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ANTIOXIDANT ACTIVITY TEST OF AFRICAN LEAVES PURIFICATION EXTRACT (*Vernonia amygdalina* Del) WITH DPPH METHOD

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ABSTRACT
African leaves (*Vernonia amygdalina* Del) is a plant that can be used as a medicinal plant because it contains flavonoid compounds that are effective as natural antioxidants that can suppress free radicals. The purpose of this study was to determine the antioxidant activity of the purified extract of *Vernonia amygdalina* Del. Antioxidant activity test of purified extract of *Vernonia amygdalina* Del leaves against *1,1-diphenyl-2-picrylhydrazil* (DPPH) was measured with a UV-Vis spectrophotometer at a wavelength of 516nm using Vitamin C as a comparison. The results of the non-specific parameters of *Vernonia amygdalina* Del leaf Simplicia were drying shrinkage of 0.79±0.02%; water content of 7.33±2.08%; water-soluble essence content of 20.5±1.73 %; and ethanol of 14.5±4.35 %. The results of purified extract screening of *Vernonia amygdalina* Del leaves, contained chemical compounds, namely the class of flavonoid compounds, tannin compounds, steroid compounds, and saponin compounds. Testing for flavonoids using the Thin Layer Chromatography method on the purified extract contained two spots with Rf values of 0.68 and 0.5 while in quercetin there was one spot with an Rf value of 0.68, thus the purified extract of *Vernonia amygdalina* Del leaves contained flavonoids. *Vernonia amygdalina* Del leaf purified extract has a very strong antioxidant activity with an IC50 value of 13.54±0.10 µg/mL. The results showed that the purified extract of *Vernonia amygdalina* Del leaves had very strong antioxidant activity.

Keywords: Antioxidants; African leaves (*Vernonia amygdalina* Del); purified extracts; Flavonoids

1. INTRODUCTION

Antioxidants are compounds that can neutralize free radicals and protect the body from various kinds of degenerative diseases such as diabetes, hypertension, cholesterol, gout, inflammation, elimination of toxins in the body, rheumatism, insomnia, pins, and needles, and so on (Ashok *et al*., 2022). Antioxidants can reduce oxidative stress by absorbing free radicals thereby preventing oxidative reactions. Sources of natural and synthetic antioxidants can maintain the balance of oxidative reactions in biological systems so that they can become an important aspect of cellular defense mechanisms against oxidative stress (Baliyan *et al*., 2022).

Medicinal plants that can be used in traditional medicine which are efficacious for counteracting free radicals, namely *Vernonia amygdalina* Del leaves which have pharmacological effects to cure various diseases including diabetes, hypertension, cholesterol, gout, and inflammation. Based on research from the ethnobotanical survey of western Nigeria, *Vernonia amygdalina* Del leaves are used in the treatment of measlesand have extraordinary bioactivity against diseases associated with oxidative stress (Ugbaja et al., 2021).

The active compounds found in *Vernonia amygdalina* Del leaves include flavonoid compounds, glycoside compounds, steroid/triterpenoid compounds, saponin compounds, and tannin compounds. The content of chemical compounds that have the potential as antioxidants is phenolic compounds such as flavonoid compounds and polyphenolic compounds or tannin compounds (Alara *et al*., 2017).

Based on the research by Erukainure et al, *Vernonia amygdalina* Del leaf infusion has antioxidant activity (Erukainure et al., 2019). Previous research on the methanol extract of *Vernonia amygdalina* Del stem bark had mild antioxidants (IfedibaluChukwu et al., 2020) and based on research conducted by Oriakhi stated that the ethanol extract of *Vernonia amygdalina* Del leaves had an antioxidant activity with an IC50 of 111.8 µg/mL (Oriakhi et al., 2013).

Based on previous studies, this study will test the antioxidant activity of purified extracts of *Vernonia amygdalina* Del leaves. In this research, the extract will be purified. Purified extracts are a way to ensure that the components of purely natural ingredients do not contain other chemical components that are not needed. In this way, it maintains some of the chemical content of the extract which has a synergistic effect so that it can maximize the treatment process (Malik et al., 2013).

One of the tests that are often used is by immersing the radical compound DPPH (*1,1-diphenyl-2-picrylhydrazil*) which is a free radical that is stable at room temperature and is often used to evaluate the antioxidant activity of several compounds or natural extracts (Irivibulkovit et al., 2018). Based on this, a study was conducted to test the antioxidant activity of purified extracts of *Vernonia amygdalina* Del leaves using the DPPH (*1,1-diphenyl-2-picrylhydrazil*) free radical entrapment method.

2. METHODS

2.1. Material

The tools used were a UV-Vis Spectrophotometer (Shimadzu, UVmini-1240); glassware; Analytical balances (OHAUS); Oven (memmert). The materials used were *Vernonia amygdalina* Del Leaves (Cirebon, Indonesia); Methanol p.a(Merck); Vitamin C (Global Science Primary); DPPH (*1,1-diphenyl-2-picrylhydrazil*) (Sigma-Aldrich); 96% ethanol (Brataco); quercetin (Sigma-Aldrich); *Moisture Analysis Balance* (Ohaus).

2.2. Sample Preparation

Fresh *Vernonia amygdalina* Del leaves were weighed as much as 1.5 kg, washed under running water, drained, and dried in an oven at 40 ºC for 24 hours (Wulandari et al., 2022). The simplicia obtained was then tested for simplicia characteristics.

2.3. Simplicia Characterization Test

2.3.1. Microscopic and Macroscopic Tests

Macroscopic and microscopic examination of dried simplicia *Vernonia amygdalina* Del leaves was carried out by examining the shape, size, color, smell, and taste with the five senses and carried out by observing the simplicia fragment identification in cell form using aquadest on a microscope.

2.3.2. Dry Shrinkage

The drying shrinkage parameter is carried out by weighing 1 gram of simplicia in a shallow weighing bottle with a lid that has been heated and tares previously put in the drying chamber, then opening the lid, and drying it at 105ºC until the weight remains constant. Before drying, the closed bottles are cooled in a desiccator to room temperature (Kementerian Kesehatan Republik Indonesia, 2017).

2.3.3. Water Content

Examination of the parameters for water content is carried out by weighing 1 gram of simplicia powder into the *Moisture Analysis Balance* tool. Record the water content formed (Islamiarti, 2021).

2.3.4. Determination of Water-Soluble Levels

The parameters for this test were carried out by weighing 5 grams of simplicia powder, then putting it into a clogged flask, adding 100mL of chloroform saturated water (1mL of chloroform in distilled water up to 400mL), shaking several times for the first 6 hours, leave for 18 hours. Then do the filtering and 20mL of the filtrate is evaporated to dryness in a shallow bottomed cup which is heated to 105 ºC and tare, heat the remainder at 105 ºC. Calculate in % water soluble essence (Kementerian Kesehatan Republik Indonesia, 2017).

2.3.5. Determination of Ethanol Soluble Levels

The parameter of the ethanol-soluble extract was carried out by weighing 5 grams of simplicia powder, then 100 mL of ethanol (95%) was added to the corked flask and shaken for six hours, then left for eighteen hours. Filtered, 20mL of the filtrate was taken and evaporated. The remaining filtrate was evaporated and then heated at 105 ºC. Calculate the levels in % of ethanol-soluble essence (Kementerian Kesehatan Republik Indonesia, 2017).

2.4. Extraction

The extract was prepared by maceration using 96% ethanol for 5 days. for 5x24 hours protected from light while frequently stirring, then wiped, and squeezed after that wash the dregs with a liquid solvent. The filtrate obtained was concentrated using a *rotary evaporator* to obtain a thick extract. The *Vernonia amygdalina* Del leaf extract was then purified with n-hexane and water (1:1) in a separating funnel, shaken and left to form two layers, then evaporated to obtain a purified *Vernonia amygdalina* Del leaf extract (Iryani et al., 2021).

2.5. Phytochemical Analysis

In the Alkaloid Test, as much as 0.5 grams of the extract was added to 1mL of 2N HCL and 9 mL of aquadest then heated for two minutes, cooled, and filtered, took 3 drops of each test tube added reagent Mayer, Bouchard, and dragendrof (Ladeska & Dingga, 2019).

In the Flavonoid Test, 2 mL of extract was added with a little magnesium powder and 2 mL of HCl 2 N (Wahid & Safwan, 2020). In the Tannin Test, weighing 0.5 grams of the extract was added to 10mL of distilled water, then filtered and diluted with distilled water. Take 2mL and add 1-2 drops of 1% ferric III chloride.

In the Triterpenoid/Steroid Test by weighing the extract plus 20mL of n-hexane then it was macerated for two hours then filtered, and evaporated and the remainder was added with two drops of anhydrous acetic acid and 1 drop of H2SO4p.

In the Saponin Test, weigh the extract and then add 10 ml of hot water, shake and add 1 drop of 2N HCl (Putra et al., 2018).

2.6. Identification of Flavonoids by Thin Layer Chromatography Method

Flavonoid content test of mobile phase n-butanol: acetic acid: water (4:1:5) and silica gel GF254 stationary phase. Samples of purified *Vernonia amygdalina* Del leaf extract and quercetin standard solution in silica gel GF254 stationary phase were spotted on the Thin Layer Chromatography plate and then put into a chamber that had been saturated with filter paper which already contained a mixed mobile phase of n-butanol: acetic acid: water (4:1:5), close the chamber let the mobile phase travel upwards to the expansion limit. Take the Thin Layer Chromatography plate, let it dry in air, and observe the spots under 254 nm UV light. Then spray with iron (III) chloride then calculate the value of the Rf or hRf number.

2.7. Antioxidant Activity

In the antioxidant activity test using DPPH. 2mg DPPH was weighed and dissolved in methanol p.a to obtain a concentration of 80µg/mL. Pipette 2 ml of the solution, then add 1 ml of methanol and measure the absorbance at a wavelength of 400–800 nm. The vitamin C test solution and the *Vernonia amygdalina* Del leaf extract test solution was prepared by weighing 5 mg of vitamin C and the purified *Vernonia amygdalina* Del leaf extract and dissolved using methanol p.a to a concentration of 100 µg/mL then pipetting 0.1;0.2;0.3;0.4 mL and 0.5mL into a 5mL flask and add 2mL of DPPH and methanol p.a up to the mark. This solution was then incubated for 10 minutes and its absorbance was measured at the max DPPH wavelength (Iryani et al., 2021).

2.8. Data Analysis

Data analysis in this study was in the form of descriptive with simplicia characteristic tests described then conclusions were made. Antioxidant activity was tested using the DPPH (*1,1-diphenyl-2-picrylhydrazil*) method, wherein the DPPH method the sample was reacted with a DPPH radical solution. The sample absorbance was measured using UV-Vis spectrophotometry with methanol as a blank solution. Calculated based on the percent attenuation.

$$\%Inhibition = \frac{Blank\ absorbance - Sample\ absorbance}{Blank\ absorbance} \times 100\% \quad (1)$$

After obtaining the percentage of inhibition from each concentration, it is followed by calculations by linear regression using the equation y=bx+a. Antioxidant activity is expressed by determining the value (50% Inhibitory Concentration) or IC50 (Sulastri et al., 2021).

3. RESULTS AND DISCUSSION

Results of macroscopic examination of fresh *Vernonia amygdalina* Del leaves were variable, erect, often branched from the base, low-stemmed scattered leaves, leaf blades ovate, oval, elongated lancet or line shape, rough serrated edges, smooth hairy surface, brown green, has a characteristic odor, and tastes bitter. Microscopic examination of *Vernonia amygdalina* Del leaf simplicia showed transport bundles with mesh-type thickening, parenchyma with rosette-shaped calcium oxalate crystals, covering hairs, sclerenchyma, lower epidermis with stomata, and leaf mesophyll and scale hairs (**Figure 1**).

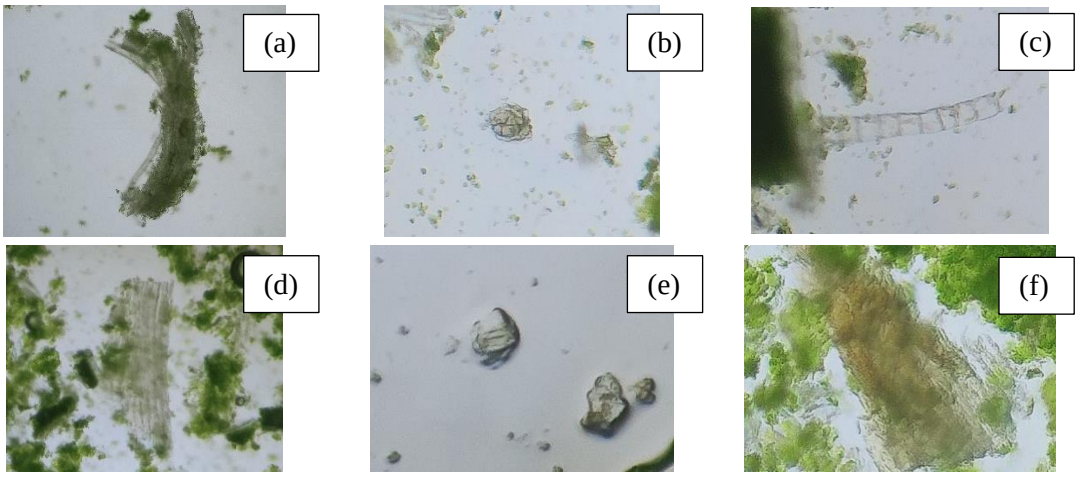

Figure 1. Microscopic Examination Results of *Vernonia amygdalina* Del Leaves. (a) Transport tufts with mesh-type thickenings, (b) Parenchyma with rosette-shaped calcium oxalate crystals, (c) Images of covering hairs, (d) Sclerenchyma, (e) Lower epidermis with stomata, (f) Mefossils of leaves and scale hairs

The non-specific parameter of drying shrinkage is carried out to provide an illustration of the maximum limit of compounds that are lost or compounds that evaporate during the drying process of simplicia. The drying shrinkage parameters for *Vernonia amygdalina* Del leaf simplicia were 0.79 ± 0.02% (**Table 1**). According to the Indonesian Ministry of Health, (2017) the requirement for drying shrinkage of *Vernonia amygdalina* Del leaf simplicia is no more than 10% due to a reduction in the number of substances contained during the drying process such as water, compounds that dissolve in other solvents, essential oils (Kementerian Kesehatan Republik Indonesia, 2017).

The non-specific parameter of water content is carried out to provide an overview of the minimum limit or range regarding the amount of water content in the material. The results of the water content parameter for *Vernonia amygdalina* Del leaf simplicia were obtained at 7.33 ± 2.08% (**Table 1**). According to the Indonesian Ministry of Health, the requirement of no more than 12.5% if it exceeds these requirements can be a good medium for microbial growth, the presence of fungi or insects, and encourages damage to occur in the hydrolysis process. That way the water content meets the specified conditions (Kementerian Kesehatan Republik Indonesia, 2017).

Determination of extract content was carried out using two solvents, namely water, and ethanol. Determination of the water-soluble essence content was carried out to determine the levels of polar chemical compounds contained in simplicia, while the ethanol-soluble extract levels were used to determine the levels of ethanol-soluble compounds, both polar and non-polar compounds. The parameter results for the water-soluble essence of *Vernonia amygdalina* Del leaf simplicia were 20.5 ± 1.73%, while the ethanol-soluble extract obtained was 14.5 ± 4.35 % (**Table 1**). Both meet the requirements, namely for a water-soluble essence content of not less than 18.8% and an ethanol-soluble essence content of not less than 11.8% (Kementerian Kesehatan Republik Indonesia, 2017). The content of the essence is soluble in water with yields and higher value requirements than the content of the soluble essence in ethanol because polar compounds are more soluble in water solvents than ethanol, and water-insoluble compounds will dissolve in ethanol solvents (Kementerian Kesehatan Republik Indonesia, 2017).

Table 1. Value of Specific Parameters and Non-Specific Parameters of Simplicia

Parameters	Mean ± SD (%)	Standard
Dry shrinkage	0.79 ± 0.02	≤ 10%
Water content	7.33 ± 2.08	≤ 12.5%
Water Soluble Levels	20.5 ± 1.73	≥ 18.8%
Ethanol Soluble Levels	14.5 ± 4.35	≥ 11.8%

From the results of testing the simplicia characteristics, it was found that the simplicia met the parameters of the simplicia quality standard. The research was continued by making extracts with the maceration method. The thick extract of *Vernonia amygdalina* Del leaves was then extracted using a mixture of distilled water and N-hexane in the same ratio. The purpose of adding the solvent is to remove impurities that are attracted during extraction such as fats and pigments that are not needed (Iryani et al., 2021). The results of the phytochemical screening test are listed in **Table 2**.

Table 2. Phytochemical screening results of purified extracts of Vernonia amygdalina Del

Secondary metabolite	Reagent	Results	Conclusion
Alkaloids	Mayer, Lieberman, Dragendroff	No precipitate Green Brick red precipitate	Negative
Flavonoids	Mg+HCl Pekat	Orange	Positive
Tannins	$FeCl_3$ 1%	Black Green	Positive
Triterpenoids/Steroids	Lieberman-Burchard	Bluish Green	Positive
Saponins	HCL 2N	Foaming	Positive

Based on the results of the phytochemical screening test of purified extracts of *Vernonia amygdalina* Del leaves, there were several metabolites, namely flavonoids, tannins, steroids, and saponins. In the alkaloid test of purified *Vernonia amygdalina* Del leaf extract using Mayer and Lieberman reagents, no white/yellow and brown/black precipitate was formed, only Dragendroff reagent which contained a brick red precipitate, the result was that the purified *Vernonia amygdalina* Del leaf extract did not contain alkaloids which are generally semi-polar, only bound by non-polar solvents such as n-hexane.

The results of the flavonoid test were identified by the formation of an orange color, this indicated that the purified extract of *Vernonia amygdalina* Del leaves positively contained flavonoids. Mg powder and concentrated HCl function to reduce the benzopiron nucleus contained in the flavonoid structure so that a color change to red or orange is formed (Jannah et al., 2017).

The results of the tannin test were identified by the formation of a green-black color, this indicated that the purified extract of *Vernonia amygdalina* Del leaves positively contained tannins. Tannin compounds are compounds that are polar because of the presence of OH groups, because when FeCl3 is added a color change will occur such as dark blue or green-black which indicates the presence of hydrolyzed tannins (Wahid & Safwan, 2020).

The results of the triterpenoid/ steroid test were identified by the formation of a bluish-green color, this indicated that the purified extract of *Vernonia amygdalina* Del leaves positively contained steroids. The addition of anhydrous acetic acid with concentrated sulfuric acid is based on the ability of steroid compounds to form a green-black color. Steroids are compounds that can be extracted with non-polar or semi-polar solvents (Nugrahani et al., 2016).

Saponin test results are known by the formation of foam in water. The saponin test was carried out by adding hot water, and shaking vigorously. and foam will form on a stable surface for 10 minutes. The foam formed indicates the presence of glycosides which can form foam in water. This shows that the purified extract of *Vernonia amygdalina* Del leaves positively contains saponins.

3.1. Identification of Flavonoids by Thin Layer Chromatography Method

Identification of the flavonoid content in the purified extract of *Vernonia amygdalina* Del leaves was carried out using the Thin Layer Chromatography (TLC) method. The stationary phase used is silica gel G which is polar and will be attached to an aluminum plate while the mobile phase used is n-butanol: acetic acid: water which is non polar, semi polar, and polar. The comparison of the three mobile phases used is 4:1:5. In the spotting process on the Thin Layer Chromatography plate, the sample to be stained was a purified extract of *Vernonia amygdalina* Del leaves which had been dissolved in 96% ethanol and quercetin as standard. Quercetin was chosen as the standard because it includes flavonol compounds, namely flavonoids with ketone groups (Panche et al., 2016). The silica gel Thin Layer Chromatography plate was activated in an oven at 110 °C for 30 minutes to remove the water present on the Thin Layer Chromatography plate. Furthermore, all samples were spotted on the Thin Layer Chromatography plate with a distance of 1 cm every 3 times, dried by aerating, and then eluted, if there are stains after elution, calculate the Rf value. There were 3 stains formed after elution, including 2 stains on purified extracts of *Vernonia amygdalina* Del leaves and 1 spot on quercetin as a reference standard. The Rf value of each stain spot can be calculated, the Rf of the purified extract of *Vernonia amygdalina* Del leaves obtained is 0.68 and 0.5 and the Rf of quercetin obtained is 0.68, which means that the purified extract of *Vernonia amygdalina* Del leaves contains quercetin flavonoids because the Rf value is the same as the quercetin reference standard. There are 2 spots with Rf values of 0.72 and 0.5 with a development distance of 8 cm on the Thin Layer Chromatography plate. The stains obtained in the elution process were then observed under 254nm UV light to obtain Rf values with the same results. Furthermore, spraying using FeCl3 reagent. This spraying is done with the

aim that the spots that are not visible on the UV lamp can be seen after spraying. If the sample is positive for flavonoids, a blue-black, the green-black stain will appear (Pyka, 2014). When the plate was sprayed with FeCl3, green-black spots appeared indicating that the sample contained flavonoid compounds with the same Rf value, thus the results of the study showed that the purified extract of *Vernonia amygdalina* Del leaves contained flavonoid compounds because the Rf value and spot stains were the same as quercetin. as a standard of comparison (**Figure 2**).

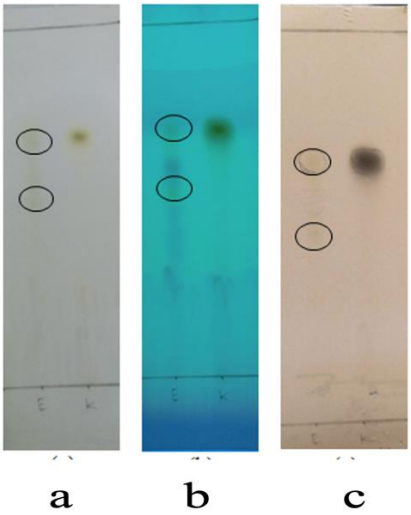

Figure 2. Thin Layer Chromatography Results of Purified Extract of *Vernonia Amygdalina* Del Leaves and Reference Standard. (a) Before UV irradiation, (b) after 254nm UV irradiation, (c) after spraying with FeCl3 reagent.

3.2. Antioxidant Activity

Antioxidant activity test on purified extract of *Vernonia amygdalina* Del leaves was carried out using the DPPH method with UV-Vis spectrophotometry. This method was chosen because it is simple, easy, fast, sensitive, and requires only a small sample. The comparator used in this method is vitamin C because it functions as a secondary antioxidant that has free hydroxy groups that act as free radical scavengers, and has very high antioxidant activity (Damanis et al., 2020). As an initial step, the maximum wavelength is determined first using a blank solution with a wavelength range of 400-800nm using a UV-Vis spectrophotometer. The measurement results show that the DPPH solution in methanol produces a wavelength of 516 nm with an absorbance value of 0.840. The maximum wavelength meets the requirements in the DPPH maximum wavelength range of 515-517 nm. Then the operating time is carried out to determine the time in which a reaction process takes place stably. Measurements use a time range of 0 to 50 minutes so that a stable time is obtained in the range of 0 to 10 minutes which will be used for the incubation time of purified extract samples of *Vernonia amygdalina* Del leaves and Vitamin C and the highest absorbance value obtained at 50 minutes with a value of 0.867 will be used for blank absorbance values. After that, measuring the antioxidant activity by looking for the absorbance value of the sample on purified *Vernonia amygdalina* Del leaf extract and vitamin C with a concentration of 2,4,6,8,10 ppm, there was a change in reaction during the process which was marked by a purple color change in DPPH to yellowish color change occurs because the conjugated double bond in DPPH will decrease. After all, the antioxidant compound has captured one electron (Baliyan et al., 2022). The results of measuring the antioxidant activity obtained were from the absorbance of the control and the absorbance of the sample which will later be used to determine the percentage of inhibition and IC50 which can be seen in **Table 3** with linear regression values in **Figure 3** and **Figure 4**.

Table 3. Value of Antioxidant Activity of Purified Extract of *Vernonia amygdalina* Del and Vitamin C

Extract	Concentration (ppm)	Mean±SD Inhibition (%)	Value IC_{50}±SD (µg/mL)
Vernonia amygdalina Del. purified extract	2	16.79 ± 0.06	13.54 ± 0.10
	4	20.57 ± 0.31	
	6	28.17 ± 0.06	
	8	32.48 ± 0.06	
	10	40.40 ± 0.11	
Vitamin C	2	15.22 ± 0.00	4.78 ± 0.03
	4	42.57 ± 0.06	
	6	68.28 ± 0.00	
	8	93.07 ± 0.00	
	10	94.07 ± 0.06	

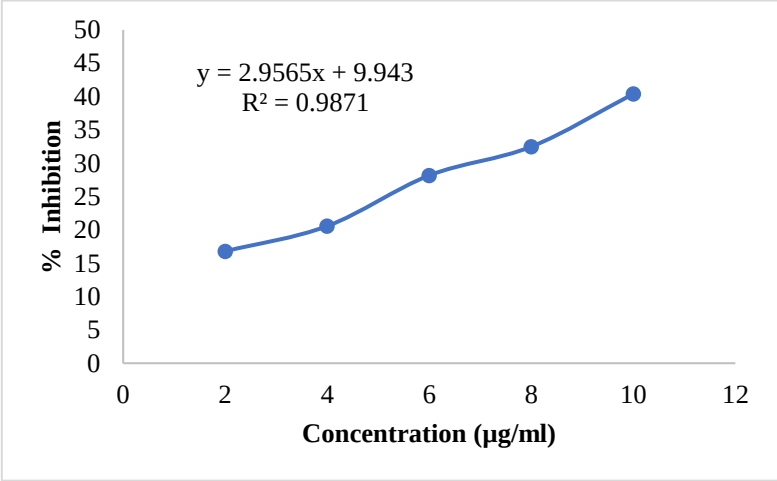

Figure 3. Linear Regression Results Of Purified Extract Of *Vernonia Amygdalina* Del Leaves With DPPH Method

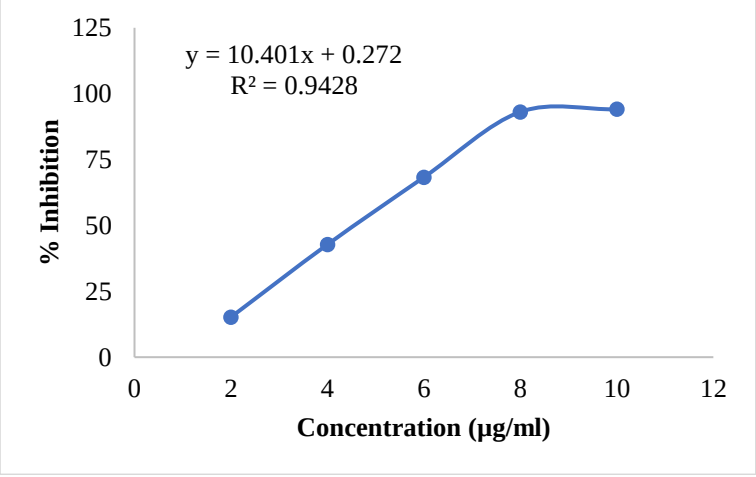

Figure 4. Linear Regression Results of Vitamin C with the DPPH Method

After measuring the absorbance for the sample and reference standard by replication 3 times, the absorbance value of each concentration and sample tested was obtained so that the percentage inhibition value could be determined, as seen in **Table 3** where the higher the concentration of purified *Vernonia amygdalina* Del leaf extract and vitamin C, the higher of inhibition, the percentage of inhibition will be even higher, which means that the higher the concentration with the amount of active antioxidant compounds, the ability to inhibit free radicals will increase, thus indicating a high percentage of inhibition at this optimum concentration. It can be seen that the IC50 in the purified extract of *Vernonia amygdalina* Del leaves are 13.54 ± 0.10 µg/mL which

can be categorized as having a very strong antioxidant activity as well as vitamin C as a comparison which has a definite IC50 value of 4.78 ± 0.03 µg/mL has very strong antioxidants. IC50 value <50µg/mL includes having a very strong antioxidant (MR & A, 2017). Compounds that have very strong antioxidants contain free radical scavenging compounds, namely flavonoids and other phenolic compounds. Flavonoids can convert free radicals into compounds that are stable and less reactive. Because these compounds contain hydroxyl groups, these compounds have antioxidant properties which act as free radical scavengers and reducing agents so that flavonoids act as hydrogen donors for free radicals. Antioxidants play a role in preventing tissue damage caused by free radicals by minimizing the formation of radicals, soaking, or increasing decomposition.

4. CONCLUSION

Based on the results of the study it can be concluded that in the antioxidant activity test the purified extract of *Vernonia amygdalina* Del leaves had an IC50 value of 13.54 ± 0.10 µg/mL and vitamin C showed an IC50 result of 4.78 ± 0.03 µg/mL. *Vernonia amygdalina* Del purified extract and vitamin C have very strong antioxidant activity. Further research is needed to determine the antioxidant activity of purified extract preparations of *Vernonia amygdalina* Del leaves.

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6. CONFLICT OF INTEREST

The author states that there is no conflict of interest in conducting this research.

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