violet color. The DPPH radical method is a measurement of antioxidant activity using only a small sample amount and a short time. The antioxidant activity of a compound is indicated by the inhibition of DPPH absorption at a wavelength of 515-517 nm. DPPH has strong absorption at a wavelength of 515-517 nm, producing a dark violet color. This method was first proposed by Marsden Blois in 1958 (Molyneux, 2004).

The obtained wavelengths were used to measure the antioxidant activity of n-hexane, acetone, and ethanol extracts from gambir leaves. The maximum DPPH wavelength obtained was 515 nm with an absorbance of 0.627. The magnitude of antioxidant activity is indicated by the IC50 value, which is the concentration of the sample solution needed to inhibit 50% of DPPH free radicals. In addition, antioxidant activity also depends on its chemical content.

In testing the antioxidant activity of n-hexane extract of gambir leaves, the absorbance of the solution was 0.374, 0.314, 0.238, 0.152, 0.094 and the percentage of free radical scavenging activity was 40.3508%, 49.9202%, 62.0414%, 75.9346%, 85.0079%. The IC50 value or free radical scavenging activity of 50% was obtained from the n-hexane extract of gambir leaves (*Uncaria gambir* (Hunter) Roxb) at a concentration of 1948.6277 µg/mL. Can be seen in Figure 1.

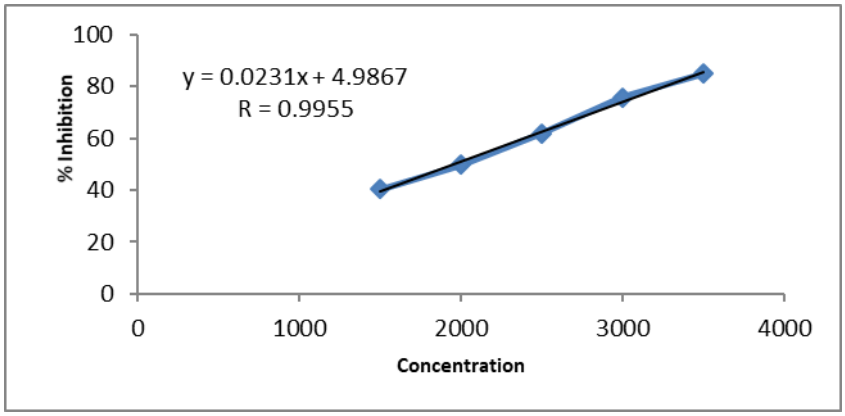

Figure 1. Relation Curve Between Concentration and % Inhibition of Gambir Leaves N-Hexane Extract

Then testing the antioxidant activity of acetone extract of gambir leaves, obtained absorbance of the solution 0.520, 0.428, 0.360, 0.290, 0.220 and obtained percentage of radical scavenging activity 19.0653%, 31.7384%, 42.5837%, 53.748%, 64.9122%. The IC50 value or free radical scavenging activity of 50% was obtained from acetone extract of gambir leaves (*Uncaria gambir* (Hunter) Roxb) at a concentration of 56.6772 µg/mL. Can be seen in Figure 2.

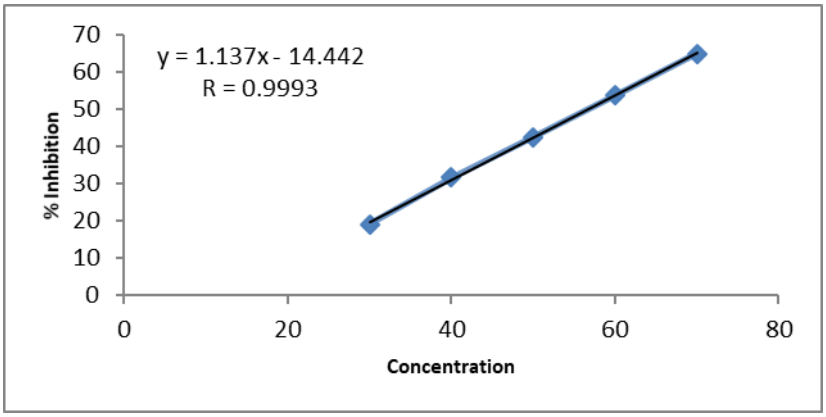

Figure 2. Relation Curve Between Concentration and % Inhibition of Gambir Leaves Acetone Extract

Furthermore, testing the antioxidant activity of ethanol extract of gambir leaves, obtained absorbance of the solution 0.416, 0.353, 0.308, 0.328, 0.183 and obtained percentage of radical scavenging activity 33.6523%, 43.7001%, 50.8071%, 62.0414%, 70.8133%. The IC50 value or free radical scavenging activity of 50% was obtained from ethanol extract of gambir leaves (*Uncaria gambir* (Hunter) Roxb) at a concentration of 135.2190 µg/mL. Can be seen in Figure 3.

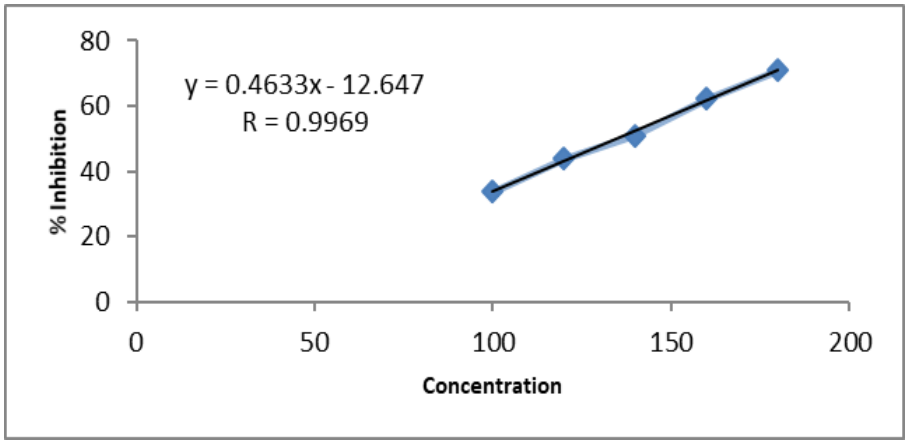

Figure 3. Relation Curve Between Concentration and % Inhibition of Gambir Leaves Ethanol Extract

Comparative testing of antioxidant activity, namely vitamin C because vitamin C is known as a very strong antioxidant, obtained the absorbance results of the solution of 0.454, 0.415, 0.372, 0.328 and 0.288 and obtained the percentage of radical scavenging activity of 27.5917%, 33.8118%, 40.6698%, 47.6874%, 54.0669%. The IC50 value or free radical scavenging activity of 50% was obtained by standard vitamin C at a concentration of 8.7636 µg/mL. Can be seen in Figure 4.

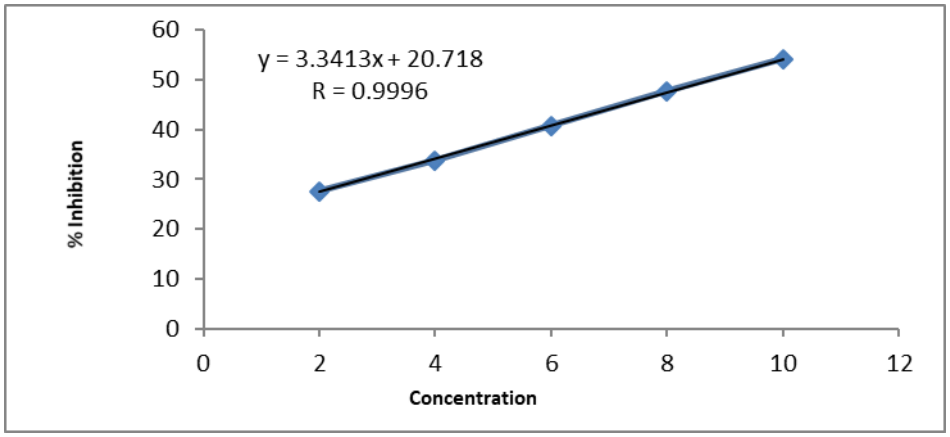

Figure 4. Relation Curve Between Concentration and % Inhibition of Vitamin C

Judging from the absorbance results, it can be seen that the greater the sample concentration, the smaller the absorbance value obtained. This is because the higher the antioxidant compound that is able to reduce or ward off radicals in DPPH and the percentage inhibition value will be greater (Bahriul *et al.*, 2014).

4. Conclusion

Based on the research conducted, it can be concluded that:

1. The results of the phytochemical screening conducted showed positive results for the chemical compounds contained in the hexane extract, namely terpenoids and steroids. The acetone extract showed positive results for phenols, flavonoids, alkaloids, tannins, and terpenoids. While the ethanol extract showed positive results for phenols, flavonoids, alkaloids, tannins, terpenoids, and saponins.
2. The results of the antioxidant activity of the n-hexane extract obtained an IC50 value at a concentration of 1948.6277 µg/mL (weak antioxidant>150 µg/mL), in the acetone extract obtained an IC50 value at a concentration of 56.6772 µg/mL (strong antioxidant 50-100 µg/mL), while in the ethanol extract obtained an IC50 value at a concentration of 135.2190 µg/mL (moderate antioxidant 100-150 µg/mL), the comparison of vitamin C obtained an IC50 value at a concentration of 8.7636 µg/mL (very strong antioxidant <50 µg/mL).
3. The results of determining the appropriate solvent and obtaining the highest antioxidant activity with the lowest IC50 value are acetone extract.

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