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Total Flavonoid Content and Antioxidant Activity of Soursop Leaves from the Three Largest Producing Areas of South Sulawesi Province, Indonesia

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ABSTRACT

Soursop leaves (Annona muricata L.) have anticancer and antioxidant activities; soursop leaves contain substances with the potential as antioxidants, such as flavonoids. Antioxidant assays and total flavonoid content from the three largest producing regions in South Sulawesi Province, namely Gowa, Takalar, and Pinrang, were carried out to obtain data on the antioxidant activity and flavonoid content of soursop leaves (Annona muricata L.) three areas. Total Flavonoid content was determined by UV-Vis spectrophotometry, and antioxidant activity was measured by the DPPH (1,1-Diphenyl-2-picryl Hydrazyl) method. According to the study, soursop leaves (Annona muricata L.) from the Gowa, Takalar, and Pinrang regions have total flavonoid levels of 7,6484 mg QE/g, 3,74429 mg QE/g, and 3,3105 mg QE/g, respectively, and IC50 values of 70.509 g/mL, 102.159 g/mL, and 99.246 g/mL, respectively. The results showed that soursop leaves (Annona muricata L.) from the Gowa area had the highest flavonoid content and antioxidant activity. Gowa region could be the best source of soursop leaves for developing soursop as an herbal remedy.

Keywords: Soursop Leaf; Flavonoid Levels; Antioxidant; DPPH

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INTRODUCTION

Antioxidants are substances that, at small concentrations, are significantly able to inhibit or prevent oxidation of substrates caused by free radicals (1). Free radicals are highly reactive molecules because they have unpaired electrons in their outer orbitals, which allows them to react with body cell molecules by binding the electrons of the cell molecules. Free radicals that are produced continuously during normal metabolic processes are considered to be the cause of damage to the function of body cells, which ultimately triggers the onset of degenerative diseases (2).

Changes in people's lifestyles have an impact on the emergence of various diseases, especially cancer, and its treatment is still arguably less than perfect, namely using chemotherapy, which can damage body cells and eventually die. In Indonesia, the prevalence of cancer is also quite high. According to Riskesdas 2013 data, the prevalence of cancer in Indonesia is 1.4 per 100 population or around 347,000 people (3). One of the plants that contain anti-cancer compounds, according to research, is soursop leaves (Annona muricata L) because it contains flavonoid compounds and has a strong antioxidant with an IC_{50} value of 7 ppm (3).

Soursop can grow in a fairly wide range of climates, in the lowlands (0 m above sea level) to 1,200 m above sea level. In addition, this plant can grow on various types of soil, both nutrient-rich and well-watered, as well as marginal lands such as acidic soil, dry soil, and sandy soil. Soursop habitat can grow on all types of soil with a pH between 5-7 (4). Soursop plants will grow very well at a climate altitude of 22-28^{o}C, with relative humidity of 60-80% and rainfall ranging from 1500-2500 mm per year (5).

Flavonoids are one of the most widely found groups of secondary metabolite compounds in plant tissues. Flavonoids belong to the class of phenolic compounds with the chemical structure C6-C3-C6. The flavonoid skeleton consists of one aromatic ring A, one aromatic ring B, and the middle ring in the form of an oxygen-containing heterocyclic and oxidized form of this ring is used as the basis for the division of flavonoids into sub-subgroups. The numbering system is used to distinguish the position of carbon around the molecule (6).

Flavonoids are one class of secondary metabolites produced by plants that are included in the large group of polyphenols. These compounds are found in all parts of the plant including leaves, roots, wood, skin, pollen, nectar, flowers, fruits, and seeds. Flavonoids have the ability to capture free radicals and inhibit lipid oxidation (6).

According to Aminah et al.'s research, 2016 the ethanol extract of soursop leaves (Annona muricata L.) from three regions has different antioxidant activity potential, samples from North Mamuju area have an IC_{50} value of 1,512 µg/mL, Makassar has an IC_{50} value of 1,380 µg/mL and Jeneponto has an IC_{50} value of 1,420 µg/mL. From the three regions, each sample used has a different level of antioxidant activity. The difference in activity levels is caused by several factors, including the geographical location of the plant, climatic factors including temperature, air and humidity,

essential factors such as light, water, and soil nutrients. As well as pest or disease and weed disturbance factors (7).

Factors that affect the production of secondary metabolites which include environmental factors are temperature stress in this case the Gowa Regency area is 54.28% at an altitude of 100 - 1000 masl, has a wet tropical climate with the average air temperature of Gowa Regency in 2018 is 17.15^{o}C (8). While the Takalar Regency area is at an altitude of 0 - 1000 masl, with a tropical climate with an air temperature between 22.2^{o}C and 33.9^{o}C (9). And for Pinrang Regency, it has an altitude of 0 - 1000 masl, with rainfall occurring from December to June with the highest rainfall occurring in March and normal average temperatures between 27°C with air humidity of 82% - 85% (10).

Based on statistical data, soursop production in South Sulawesi Province by Regency / City (Quintal) in 2020. It is known that there are three largest soursop-producing areas in South Sulawesi Province, namely Gowa, Takalar, and Pinrang districts (11). This research was conducted on the determination of the total flavonoid content of soursop leaf ethanol extract (Annona muricata L.) from three regions and antioxidant testing of soursop leaves (Annona muricata L.) using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method, from several production centers of soursop (Annona muricata L.) in South Sulawesi Province to determine antioxidant activity and obtain data on the highest antioxidant activity of soursop leaves (Annona muricata L.) from three different growing places in South Sulawesi Province.) in South Sulawesi Province to determine antioxidant activity and obtain data on the highest antioxidant activity of soursop leaves (Annona muricata L.) from three different growing places in South Sulawesi Province. These three regions could be used as a source of raw materials for soursop in developing herbal medicine because it has the potential as anticancer.

MATERIALS AND METHODS

General

The tools used were stirrers, porcelain cups, glassware (Pyrex), micropipettes (Memmert), scale pipettes, dropper pipettes, tube racks, horn spoons, a set of UV-Vis spectrophotometer (Genesys 10S Uv-Vis), a set of rotavapor (Ika® RV 10 basic), test tubes, water bath, and analytical scales (Caratseries).

The materials used in this study include soursop leaf extract (Annona muricata L.), 96% ethanol, DPPH (1,1- diphenyl-2-picrylhydrazyl), filter paper, quercetin, Mayer, Dragendorf, Bouchard, $FeCl_3$, HCl 2 N and NaOH 1 N, Mg powder, concentrated HCl, aluminum chloride ($AlCl_3$), potassium acetate (CH_3COOK), aluminum foil, and distilled water.

Sample Preparation

Soursop (*Annona muricata* L.) old leaf sampling was taken in three areas, first in Tamarunang Village, Somba Opu Subdistrict, Gowa Regency. The second is in region of Pa'batangan Village,

Mappakasunggu Subdistrict, Takalar Regency, and the last is in Laleng Bata Village, Paleteang Subdistrict, Pinrang Regency in South Sulawesi Province.

Preparation of Extract

Soursop leaf powder (*Annona muricata* L.) that has been crushed is weighed and then put into a maceration container and added with 96% ethanol solvent until the powder is soaked, carried out at room temperature for 3 x 24 hours the results obtained as much as Gowa 34.097 g, Pinrang 18.414 g, and Takalar 17.884 g.

Phytochemical Screening

1) Alkaloid Test

Each extract was dissolved in ethanol solvent, and then the results obtained were filtered to obtain the filtrate. The filtrate was divided into three parts of 5 ml each and then added with

a. Mayer's reagent to form a white or yellow clumpy precipitate that dissolves in methanol.
b. Dragendorf reagent formed an orange-brown precipitate.
c. Bourchardat reagent forms a brown-to-black precipitate.

Positive Alkaloid if two or three parts of the precipitate (12).

2) Flavonoid Test

A total of 5 mL of extract was dissolved in ethanol, and then Mg powder was added and dripped with 5 drops of concentrated HCl. If the result is red yellow or orange, it means that it is positive for flavonoids (12).

3) Tannin Test

A total of 5 mL of extract dissolved in ethanol is added with $FeCl_3$ reagent. Extracts containing tannin will be blue or blackish green (12).

4) Saponin Test

The ethanol extract of each sample was added to 10 ml of hot distilled water and dissolved first while heated in a water bath and then shaken vigorously. If no foam is formed, it is negative, but if it remains bubbly after standing for 10 minutes and then adding 2 N HCl, the foam does not disappear, it is positive for saponins (12).

5) Quinone Test

A solution of each extract in ethanol solvent is added with a few drops of 1 N NaOH solution. If a red color forms, it indicates the presence of quinones (12).

6) Phenolic Test

A total of 1 mg of extract was added 2 drops of FeCl3 1%. Positive extracts contain phenols if they produce concentrated green, red, purple, blue or black colors (13).

Determination of Total Flavonoid Content of Ethanol Extract of Soursop Leaf

The total flavonoid content of the extract was measured by Chang method with some modifications (14). Standard quercetin made by 10, 20, 30, 40, 50 µg/mL from stock solution 1000

µg/mL. Briefly, 1 mL of each standard and sample was poured into a centrifuge tube, respectively; this was followed by adding 0.2 mL of $AlCl_3$ 10% and 0.1 mL of CH_3COOK 1M. The content of the centrifuge tube was mixed thoroughly with a vortex mixer for two to three minutes and allowed to stand for 30 minutes at room temperature. Absorbance was then measured at 435 nm by UV Visible spectrophotometry. Quercetin was used as a standard reference for the quantification of total flavonoids. Total flavonoid content is expressed in each extract's grams of quercetin equivalent (QE).

Antioxidant activity analysis by DPPH (1,1-diphenyl-2-picrylhidrazyl) method

Antioxidant activity testing on soursop leaf extract (Annona muricata L.) 96% ethanol extract with DPPH (1,1-diphenyl-2-picrylhydrazyl) method refers to the procedure of Aminah et al. (2016) with some modifications. Maximum wavelength measurements were made by measuring the DPPH solution that had been incubated for 30 minutes at 37°C and measured at a wavelength of 450-650 nm.

Data Analysis

The percentage of free radical binding is calculated by the formula:

% Free radical binding =

$$\frac{Abs.\ Standar - Abs.\ Sampel}{Abs.\ Standar} \text{ x } 100\%$$

The IC50 value was calculated using a linear regression equation, with sample concentration as the x-axis and % inhibition as the y-axis. From the equation: y = a + bx, the IC50 value can be calculated using the formula: $IC_{50} = \frac{(50-b)}{a}$

y = 50 (50% oxidation inhibitor)

x = IC50 (a number indicating the concentration of the extract that is able to inhibit the oxidation process by 50%)

a = slope

b = intercept

RESULTS

The extraction percentage of soursop extract yield from three regions is seen in Table 1.

Table 1. Soursop leaf extract % yield data *(Annona muricata L.)*

Sample	Region	Dried sample weight (g)	Extract weight (g)	Rendement (%)
Soursop Leaf	Takalar	280	17,884	6,387
	Pinrang	315	18,414	5,845
	Gowa	650	34,097	5,246

The soursop rendement from Takalar region is higher than Pinrang and Gowa areas. Rendement was in line with the compound which contained in the extract.

Table 2 shows that the ethanol extract of soursop leaves from Pinrang is negative in alkaloid testing with Mayer and Bouchard at reagents, and the ethanol extract of soursop leaves from Takalar is negative in saponin testing.

Table 2 Phytochemical test results of Soursop Leaf extracts from three regions

Secondary metabolites content	Ekstrak Ethanol Leaf Soursop		
	Gowa	Takalar	Pinrang
Alkaloid:			
-Mayer	+	+	-
-Dragendorf	+	+	+
-Bouchardat	+	+	-
Phenol	+	+	+
Saponin	+	-	+
Quinone	+	+	+
Flavonoid	+	+	+

Notes: (+) positive result, (-) negative result

Table 3. Total flavonoid content of ethanol extract of Soursop Leaf (Annona muricata L.) from three regions

Sample	Region	Flavonoid Total (%)
Ethanol Extract of Soursop Leaf	Gowa	0,76484
	Takalar	0,374429
	Pinrang	0,33105

Total flavonoid content in the Gowa area is the highest compared to other regions. Takalar and Pinrang regions have almost similar values in total flavonoid content

The result of antioxidant calculation of standard quercetin, Gowa, Takalar, and Pinrang region were seen in Table 4 – Table 7.

Table 4. Calculation of % inhibition of quercetin

Concentration (ppm)	Absorbance (515)	% Inhibition	IC_{50} (µg/mL)
1	0,562	26,822	
2	0,518	32,552	
3	0,479	37,63	5,100
4	0,432	43,75	
5	0,387	49,609	

The test results in Table 4 – Table 7 show that the antioxidant activity of quercetin was the highest. In the soursop, The highest activity is from Gowa, then Pinrang, and the lowest is Takalar

Table 5. Calculation of % Inhibition of Soursop Leaf ethanol extract (Gowa)

Concentration (ppm)	Absorbance (516)	% Inhibition	IC_{50} (µg/mL)
20	0,483	8,867	
40	0,359	32,264	70,509
60	0,278	47,547	
80	0,200	62,264	
100	0,197	62,830	

Table 6. Calculation of % Inhibition of Soursop Leaf ethanol extract (Takalar)

Concentration (ppm)	Absorbance (514)	% Inhibition	IC_{50} (µg/mL)
20	0,504	26,315	
50	0,425	37,865	102,159
90	0,354	48,245	
140	0,266	61,111	
200	0,18	73,684	

Table 7. Calculation of % Inhibition of Soursop Leaf ethanol extract (Pinrang)

Concentration (ppm)	Absorbance (514)	% Inhibition	IC_{50} (µg/mL)
60	0,427	37,573	
80	0,367	46,345	
110	0,316	53,801	99,246
150	0,25	63,45	
200	0,158	76,9	

DISCUSSION

Antioxidants can be defined as substances that can delay or prevent the occurrence of free radical reactions. To prevent the occurrence of free radicals, antioxidants are needed in the body. Antioxidants needed are sourced from outside sources such as vegetables, fruits, and certain plants. One plant that is proven to have antioxidants is soursop leaves (Annona muricata L). A plant can have potential as a medicine because there is a secondary metabolite process. Secondary metabolites are the result of the plant's adaptation process to the environment or stress. Plants produce secondary metabolites that vary in structure, function, and levels.

Differences in environmental conditions can also cause differences in the type and number of secondary metabolites contained in plants that grow in certain areas with other areas (13). Several factors can affect the levels of a compound in plants including the geographical location of the plant,

climatic factors which include temperature, air, and humidity, and essential factors such as light, water, and soil nutrients. In environmental conditions with high temperatures, there will be an increase in free radicals in the form of reactive oxygen species (ROS) in plants that are reactive in plant tissues, triggering cell damage. As a form of adaptation to high ambient temperatures, plants will produce compounds that are antioxidants. At higher temperatures, it will produce a higher total flavonoid as an extra defense synergy against environmental stress (15). As well as other factors that can affect the level of a compound in the plant, namely pest or disease and weed interference.

Soursop is the easiest plant species to grow among other Annona species and requires a warm and humid tropical climate. This plant can grow at an altitude of up to 1200 m above sea level. Soursop plants will grow very well in climatic conditions with a temperature of 22-28C, with humidity and rainfall ranging from 1500-2500 mm per year (5).

Antioxidant activity in a plant depends on the compound content of the plant. Some studies show that altitude is one of the factors that affect the growth of a plant. The phytochemical content of secondary metabolites such as flavonoids from a plant will be different in each region because it is influenced by several environmental factors including light, temperature, pH, and altitude of the growing place which will affect the phytochemical content of a plant. Besides this, the processing of raw materials can also affect the extracted chemical content. The type of solvent used can affect the compounds extracted from a plant. The effect of altitude on plants is closely related to environmental factors, such as temperature. The higher air temperature in the lowlands causes the water vapor capacity to increase, so that the relative humidity of the air decreases, especially during the day. In addition, the sunlight intensity factor, the low value of sunlight intensity can be caused by the presence of shade such as clouds, trees, or other forms of shade. In addition, the influence of the harvest age of the sample can also affect the content of compounds in the sample (16).

This study used soursop leaves (Annona muricata L.) taken from three areas, namely Gowa, Takalar, and Pinrang. Sampling was carried out by taking fresh old soursop leaves manually in the yard of a resident's house, then making soursop leaf dried powder to facilitate the extraction process.

Soursop leaf dried powder (Annona muricata L.) from the three regions was extracted by maceration method. Maceration was chosen because of its simple processing and equipment, which does not use heating so that it can prevent the decomposition of active substances contained in the sample due to the influence of temperature and compounds that cannot withstand heating (17). The maceration process is carried out 3x24 hours with remaceration or replacement of new solvents every 24 hours which aims to extract the compounds contained in the sample thoroughly. The solvent used in the maceration process is 96% ethanol, ethanol solvent is used because it is universal, polar, and easy to obtain. Ethanol 96% was chosen because it is selective, non-toxic, has good absorption, and high absorption ability so that it can extract non-polar, semi-polar, and polar compounds. The 96% ethanol solvent is easier to penetrate the cell wall of the sample than the ethanol solvent with a lower concentration, resulting in a concentrated extract (18).

Ethanol extracts of soursop leave from the three regions were subjected to phytochemical tests. In the screening test, alkaloids were identified by adding HCl first. Alkaloids are secondary metabolites that are alkaline in soluble in their salt form, so it is necessary to add acid first. Furthermore, Mayer and dragendorf reagents are used, where in the Mayer reagent a positive reaction will be indicated by the formation of a white precipitate, in the dragendorf reagent an orange precipitate will form, while in the bouchardat reagent a brown to black precipitate will form. Alkaloids have nitrogen atoms that have free electron pairs, so they can form coordination covalent bonds with K + ions from the potassium tetraiodomercurat (II) reagent (Mayer), potassium tetraiodobismutat (Dragendorf), and potassium iodide and iodine (Bouchardat) to form potassium-alkaloids (19).

Tannins are polyphenolic compounds that have many benzene and hydroxyl groups. In the screening test, with the addition of $FeCl_3$, phenol or tannin will react with a green or blue-black color change. This occurs due to forming a coordination covalent bond between iron (III) ions and hydroxyl groups (19).

Flavonoids are polar compounds that have many hydroxyl groups. In its identification, concentrated Mg + HCl powder is used which will form H2 bubbles. The addition of concentrated HCl will hydrolyze the flavonoid glycoside into its aglycone form which then forms a complex with Mg resulting in a red, yellow or orange color change. In addition, flavonoids can be identified using NaOH solution which will give a reaction with a reddish orange color change. Flavonoids have free o-hydroxy groups which with NaOH will form quinoid compounds that are reddish orange in color (19).

Saponins are polar compounds that dissolve in water. Saponins also have non-polar groups, namely terpenoids / steroids. Compounds that have polar and non-polar groups can be surface active so that with shaking using water, they will form micelles and colloidal solutions that will look like foam (19).

The quinone test was carried out using a 1 N sodium hydroxide (NaOH) solution reagent. 1N NaOH reagent serves to deprotonate the phenol group on quinone so that phenolic ions are formed. This phenolic ion can absorb certain light and cause a red color (19).

The difference in the results of phytochemical tests in Table 2 is due to differences in the area where it grows, which can result in differences in the content of secondary metabolites from soursop leaves.otal flavonoid content of soursop leaf ethanol extract was determined using quercetin as the standard. The results of measuring the absorbance of the standard solution at various concentrations of the calibration curve, obtained a linear regression equation which is a = 0.235; b = 0.0146; R2 = 0.9919 with r = 0.9959. The linear calibration curve has a relationship between the concentration of the gallic acid solution and the absorbance value if the r value is close to one (3).

In the measurement of total flavonoid compounds, each region was replicated three times for data accuracy purposes. The results of this study obtained total flavonoid content in soursop leaf

ethanol extract from Gowa 7.6484 mg QE/g, Takalar 3.74429 mg QE/g, and Pinrang 3.3105 mg QE/g (table 3). Research conducted by Yani revealed that the flavonoid level by the different solvent result in 96 % ethanol, ethyl acetate, and hexane, respectively, 68,9049 mgQE/g, 97,2381 mgQE/g, 73,6667 mgQE/g (20). The chemical components of a plant depend on where it grows, climate, high altitude, rainfall, intensity of sunlight, including flavonoids.

Antioxidant testing of soursop leaf ethanol extract (Annona muricata L.) was carried out using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. The DPPH method is used because it is a method that can be used to determine the antioxidant activity in the sample to be tested by looking at its ability to counteract DPPH free radicals. The advantages of this DPPH method are that the method is simple, easy, fast, sensitive, and requires a small number of samples. It is easy to apply because the DPPH radical compound used is relatively stable compared to other methods. The principle of this method is the donation of hydrogen atoms (H+) from the substance tested to the DPPH radical into a non-radical compound diphenyl picryl hydrazine which will be indicated by a color change. The color change that occurs is a color change from purple to yellow, where the intensity of the DPPH color change is directly proportional to the antioxidant activity to reduce the free radicals (21).

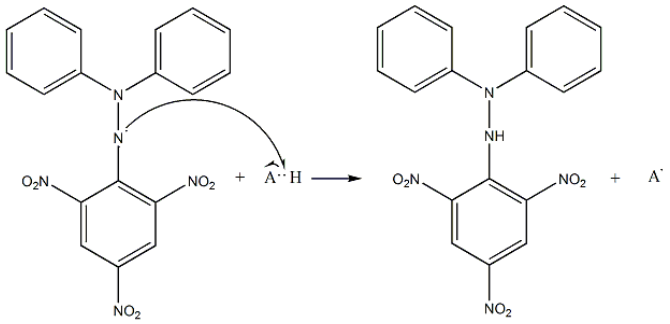

Figure 1. DPPH Reaction with Antioxidants

Before measurement of antioxidant activity using UV-VIS spectrophotometer at the maximum wavelength of DPPH, determination of the maximum wavelength is done to determine the λ which has the highest absorption of DPPH. Where measured by means of 35 ppm DPPH measured wavelength 450-650 nm and obtained the maximum wavelength at a wavelength of 515 nm with absorbance 0.768. Sample measurements must be made at the maximum wavelength so that the sensitivity is maximized and minimizes errors because at that wavelength the change in absorbance for each unit of concentration is the greatest.

All of the samples measurements were made by 5 concentration series, and obtained a regression curve. then the calculation of the IC_{50} value was carried out..

A compound is said to be a very strong antioxidant if the IC_{50} value is < 50 µg/mL, strong if the IC_{50} value is 50-100 µg/mL, moderate if the IC_{50} is 101-150 µg/mL, while if the IC_{50} value is > 151 µg/mL it is said to be a weak antioxidant.

The results obtained in testing the antioxidant activity of soursop leaf ethanol extract (Annona muricata L.) are The measurement of quercetin standard solution has an IC_{50} value of 5.100 µg/mL is a very strong antioxidant. While, the sample from Gowa has an IC_{50} value of 70.509 µg/mL is a strong antioxidant (table 6), the sample from Takalar has an IC_{50} value of 102.159 µg/mL is a moderate antioxidant (table 7) and the sample from Pinrang (table 8) has an IC_{50} value of 99.246 µg/mL is also a strong antioxidant. From the test results that have been carried out, it can be seen that the antioxidant activity in soursop leaf samples (Annona muricata L.) originating from the Gowa and Pinrang areas has a stronger level and potential antioxidant activity compared to those from the Takalar area, but is still lower than quercetin because the IC_{50} value obtained is smaller. In previous research conducted by Rivai et at. (2013) showed antioxidant test results of 96% ethanol extract of soursop leaves with DPPH method of 70.89 µg/mL which is classified as strong, and in another study conducted by Aminah et al. (2016) regarding the ethanol extract of soursop leaves (Annona muricata L.) originating from three regions has different potential antioxidant activity as well, samples originating from the North Mamuju area have an IC_{50} value of 1.512 µg/mL, Makassar has an IC_{50} value of 1.380 µg/mL and Jeneponto has an IC_{50} value of 1.420 µg/mL the results of the determination of flavonoid levels in each solvent were 68,9048 mgQE/g, 97,2381 mgQE /g and 73,6667 mgQE/g.

The results obtained show that differences in growing places can give different results. In addition, it is known that there is a relationship between antioxidant activity and the flavonoid content of soursop plants. The higher the flavonoid content, the higher the antioxidant activity. The Gowa area can be used as a source of raw materials for medicinal plants for soursop, which can be developed into original Indonesian modern medicine.

CONCLUSION AND RECOMMENDATIONS

Based on the research results, it can be concluded that soursop (Annona muricata L.) leaf ethanol extracts from three regions have different total flavonoid levels. Total flavonoid levels in soursop leaf ethanol extract from Gowa 7.6484 mg QE/g, Takalar 3.74429 mg QE/g, and Pinrang 3.3105 mg QE/g. The soursop leaf ethanol extract (Annona muricata L.) from the Gowa area has an IC_{50} value of 70.509 µg/mL, Takalar has an IC50 value of 102.159 µg/mL and Pinrang has an IC_{50} value of 99.246 µg/mL. Gowa region could be the best source of soursop leaves to develop soursop as an herbal remedy

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