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Original Article

ANTIOXIDANT ACTIVITY OF FLAVONOID RICH FRACTION OF (*VERNONIA AMYGDALINA* DELILE.) LEAVES

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ABSTRACT

Objective: Atoms that are unstable and harmful to other molecules are referred to as free radicals. Flavonoid molecules have the ability to act as antioxidants, which can help avoid oxidative stress brought on by the high levels of free radicals that are found in the body. Antioxidants are chemicals that bind to free radicals and neutralize oxidized substances by giving electrons. The main objective of this comprehensive investigation was to assess and quantify the extent of antioxidant activity manifested by fractions abundant in flavonoids extracted from *Vernonia amygdalina* Del.

Methods: Refluxing n-hexane solvent and then continuing with methanol were the steps that taken in the process of extracting *Vernonia amygdalina*. The methanol extract was subjected to fractionation utilizing the liquid-liquid fractionation method, employing a series of solvents to isolate distinct fractions with varying chemical compositions. After determining the overall flavonoid content of each extract and fraction, an evaluation was conducted on the antioxidant activity of the samples containing the maximum flavonoid content.

Results: The dichlormethane fraction had the highest flavonoid content with 69.56±0.22 mg/g QE. Then the DCM fraction was tested for antioxidant activity, and the results obtained for the DPPH method were 117.33±0.56 and the CUPRAC method was was 84.80±0.73.

Conclusion: The results showed that DCM fraction is the highest flavonoid fraction and exhibits potent antioxidant properties.

Keywords: African leaves, *Vernonia amygdalina* del., Antioxidant, Flavonoid rich fraction

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INTRODUCTION

Antioxidants, which are also known as free radical oxygen species, are substances that can be found in nature or manufactured chemically. They block or postpone the oxidation process, or they suppress the reaction that is caused by oxide or peroxide species. These species are responsible for causing damage to cells when reactive oxygen is present [1]. Reducing free radical damage can help treat degenerative disease like cancer. In order to eliminate free radicals and prevent then form impeding the body's metabolism, antioxidant chemicals can combat free radicals by giving unstable free radicals electrons [2]. Antioxidants are classified into two groups based on where they coma from: endogenous antioxidants, which are enzymes found within the body that have antoxidant qualities, like glutathione peroxidase (Gpx), catalase (Cat), and superoxide dismutase (SOD); and exogenous antioxidants, which come from food or sources outside the body [3].

Vernonia amygdalina (VA) is a member of the Asteraceae family. It has a vast distribution over Africa. The leaves of this plant are utilized in African traditional medicine. Numerous bioactive phytochemicals, such as flavonoids, phenolic acid, terpenes, coumarins, saponins, fatty acids, and sesquiterpene lactones, are present in the leaves of VA [4], and terpenoids that kill parasites that cause malaria, anti-amoebic malaria, anti-amoeba, anti-tumor, antimicrobial [5]. Multiple research have demonstrated the therapeutic potential of VA, including antioxidant, antibiotic, anticancer properties, nephroprotective, antihyperlipidemic, immunological effects, anthelmintic, anti-obesity [6, 7]. The quantity of hydroxy groups in phenolic and flavonoid components' molecular structures determines between their antioxidant activity in terms of reducing free radicals. The connection between phenols' and flavonoids' chemical structures and antioxidant activity [8].

Polyphenolic substances called flavonoids have a critical function in shielding plants from oxidative stress, bacteria, and UV radiation. By serving as reducing agents in a number of processes, flavonoids can exhibit antioxidant activity. The scavenging of Reactive Oxygen Species (ROS), inhibition of oxidases that produce superoxide anion, chelation of trace metals, and activation enzymes are some of the fundamental processes of flavonoids [9]. The direct way that flavonoids function as antioxidants is by giving hydrogen ions to counteract the harmful effects of free radicals. By activating nuclear factor erythroid 2 relatied factor 2 (Nrf2), flavonoids, as antioxidants, indirectly increase the expression of antioxidant genes by increasing the number of genes involved in the synthesis of endogenous antioxidant enxymes, such as the SOD gene [10]. Colorimetry is commonly employed to ascertain the Total Flavonoid Content (TFC) of plants subsequent to solvent extraction. The colorimetric assay using aluminium chloride is widely used to determine TFC in plant extracts. $AlCl_3$ is employed as a complexing agent [11].

The DPPH (*2,2-Diphenyl-1-picrylhydrazyl*) assay is a quick and uncomplicated approach to evaluate the ability of an antioxidant to remove scavenging chemicals. When DPPH is oxidized, it gives methanol a rich violet hue. When and antioxidant substance provides an electron to DPPH, it reduces and turns from a deep violet to a yellow tint. Deep violet in hue, DPPH solutions have a significant absorbance at 517 nm. The process of scavenging the free radical DPPH reveals the test samples' capacity to scavenging free radicals or their antioxidant potential, indicating its efficacy in

preventing, intercepting, and repairing damage to biological systems [12].

CUPRAC (*Cupric Ion Reducing Antioxidant Capacity*). In this method, the reagent used is Cu(II) neocuprine (Cu(II)-$(Nc)_2$) for chromogenic oxidation so that the reduction of Cu(II) ions can be measured. The reason for the widespread usage of this approach is the utilization of CUPRAC as the reagent, which exhibits selectivity and possesses a low reduction potential value. Consequently, it is simple to execute and has a little expense [13]. Considering the association observed between the total flavonoid concentration and antioxidant activity. The purpose of this study was to establish a basis for future research on the creation of more affordable and safer natural antioxidants for use in therapeutic and pharmaceutical formulations.

MATERIALS AND METHODS

Plants and chemicals material

Vernonia amydgalina leaves were gathered form Universitas Sumatera Utara, Faculty of Pharmacy in Indonesia. Aluminium chloride (Smart lab), ammonium acetate (Smart lab), distilled water, dichloromethane, DPPH, ethyl acetate, methanol, neocuprine, chloride dihydrate, quercetine (TCI), sodium acetate.

Extract preparation

The initial stage of the extraction process involved subjecting the solvent n-hexane to reflux, followed by the subsequent addition of methanol. Subsequently, the methanol extract underwent a fractionalization process utilizing a liquid-liquid extraction technique, wherein dichloromethane, ethyl acetate, and n-butanol were employed as distinct solvents for partitioning and isolating specific components from the extract.

Determination of total flavonoid content

10 mg of the n-hexane and methanol extract, diclorometh ane, ethyl acetate, n-butanol, and the residual fraction of VA was meticulously dissolved in 10 ml of methanol. Subsequent to this, a precise volume of 0.5 ml from the resulting solution was meticulously pipetted and amalgamated with 1.5 ml of methanol, 0.1 ml of a 10% aluminum chloride solution, 0.1 ml of a 1 M sodium acetate solution, and 2.8 ml of distilled water. This intricate amalgamation was then allowed to stand undisturbed for a duration of 30 min, creating an environment conducive to optimal reaction conditions.

The ensuing measurement, conducted at a wavelength of 436 nm, was executed with meticulous attention to detail. It is important to mention that three replicates of each measurement were conducted to confirm the reliability and consistency of the acquired results. The flavonoid concentration was measured and then converted into milligrams of quercetin equivalent per g of extract using the accurate linear regression method for substitution, which improved the analytical precision and reliability of the results. This all-encompassing methodology was designed to elevate the precision and comprehensiveness of the disclosed flavonoid concentrations, thereby bolstering the overall dependability of the experimental results.

Antioxidant activity

DPPH (*2,2-Diphenyl-1-picrylhydrazyl*) scavenging activity

After dissolving a 10 mg extract of VA in 10 ml of methanol, the volume was adjusted with methanol until it reached the mark line. This resulted in a concentration of 1000 µg/ml, which is referred to as main solution I. Methanol was used to modify the volume until it reached the mark line. It was placed into a flask with a volume of 5 ml using a pipette, and the flask had a capacity of 10 ml. After that, the flask was filled with methanol until it reached the mark line, and the contents were aggressively stirred until they reached a consistency that was consistent throughout. The primary solution II, which was the solution that was produced, had a concentration of 500 micrograms per milliliter.

The next step involved the preparation of the main solution II, which involved the creation of five distinct concentrations, specifically 12.5, 25, 50, 100, and 200 µg/ml. In order to make a solution, 10 mg of DPPH were dissolved in fifty milliliters of methanol. After that, the solution that was obtained from each series was mixed with 1 ml of DPPH under vigorous conditions, and then it was allowed to incubate for thirty minutes at a temperature of twenty-seven degrees Celsius. In the subsequent step, the sample was measured with a spectrophotometer at a wavelength of 517 nm in order to determine its quantity.

CUPRAC (*Cupric Ion Reducing Antioxidant Capacity*) scavenging activity

250 ml volumetric flask was filled with 0.4262 gs of $CuCl_2.2H_2O$ (copper (III) chloride) to make $CuCl_2$ (copper(II) chloride) solution. Distilled water was used to dissolve the powder. Dissolve 19.27 g NH4Ac (ammonium acetate) in distilled water in a 250 ml volumetric flask to make ammonium acetate pH 7. Dissolved 0.039 g neocuprine (Nc) in a 25 ml volumetric flask and dilute with ethanol.

10 mg of VA extract was dissolved in 10 ml of methanol, and then the volume was adjusted with methanol till reaching the mark line, resulting in a concentration of 1000 µg/ml, referred to as main solution I. I was transferred into a 10 ml flask using a pipette, with a volume of 5 ml. The flask was then filled with methanol until reaching the mark line, and the mixture was vigorously agitated until it became uniform. The resulting solution, known as main solution II, had a concentration of 500 µg/ml. Then form the main solution II, 5 series of concentrations were made, which are (12.5; 25; 50; 100; 200) µg/ml. Then each series of the samole was pipetted into a volumetric flask, then 1 ml of each Cuprac reagent ($CuCl_2.2H_2O$, neocuproin and buffer ammonium acetate) was added. Inject 0.1 ml of distilled water into the flask, then agitate vigorously and let it to sit undisturbed for 30 min at a temperature of 27 °C. Subsequently, the measurement was conducted utilizing a spectrophotometer set at a wavelength of 452 nm.

RESULTS AND DISCUSSION

Total flavonoid content

Secondary metabolites found in plants can be broken down into three primary categories: alkaloids, phenols, and terpenoids among others. Flavonoids are a subgroup of phenols, and more than half of the phenol classes that have been found are flavonoids. This category of secondary metabolites, known as flavonoids, is liable for a diverse range of functions within the medical industry. The assessment of Total Flavonoid Content (TFC) in plants is a crucial step in understanding their chemical composition and potential health benefits [11].

This evaluation is commonly conducted through colorimetric analysis, a widely adopted technique that comes into play post the completion of the solvent extraction process. Among the various colorimetric assays available, the aluminum chloride method stands out as a particularly favored and extensively employed approach for quantifying the TFC in plant extracts [11]. Aluminum (III) plays a pivotal role as a complexing agent within the context of this testing procedure, where its unique chemical properties and coordination capabilities are harnessed to form stable complexes, thereby facilitating the intricate and precise analysis of the substances under investigation.

Table 1: Total flavonoid content of *Vernonia amygdalina* delile

Extract	Total flavonoid content (mg QE/g extract)
N-Hexane extract	14.89±0.43
Methanol extract	45.55±0.33
Ethyl acetate fraction	47.21±0.43
Diclorometh ane fraction	69.56±0.22
N-butanol fraction	64.64±0.33
Residual fraction	25.67±0.33

Results are given as mean±SD, n=3

Table 1 displays the TFC of each extract. The diclorometh ane fraction of VA had the most significant TFC at 69.56±0.22 mg QE/g extract, followed by the butanol fraction of VA at 64.64±0.33 mg QE/g extract, and the ethyl acetate fraction of VA at 47.21±0.43 mg

QE/g extract. Flavonoid in VA reported act as free radical scavengers and have a potential in lowering the risk of coronary heart disease, cardiovascular and cancer [14].

Fragrant compounds are found in flavonoids. These aromatic compounds are conjugated in such a way that they exhibit substantial absorption bands in the visible light spectrum, as well as in the ultraviolet and visible light spectral regions [15]. Because it contains flavonol group flavonoid compounds that have keto groups at C4 and hydroxyl groups at C-3 or C-5 atoms, quercetin is used as a standard. This is because it contains either of these structures. Furthermore, flavones and flavonols are located in close proximity to these hydroxyl groups [16].

As part of the experiment, $AlCl_3$ is added to the sample solution. This has the capability to lead to the creation of a complex and induce a wavelength shift into the visible spectrum, evident by a discernibly more yellow color during the test. The addition of sodium acetate becomes imperative to ensure the preservation of the wavelength within the visible range [17]. On the flavone or flavonol complex, the chemical AlCl3 has the potential to have a reaction with the keto group that is situated at carbon 4, as well as with the hydroxyl group that is placed at either carbon 3 or carbon 5. The production of a stable complex product or compounds is the end result of this reaction which takes place [18].

Antioxidant activity

Many researchers have reported on the antioxidant activity of *Vernonia amygdalina* [19]. The analysis of African leaves extract reveals that it possesses phenolic and flavonoid components, which are recognized for their significant antioxidant properties. Therefore, this plant extract exhibits intriguing potential as a natural source of antioxidants [20].

Based on table 1, in this work, the DCM fraction, which is a fraction high in flavonoids, was utilized for antioxidant testing. The DCM fraction was generated by determining the flavonoid content.

Table 2: The results of antioxidant activity of *Vernonia amygdalina* flavonoid rich fraction

Antioxidant	Concentration	Absorbance	% Inhibition	Regression equation	*IC_{50}
DPPH	12.5	1.145	19.48	y = 0.3415x+9.3229	117.33±0.56
	25	1.137	20.04		
	50	1.038	27.00		
	100	0.763	46.34		
	200	0.350	75.39		
CUPRAC	12.5	0.174	13.22	y = 0.3759x+18.405	84.80±0.73
	25	0.248	39.11		
	50	0.300	49.67		
	100	0.556	72.84		
	200	0.805	81.24		

*Results are given as mean±SD, n=3

For the purpose of determining the amount of antioxidant activity that dichlormethane fraction extract of African leaves possesses, the IC_{50} calculation is typically utilized. The term "IC_{50}" refers to the concentration of an antioxidant molecule that results in a reduction of DPPH activity that is equal to fifty percent. Making use of the linear regression equation that was obtained allows for the calculation of the IC50 value. Fig. 1 depicts the linear regression equation of the flavonoid-rich proportion of Vernonia amygdalina with DPPH, and fig. 2 depicts the CUPRAC technique.

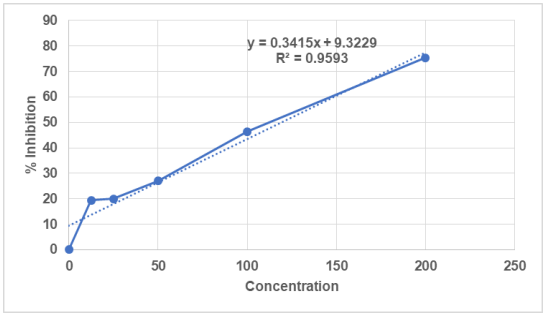

Fig. 1: The linear regression equation for determining the antioxidant activity of the flavonoid-rich fraction of *Vernonia amygdalina* using the DPPH method

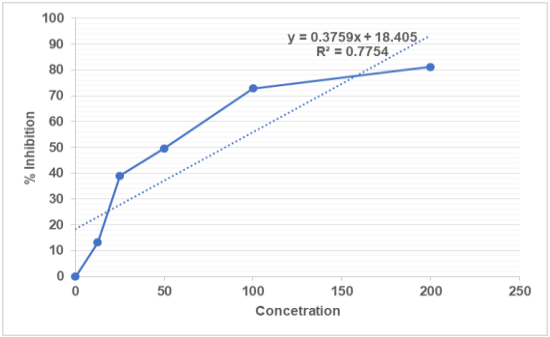

Fig. 2: The linear regression equation for determining the antioxidant activity of the flavonoid-rich fraction of *Vernonia amygdalina* using the CUPRAC method

The IC_{50} represents the concentration at which 50% of the starting concentration is dectreased. The smaller IC_{50} value indicates better antioxidant activity [13, 21]. *Diphenyl-picryl-hydrazine,* a yellow prodict, is produces when the purple color of the DPPH solution is reduced using the DPPH test procedure. The degree of hue corresponds to the sample's concentration of reducing components anad antioxidant capabilities working in concert [1]. The unpaired electrons of the DPPH radical absorb significantly at 517 nm, resulting in a deep purple color. However, as the odd electron pairs with another electron, the initial color progressively changes color to pale yellow and the absorbance value decreases [22]. As the concentration of the sample increases, so does the antioxidant activity. This is due to the fact that at high concentrations, the ability of the antioxidants to scavenge free radicals is greater [23].

The reagent used in the CUPRAC antioxidant activity test is neocuproin (Nc), which is a chromogenic oxidizing agent and forms a chelate with Cu2+, resulting in the formation of reduced Cu^{2+}-Nc, which has a blue color. The antioxidant activity can be measured by the ability of Cu^{2+}-Nc to become Cu^{+}-Nc [24, 25]. A yellow hue develops as an indication of this. The Cu^{+}-Nc chelate formed is stable. It is not easily altered by environmental changes such as air, light, or pH. This color change causes a change in absorbance due to changes in the amount of light that can be absorbed. This is measured using a UV-Vis spectrophotometer [13].

Based on table 2, The IC_{50} result of the DCM fraction with DPPH method was 117.33±0.56, and with CUPRAC method was 84,80±0,73 which shows that the DCM fraction of *Vernonia amygalina* has medium to strong antioxidant activity. Previous studies have established that an antioxidant is classified as highly potent if its IC_{50} value is below 50 µg/ml, potent if it falls between 50 and 100 µg/ml, moderate if it ranges from 100 to 150 µg/ml, and weak if it is between 151 and 200 µg/ml [26]. Flavonoids are a prevalent group of organic compounds that are highly plentiful in plants. They are the most naturally occurring phenolic compounds that can assist maintain a healthy body. It has been discovered that flavonoids are particulary effective at scavenging free radicals, which is the factors that leads to their activity as antioxidants [27]. This indicates that the flavonoid-rich fraction will have stronger antioxidant capabilities. These findings clearly show that the flavonoid-rich fraction of *Vernonia amygdalina* possesses a large number of electron-and hhygrogen-contributing active chemicals and is thus a strong source of antioxidants [28].

CONCLUSION

The results showed that DCM fraction is the highest flavonoid fraction and exhibits potent antioxidant properties.

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AUTHORS CONTRIBUTIONS

Rosalina V: Data Curation, Formal Analysis Writing – Original Draft Preparation; Hasibuan P. A. Z: Funding Acquisition, Supervision, Validation; Satria D: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, writing – Review and Editing; Meiyanto E: Methodology, Project Administration; Putra D. P: Data Curation, Methodology, Analysis; Chatri M: Formal Analysis, Invertigation; Septisetyani E. P: Data Curation, Project administration, Methodology.

CONFLIC OF INTERESTS

The authors affirm the absence of any conflict of interest.

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