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OPTIMIZATION OF ANTIOXIDANT ACTIVITY OF 96% ETHANOL EXTRACT OF *Chromolaena odorata* L. ABTS RADICAL PROPHYLACTIC

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ABSTRACT

Choromolaena odorata L. is one of the plants used as an antioxidant. This research aims to determine the optimization of the antioxidant activity of 96% ethanol extract of C. odorata L. leaves using the ABTS method, a variety of drying techniques. Previous research has tested antioxidant activity using one drying technique, whereas this research uses a variety of drying techniques because the sample drying technique will affect its activity. Simplicia is obtained from various drying techniques. Then the simplicia was organoleptically tested and its water content was measured using the distillation method. The ethanol extract of Tacklean leaves was carried out using the maceration method using 96% ethanol solvent, then phytochemical screening was carried out using the TLC method and the antioxidant activity test of the ethanol extract of C. odorata L leaves was carried out using the ABTS method. The formation of the ABTS radical

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cation [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) forms the basis of one of the spectrophotometric methods that has been applied to the measurement of total antioxidant activity of plants. The research results from the three drying techniques (50°C oven, direct sun drying, and air drying) showed organoleptic results in successive colors (brown, chocolate and dark green), while for shape and odor, namely dry leaves and a distinctive odor. The water content of simplicia ranges from 7.52-9.31%, soaked extract 8.4-10.3% and IC_{50} 23.287-29.064 µg/mL and quarcetin as a comparison with IC_{50} 2.292 µg/mL. Phytochemical screening results contain alkaloids, flavonoids, steroids and tannins. Oven drying at 50°C produced the greatest antioxidant activity with an IC_{50} value of 23.287 µg/mL which indicates very strong antioxidant power, although the antioxidant activity with a variety of drying techniques was all in the very strong category because it was <50 µg/mL.

INTRODUCTION

Free radicals are reactive to their electron pairs, so if they are formed in the body a chain reaction will occur which produces new free radicals. In the end, the number of free radicals will continue to increase. Oxidative stress occurs when the body is exposed to free radicals in amounts that it cannot handle. Therefore, our body needs important substances such as antioxidants which can protect the body from free radical attacks by reducing the negative impacts caused by free radicals. (Taeri et al., 2019). One of the plants used by the community as medicine is *C. odorata* L. leaves which can be used as a wound medicine and antioxidant (Utami, Imrawati, et al., 2023).

Antioxidants can prevent or inhibit the oxidation of fats, nucleic acids and other molecules. When antioxidant compounds work, they provide protons or hydrogen atoms to radical compounds, which stops the free radical chain reaction and makes them more stable. Many studies show that consuming lots of fresh fruit and vegetables that are full of antioxidants and adopting a healthy lifestyle can reduce the chance of developing degenerative diseases (Riskianto, 2021).

Research by Utami, Imrawati, et al. (2023), namely the antioxidant potential of 96% ethanol extract of *C. odorata* L. leaves using the simple drying technique using indirect sunlight has very strong antioxidant activity, this is due to the content of flavonoid compounds which have potential as antioxidants. Apart from that, various drying techniques have also been carried out on tacklelan leaves using 70% ethanol for antioxidant activity with the results of the drying method using indirect sunlight having the best activity, namely 10.645 µg/mL(Utami et. al., 2023).

Drying techniques affect the chemical compounds contained in a herbal plant, especially compounds that have antioxidant properties. The total phenolic and flavonoid content in a simplicia which has antioxidant activity, its stability is influenced by the drying process (Utami et. al., 2023).

There are several drying methods, including drying in direct sunlight, oven and air drying. According to(Utami, Imrawati, et al (2024) the drying process using an oven will produce better simplicia. Drying in an oven is better because it will reduce the water content

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more evenly. However, using temperatures that are too high can result in biochemical changes that reduce the quality of the simplicia produced. Meanwhile, the air dry method is considered cheap, can retain bioactive compounds in a simple form, but is considered less efficient in terms of time. On the other hand, long drying at room temperature also has an impact on reducing the levels of bioactive compounds in simplicia. Drying with direct sunlight also provides advantages in terms of production costs in a shorter time compared to air drying techniques, however direct sunlight can degrade the phytochemical compounds contained therein (Utami, Mubarak, et al., 2023). Indirect sun drying uses a cloth cover which functions as a protector from UV rays and can block direct sunlight from entering the sample. Therefore, good drying techniques are needed to obtain *C. odorata* L. leaf extract with high levels of antioxidant activity. Drying is carried out to obtain a water content below 10% with the aim of preventing the growth of bacteria and fungi during the storage stage (Utami, Imrawati, et al., 2024).

The antioxidant test method used in this research was the ABTS (2,2-Azinobis 3-ethyl benzothiazoline 6- sulfonic acid) free radical soaking method. This method has advantages such as specific absorbance at visible wavelengths and faster reaction times. In addition, ABTS can be dissolved in organic solvents or water so it can detect compounds that are both lipophilic and hydrophilic. (Utami, Yulianty, et al., 2024).

Based on the description above, research was carried out which aimed to determine the effect of drying simplicia on the antioxidant activity of tacklen leaves using three drying methods, including oven drying at 50°C, direct sunlight and air drying.

Methodology

Types of research

The research carried out was laboratory scale experimental research. This research was carried out at the Pharmaceutical Biology Laboratory, the Research Laboratory of the Faculty of Health Sciences, Almarisah Madani University and the Phytochemical Laboratory, Hasanuddin University, Makassar.

Materials

The tools used in this research were aluminum foil, stirring rod, maceration vessel, porcelain cup, funnel, beaker (Iwaki®), scissors, filter paper, measuring flask (Iwaki®), micropipette (dragonLab®), balance analytical (Mettler toledo®), oven, dropper pipette, capillary tube, horn spoon, UV-Vis spectrophotometer (T60®), jar and vial. The materials used in this research were *C. odorata* L. leaf, distilled water, aluminum chloride, dragendroff, ethanol 96%, absolute ethanol (Brand Germany®), ethyl acetate, $FeCl_3$ 5%, concentrated HCl, potassium persulfate, quercetin, chloroform, Liebermann-Burchard, methanol, n-hexane, TLC plate (KGaA®), ABTS powder (Sigma®) and toluene.

Sample processing

The obtained *C. odorata* L. leaves are sorted wet, washed thoroughly with running water, drained. *C. odorata* L. leaves are chopped and dried using 3 variations of drying techniques, samples are dried using direct sunlight by spreading the samples on sacks, drying is carried out for 2 days from 9-16, occasionally turning them back and forth then lifting them

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and on the following day it is done in the same way. the same, the samples are spread on a cloth covered with sacking then air dried in a room with the lights on for 144 hours or 6 days, and the samples are dried using an oven done by gutting the oven covered with paper then spreading the samples on top, oven drying using temperature 50°C for 8 hours. After all the samples are dry, then put them in plastic and tie them tightly. The dried samples were then sorted dry and weighed. Dried simplicia is powdered using a blender and sifted using a sieve then stored in a glass container (Utami dkk. 2023).

Determination of Water Content

Determination of water content of simplicia is done by distillation of toluene. In this case, the toluene used is saturated first. Then 5 grams of each simplicia was put into a round bottom flask and saturated toluene was added. The round bottom flask containing the simplicia is then heated until the toluene boils. After the toluene boils, the distillation speed is set at 2 drops/second, then the distillation speed is increased to 4 drops/second, after all the water has been distilled, heating continues for 5 minutes. Allow the cooling receiver tube to cool to room temperature. The volume of water is read after the water and toluene have completely separated (Utami, Imrawati, et al., 2024).

$$\% \text{ warter content} = \frac{volume\ of\ distilled\ water\ (ml) x\ BJ\ water}{sample\ weight\ (g)} \times 100\%$$

Sample Extraction

Extract preparation was carried out by maceration with 96% ethanol solvent, 300 g of simplicia powder was put into a glass container, poured with ± 1000 mL of 96% ethanol, covered and left for 3 x 24 hours, protected from light and stirred occasionally. After that, the mixture is filtered until the filtrate and residue are obtained. Then remaceration was carried out on the residue with ± 500 mL of 96% ethanol, covered and left for 1 day, then filtered into a Pirex and evaporated until a thick extract was obtained. The thick extract obtained was weighed and the yield was calculated using the equation (Maryam et al., 2023):

$$\% \text{ yield} = \frac{Extract\ Weight}{Sample\ Weight} \text{ x } 100\%$$

Phytochemical screening using TLC

Screening was carried out using F_{254} silica gel plates. The TLC plate was made with dimensions of 1 cm x 7 cm using a pencil (bottom of the plate 1 cm and top 0.5 cm). The F254 silica gel TLC plate was activated first using an oven at 110°C for 30 minutes to remove moisture from water adsorbed on the plate. The eluent mixture is put into the chamber then closed tightly and the saturation process is carried out using filter paper as a benchmark. This saturation is carried out to equalize the vapor pressure of the eluent so that the separation can run well (Imrawati dkk. 2023).

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C. odorata L. leaf ethanol extract from various drying techniques was taken 25 mg and dissolved in ethanol. Then the extract was spotted at a distance of 1 cm from the bottom edge of the plate with a capillary tube. The extract that has been spotted on the TLC plate is then eluted with an eluent. The TLC plate is inserted into a chamber containing saturated eluent, then the chamber is closed tightly until the eluent reaches the top line distance. Then the plate is removed, dried and sprayed with reagent. The results of the stain appearance can be seen using a 366 nm UV lamp (Imrawati dkk. 2023).

This test was carried out on several compounds, among others (Mus, Suwahyuni dkk. 2023) :

1. Alkaloids

Weighed 25 mg of *C. odorata* L. leaf extract then dissolved it in 95% ethanol, the solution was spotted on a TLC plate using a capillary tube and eluted in the mobile phase chloroform: methanol (9:1). After that the plate is dried and sprayed with Dragendroff's reagent. The sample is said to be positive for alkaloids if it appears orange/brown in a 366 nm UV lamp.

2. Flavonoids

Weighed 25 mg of *C. odorata* L. leaf extract, then dissolved in 95% ethanol, the solution was spotted on a TLC plate using a capillary tube and eluted in the mobile phase n-hexane: ethyl acetate (3:7). After that, the plate was dried and sprayed with aluminum chloride reagent. The sample was said to be positive for flavonoids if the stain fluoresced yellow under a 366 nm UV lamp.

3. Tannin

Weighed 25 mg of *C. odorata* L. leaf extract then dissolved in 95% ethanol, the solution was spotted on a TLC plate using a capillary tube and eluted in the mobile phase methanol: water (6:4). After that, the plate was dried and sprayed with 5% FeCl3 reagent. The sample was said to be positive for tannin if a black stain appeared in the 366 nm UV lamp.

4. Steroid

Weighed 25 mg of *C. odorata* L. leaf extract then dissolved in 95% ethanol, the solution was spotted on a TLC plate using a capillary tube and eluted in the mobile phase chloroform: methanol (9:1). After that, the plate was dried and sprayed with Liebermann-Buchard reagent, the plate was heated first and then observed under a 366 nm UV lamp. The sample was said to be positive for steroids if a blue-green stain appeared.

Antioxidant Activity Test

Preparation of ABTS Solution

The ABTS solution was made by weighing 7.1 grams of ABTS and 3.5 grams of K2S2O8, then dissolving each in 5 mL of distilled water, then mixing and incubating for 14 hours, then increasing the volume with absolute ethanol to 25 mL in a volumetric flask (Utami, Yulianty, et al., 2024).

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Blank Absorption Measurement

The test was carried out by pipetting 1 mL of ABTS solution to an adequate volume of 5 mL with absolute ethanol in a volumetric flask. The solution was homogenized and left for 30 minutes, then measured using a UV-Vis spectrophotometer at a wavelength of 667.8 nm (Utami, Yulianty, et al., 2024).

Measurement of Antioxidant Activity of Ethanol Extract of *Choromolaena odorata* L. Leaves

A stock solution of 1000 ppm was prepared by weighing 50 mg of ethanol extract of *C. odorata* L. leaves and dissolving it with 50 mL of absolute ethanol, the final volume was determined. The stock solutions were pipetted at 100 µl, 150 µl, 200 µl, 250 µl and 300 µl respectively. then 1 mL of ABTS was added and the volume was increased to 5 mL with absolute ethanol to obtain concentrations of 20 ppm, 30 ppm, 40 ppm, 50 ppm and 60 ppm. The mixture was then homogenized after 30 minutes, absorbance was measured at a wavelength of 667.8 nm (Imrawati, Utami, & Hardianto, 2023).

Antioxidant Measurement of Quercetin Comparative Solution

A stock solution of 100 ppm was prepared by weighing 10 mg of quercetin and dissolving it with 100 mL of absolute ethanol, the final volume was determined. The stock solutions were pipetted into 50 µl, 100 µl, 150 µl, 200 µl and 250 µl respectively. then 1 mL of ABTS was added and the volume was increased to 5 mL with absolute ethanol to obtain concentrations of 1 ppm, 2 ppm, 3 ppm, 4 ppm and 5 ppm. The mixture was then homogenized after 30 minutes, absorbance was measured at a wavelength of 667.8 nm.

Data Analysis

The sample absorbance data is used to find the % inhibition. The formula for finding % inhibition is as follows (Imrawati, Utami, & Insani, 2023):

$$\text{percent inhibition} = \frac{Abs\ Blank - Abs\ Sample}{Absorbance\ Blank} \times 100\%$$

Description :
Abs blank : absorbance on ABTS without sample
Abs sample : absorbance on ABTS after adding the sample
The calculation results will be entered into a linear equation.

The resulting linear equation is to obtain the IC_{50} value. The IC_{50} value is the concentration obtained when the % inhibition is 50 from the equation y = ax + b when the % inhibition = 50, so the formula for calculating the IC50 value is the equation: $x = \frac{50-b}{a}$

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RESULTS AND DISCUSSION

This research was carried out in several stages, namely sample preparation, simple organoleptic test, determination of water content, extraction using the maceration method, identification of compounds using the TLC method and the extract results were tested for antioxidant activity using the ABTS method. The samples used in this research were *C. odorata* L. leaves which were dried using 3 variations of drying techniques, namely, air drying, oven and direct sunlight.

The organoleptic test aims to determine the organoleptic characteristics of simplicia using the five senses in describing shape, color, smell and taste. The results of the simplicia organoleptic test based on the drying technique can be seen in table 1 below:

Tabel 1. Organoleptic Test Results of *Choromolaena odorata* L. Leaves

Technique Drying	**Organoleptic**		
	Shape	**Form**	**Warna**
Oven 50°C	Dried leaves	Typical	Brown
Direct sunlight	Dried leaves	Typical	Brown
Air dried	Dried leaves	Typical	Dark green

It is very necessary to determine the water content, because the water content can affect the quality of the simplicia. Water content greatly influences the quality of simplicia, because the quality requirement for the water content of a simplicia is <10%. Azeotropic distillation is distillation by evaporating a liquid without changing the composition. So there is a difference in composition between the liquid phase and the vapor phase, and this is the main requirement so that separation by distillation can be carried out. If the composition of the vapor phase is the same as the composition of the liquid phase, then separation by distillation cannot be carried out (Nadliroh & Fauzi, 2021). The principle of determining the water content of the azeotropic distillation method is the evaporation of water from materials together with an immiscible solvent in a fixed ratio. The material water vapor and solvent vapor are condensed and collected in a distillate flask. The amount of water resulting from the distillation of the ingredients can be directly determined by reading the meniscus on the distillation flask (Utami et al., 2022). A comparison of the water content of *C. odorata* L. leaves based on variations in drying and drying time can be seen in table 2 below:

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Tabel 2. Results of Water Content Measurement of *Choromolaena odorata* L. Leaves

Technique Drying	Drying Time	Water content
Oven 50°C	8 hours	7,52 %
Direct sunlight	14 hours	8,12 %
Air dried	144 hours	9,31 %

Based on Table 2. The results of measuring the lowest water content of *C. odorata* L. leaf simplicia were drying using a 50°C oven, namely 7.52%, while the highest were drying using indirect sunlight and air drying at 9.31%. Based on the results obtained, it can be concluded that the higher the temperature used, the longer it takes to dry. The higher the drying temperature, the faster the transition process. This is shown in oven drying where the temperature used is higher so it affects the water in the sample and the shorter the time needed to get the water content to the lowest (Utami, Mubarak, et al., 2023). The results of water content measurements obtained on simplicia from drying variations are in accordance with the quality requirements, namely <10%, if the water content is higher than 10% it will result in enzymatic processes and damage by microbes (Widarta & Sri Wiadnyani, 2019).

After testing the water content, the tacklean leaf simplicia was extracted using the maceration method using ethanol solvent. Ethanol is used as a solvent because it is a universal solvent, this solvent can dissolve almost all organic compounds in the sample, both polar and non-polar compounds (Hakim & Saputri, 2020). Extract yield calculations are carried out to determine the ratio of the amount of extract obtained from a material to the initial weight of the simplicia and to determine the number of bioactive compounds contained in the extracted material. (Utami, 2020). The principle of the maceration method is that the filter fluid will penetrate the cell wall, the active substance will be dissolved due to the difference in concentration between the active substance solution inside the cell and outside the cell, so that the solution with a high concentration will be pushed out of the cell. From the results of the maceration of the *C. odorata* L. leaf simplicia using 96% ethanol solvent, the yield results for each sample were obtained as follows:

Table 3. Yield of Ethanol Extract of *Choromolaena odorata* L Leaves

Technique Drying	Extract Weight (g)	Sample Weight (g)	Yield (%)
Oven 50°C	27	300	9
Direct sunlight	25,3	300	8,4
Air dried	31	300	10,3

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In table 3, the highest yield from *C. odorata* L. leaf extract was found in air-dried simplicia, namely 10.3%. The results of variations in drying techniques and drying time influence the yield of *C. odorata* L. leaves. During the drying process the decrease in yield is directly proportional to the high temperature and drying time used. According to Yuniarti et al. (2020), the higher the drying temperature causes the water content of the sample to decrease. As the water content evaporates, the resulting yield level also decreases. Yuniarti's statement is supported by Utami, et al., (2023), that the drying process causes the water content during the processing process to decrease, resulting in a decrease in yield. The yield value obtained ranged from 8.4% -10.3%. The differences in yield percentage may be due to plant age, storage conditions and time.

Followed by testing the identification of compound content using the TLC method of each extract. The results obtained from the *C. odorata* L. leaf extract were positive for containing alkaloids, flavonoids, tannins and steroids. This is in accordance with the results obtained in previous research conducted by Utami et.al., (2023) *C. odorata* L. leaves contain alkaloids, flavonoids, tannins and steroids.

The identification process using TLC aims to see the separation of the sample in the form of a typical chromatogram pattern in the extract based on the difference in polarity between the sample and the solvent and provide an initial picture of the chemical content based on the chromatogram pattern (Lestari & Santoso, 2021). The principles of TLC are adsorption, desorption and elution. Adsorption occurs when the sample solution is applied to the stationary phase, the components in the sample will be adsorbed in the stationary phase. Desorption is an event when components adsorbed in the stationary phase are forced by the eluent, elution occurs when the components are carried away by the eluent (Husna & Mita, 2020).

Table 4. Phytochemical Screening Results Using the TLC Method

Compound Classes	Reagent	Technique Drying			Stain Color
		OV	DS	AD	
Alkaloids	Dragendroff	+	+	+	Orange
Flavonoids	$AlCl_3$	+	+	+	Fluorescent Yellow
Steroids	Lieberman-Buchard+ warmup	+	+	+	Green Blue
Tannin	$FeCl_3$ 5%	+	+	+	Black

Descripsion : OV (Oven), DS (Direct Sunlight), dan AD (Air dried)

The results of the identification of compounds in TLC can be seen in table 5. The results show the presence of alkaloid compounds in the chromatogram pattern which is marked by the presence of orange colored spots when sprayed with dragendrof using the mobile phase Chloroform: methanol (9:1). The identification of flavonoids in the chromatogram pattern is marked by yellow spots after being sprayed with $AlCl_3$ using the

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mobile phase n-hexane: ethyl acetate (3:7), the results of identifying tannin compounds using the TLC test using the mobile phase methanol: water (6: 4) are marked with black spots after being sprayed with 5% $FeCl_3$ and then identify steroid compounds using the mobile phase chloroform: methanol (9: 1) marked with blue-green spots after spraying with Lieberman-Buchard reagent.

Testing of the antioxidant activity of the three samples of ethanol extract of *C. odorata* L. leaves was carried out quantitatively on ABTS by calculating the % inhibition. Based on the results of the analysis, variations in drying techniques have an effect on the antioxidant activity of tacklen leaves. The antioxidant activity of *C. odorata* L. leaves can be seen in Table 5.

Table 5. Data on Antioxidant Measurements of 96% Ethanol Extract from Varying Drying Techniques

Technique Drying	Concentration	% Inhibision	IC_{50} Value	Category
Oven 50°C	20 µg/mL	45,609	23,287 µg/mL	Very strong
	30 µg/mL	55,041		
	40 µg/mL	72,267		
	50 µg/mL	81,634		
	60 µg/mL	87,239		
Direct Sunlight	20 µg/mL	41,871	24,344 µg/mL	Very strong
	30 µg/mL	55,701		
	40 µg/mL	72,832		
	50 µg/mL	79,433		
	60 µg/mL	82,770		
Air-Dried	20 µg/mL	40,351	29,064 µg/mL	Very strong
	30 µg/mL	50,455		
	40 µg/mL	63,237		
	50 µg/mL	69,807		
	60 µg/mL	70,011		

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Graph 2 shows that the drying method has an influence on the antioxidant activity of *C. odorata* L. leaves. The highest antioxidant activity value of *C. odorata* L. leaves was found in the 50°C oven drying technique, namely 23,289 µg/mL, while the smallest antioxidant activity value of *C. odorata* L. leaves was found in the air-drying technique, namely 29,344 µg/mL, which indicates that the antioxidant activity is very strong. The smaller the IC50 value, the higher the antioxidant activity (Duenngai et al., 2024) . According to Sukardi.et.al., (2021), stated that the factors that influence the value of antioxidant activity are caused by the nature of antioxidants which are susceptible to temperature, oxygen, pH, peroxide and light. Dharma. el.al., (2020) states that temperature and drying time affect antioxidant activity because these conditions can damage the active substances contained in a material. The antioxidant activity value of the results obtained is inversely proportional to the statement Utami, Yulianty, dkk. (2024), This may be caused by differences in active components in plants, synergistic effects or antagonistic effects between the active components contained, research conditions and methods used which can influence antioxidant activity in plants.

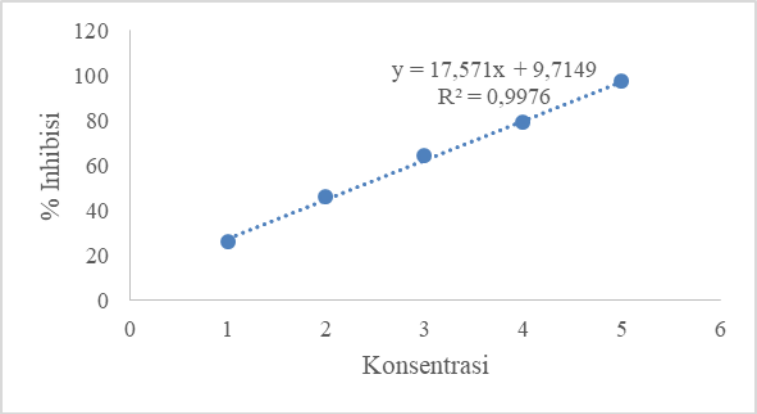

Figure 1. Quarcetin Line Equation Curve as a Comparison

Table 6. Data from Measurement Results of the Antioxidant Quercetin

Sample	Concentration	% Inhibisi	IC_{50} Value	Category
Quercetin	1 µg/mL	25,826 %	2,292 µg/mL	Very Strong
	2 µg/mL	45,955 %		
	3 µg/mL	64,173 %		
	4 µg/mL	79,048 %		
	5 µg/mL	97,133 %		

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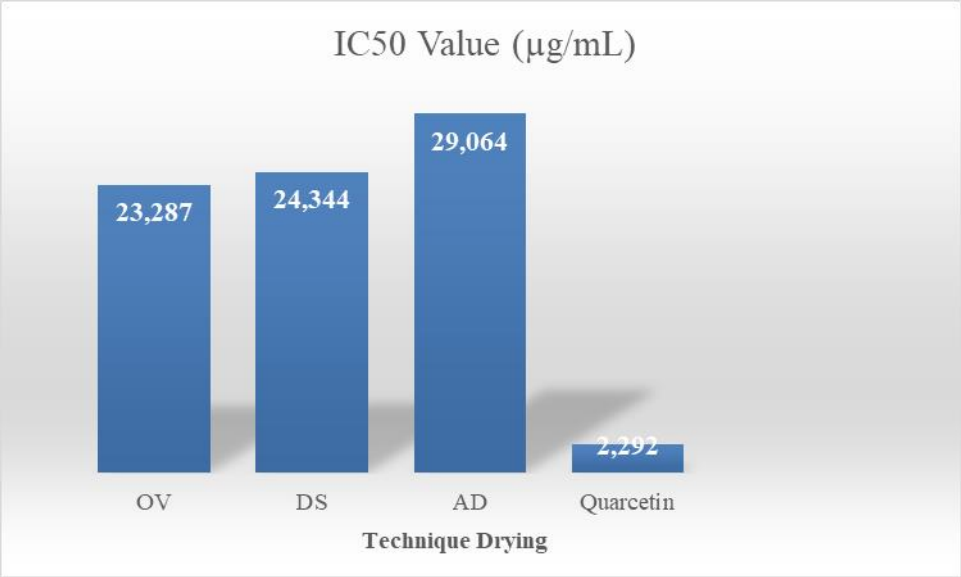

Figure 2. Graph of Antioxidant Activity of 96% Ethanol Extract of *C. odorata* L. Leaves with Varying Drying Techniques and Quarcetin Comparative

In Table 5. Based on the results of research conducted on the antioxidant activity of quercetin with a concentration of 1-5 µg/mL, the IC_{50} value was 2.292 µg/mL. This shows that the antioxidant activity of quercetin is included in the very strong category (IC_{50} < 50%). The reason for using quercetin as a comparison is because quercetin is a flavonol from a group of flavonoid and polyphenolic compounds found in almost every type of plant and standard quercetin is a natural antioxidant that has very strong antioxidant activity (Maryam et al., 2023). The yield of quercetin was 10 times smaller than all drying method treatments. This is because the extract obtained from simplicia is still a mixture of various compounds, while quercetin is a pure compound which has very strong antioxidant activity.

CONCLUSIONS

Based on the results of the research that has been carried out, it can be concluded that the optimization of the antioxidant activity of 96% ethanol extract of tacklen leaves with the IC_{50} value for each sample is as follows: samples dried in a 50°C oven were 23,289 µg/mL, samples dried in the air were 29,344 µg/mL and the direct sunlight sample was 24,344 µg/mL, which indicates that all IC_{50} values obtained indicate very strong antioxidant power.

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