ALKALOIDS EXTRACTION FROM *Vernonia amygdalina* Del. AND EVALUATE THE REDUCING POWER ACTIVITY OF THE OBTAINED EXTRACT

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ABSTRACT

The study was conducted to determine the proper conditions for alkaloid extraction from *Vernonia amygdalina* Del. and determine the antioxidant capacity of the obtained extract. The parameters such as solvent, material/solvent ratio, extraction temperature, and extraction time, were investigated. Surveyed factors include used solvents (methanol, ethanol, and water), solvent concentrations (60, 70, 80, 90, 99.7%, v/v), ratios of raw material/solvent (1/10, 1/20, 1/30, 1/40, w/v), extraction temperatures (30, 40, 50, 60, 70 ^oC) and extraction times (1, 2, 3, 4, 5 hours). The efficiency of the extraction process was evaluated via the total alkaloid content (TAC). TAC was determined by the UV-Vis spectroscopy method. In general, all surveyed factors significantly affected the extraction yield recovery. The optimal solvent for the extraction process is methanol 70%, the raw materials/solvent ratio 1/20 (w/v), and the extraction temperature of 50 ^oC for 4 hours. The antioxidant capacity of alkaloid extracts was determined via a reducing power (RP) essay with IC_{50} = 133.36 µg/mL.

Keywords: Alkaloid, antioxidant activity, extraction, *Vernonia amygdalina*

1. INTRODUCTION

Vernonia amygdalina Del., according to the *Asteraceae* family, commonly called "bitter leaf", is a perennial shrub widely distributed in tropical regions of Africa. In Vietnam, this plant is common in high and cool mountainous areas such as Lao Cai, Cao Bang, Bac Kan, and Thanh Hoa provinces. It is a popular vegetable in West Africa, commonly used for medicinal purposes to treat several diseases thanks to its effects and diverse pharmacology [1]. Many studies demonstrated biological properties due to the phytochemical compounds abundant in the plant [2-4]. These compounds, including alkaloids, steroid glucosides, sesquiterpene lactones, and flavonoids, contribute to bitter taste and biological activities. In traditional medicine, *V. amygdalina* is used for antihelminthic, antimalarial, laxative, digestive aid, antipyretic, and wounds. Bitter leaf extracts with high pharmacological properties have been shown in antimicrobial effects (antibacterial, anti-fungal, antiproliferative, etc.), anti-cancer, antioxidant, hypoglycemic, liver and kidney protection, and serum lipid regulation. These properties come from acting alone or combined with the metabolism of various phytochemicals found in the plant [5].

Alkaloids, a class of essential organic compounds, are widely distributed in nature and contain at least one nitrogen atom, which is mostly alkaline. Still, some related compounds are

neutral and even have weak acidity. Alkaloid has been used in medicine for hundreds of years and is still a well-known medicine today. This natural product plays a vital role in preventing and treating cancer and other diseases as a critical component of many natural pharmaceutical products [6]. The remarkable potential of alkaloids and plants maintained them contribute to other health and pharmacology studies [7]. All alkaloids or their free salts can be dissolved in methanol or ethanol. So, it could be used heated alcohol under reflux extraction or ultrasonic alcohol extraction. Most free alkaloids are lipophilic. The heated alcohol under reflux was applied commonly to extract free alkaloids with organic solvents such as chloroform, benzene, and ether [8].

Many researchers in the medical sciences have studied the antioxidant properties of *V. amygdalina*. Antioxidants inhibited the harmful effects of free radicals in the human body and fat loss without corresponding adverse effects compared with synthetic antioxidants [2]. More than 130 alkaloids isolated from plants, fungi, algae, bacteria, and animals have shown significant free radical scavenging capacity with primary assays (DPPH, ABTS, FRAP, RP) [9].

V. amygdalina holds excellent potential for medicine because of its significant alkaloid content and its available pharmacological properties [10-14]. According to Odiba John Oko *et al.*, (2018) showed that lactucopicrin (34.00%) and lactucin (18.91%) were the most predominant alkaloids among over 20 alkaloids in both leaf and stem extracts [13]. In addition, this plant is an abundant source, but it has not been effectively utilized. Thus, taking advantage of these raw materials to produce high medicinal products results in scientific, economic, and social value. The study was carried out to determine the primary conditions for the alkaloid extraction from *V. amygdalina*, preliminary assessment of antioxidant capacity by reducing power (RP) assay. This study offers a platform for further research on applying these compounds to functional food and pharmaceutical purposes.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Material

Matured leaves of *V. amygdalina* were collected at Hoa Loc ward, Mo Cay Bac district, Ben Tre province in June 2021. Analysis and identification of scientific names by morphological comparison method according to documents of Pham Hoang Ho [15]. After collection, it was transported within the day to the laboratory of the Faculty of Food Science Technology – Ho Chi Minh City University of Food Industry, where raw materials were removed from impurities. Then, they were drained and dried at 60 ^{o}C until the moisture content was less than 10%. The fine powder was obtained by grinding by a mechanical grinder (less than 80 mesh size) and stored in thick Polyethylene bags, placed in a sealed plastic container, and stored in normal conditions without direct light and moisture for all experiments.

2.1.2. Chemicals

Methanol 99.8% (Sigma), Ethanol 96% (Sigma), Chloroform 99.8% (Sigma), and Hydrochloric acid 38% (Sigma). Standard substance Atropine 96% (Merck), Bromocresol green (BCG) (Merck). All other chemicals used in the study met the technical requirements of analytical chemicals.

2.2. Methods

2.2.1. Alkaloid extraction investigation

5 g of the material (according to % of dry mass - dm) was put in a conical flask, adding solvents (ethanol, methanol, water) with the concentrations investigated in turn (60%, 70%,

80%, 90%, 99.8%) at raw material/solvent ratios (1/10, 1/20, 1/30, 1/40, w/v) during the times (1, 2, 3, 4, 5 hours) at the temperatures (30, 40, 50, 60, 70 ºC). In each experiment examining one factor, the remaining factors will be fixed for the experiment. The mixture was filtered and then determined the total alkaloid content by UV-Vis spectroscopy (WTW, in Germany) to select the suitable solvent for the alkaloid extraction. The experiments were repeated three times.

2.2.2. Reducing power assay - RP

RP assay is also an effective measure of antioxidant capacity besides DPPH or ABTS. The marked antioxidant activity of RP is the result of their radical scavenging activity and reducing power [16]. Gordon (1990) reported that the antioxidant activity was based on the breakdown of the free radical chain by donating the hydrogen atom. The reducing agents also react with some peroxide precursors, thereby preventing peroxide formation [17].

The iron reduction capacity (RP) was determined according to the method of Quezada *et al.* (2006) [18] with some adjustments. The reaction mixture consisted of 1 mL of the extract (with concentrations from 0 to 500 µg/mL), 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% $K_3[Fe(CN)_6]$. The reaction mixture was then incubated at 50 ºC for 20 minutes, added 1 mL of 10% CCl_3COOH, and centrifuged at 3000 rpm for 10 min. 1 mL of the clear solution was gently withdrawn, adding 1 mL of distilled water and 0.2 mL of 0.1% $FeCl_3$, and mixed well before measuring the absorbance at 700 nm via spectrophotometer. The blank is the sample diluent. The positive control was used as ascorbic acid (vitamin C) prepared at concentrations and performed as the test sample.

Percent inhibition of RP was calculated according to the formula:

$$\% \text{ RP inhibition} = [(A_1 - A_0)/A_1]x100$$

Where: A_1 is the absorbance of the sample, A_0 is the absorbance of the blank.

The antioxidant ability was assessed through the IC_{50} value, which showed 50% of the activity of reducing $K_3[Fe(CN)_6]$ complexes to $K_4[Fe(CN)_6]$ complexes.

2.2.3. Alkaloid content determination by spectrophotometric method

Based on the UV-Vis spectrophotometer method and the reaction of alkaloids with Bromocresol Green (BCG) to determine the total alkaloid content in the raw materials through the standard curve Atropine, a tropane alkaloid that exhibits a characteristic alkaloid and BCG response [19]. The resulting yellow complex was easily extracted with chloroform at pH 4.7, and the absorbance was measured in a UV-Vis spectrophotometer at 470 nm.

An atropine calibration curve was established based on the method of Fazel Shamsa *et al.*; with some suitable adjustments, briefly [20]:

1 mL of Atropine standard solution with concentrations ranging from 20 to 120 µg/mL was added to 1 mL of 2N HCl. The solution was filtered with filter paper to remove residue after 5 minutes of reaction. Next, the solution was transferred to a separating funnel, adding 5 mL of phosphate buffer (pH 4.7) and 5 mL of BCG solution. Finally, the mixture was shaken vigorously with 5 mL chloroform for 2 min and transferred to a 10 mL volumetric flask, and made up to the mark with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. The blank was prepared in the same protocol as the test sample and replaced test samples with the diluent.

Determination of alkaloid content: the extracted solution replaces the standard sample and then follows the same method of standard curve construction. From the standard curve, the equation deduces the total alkaloid content.

2.2.4. Data analysis

Experiments were repeated three times, and the results were presented as mean ± SD. Using IBM SPSS Statistics 20.0 software to analyze experimental data and evaluate the difference between samples ($p < 0.05$). The graph was drawn using Microsoft Excel 2016 software.

3. RESULTS AND DISCUSSION

3.1. Effects of solvent on the alkaloid extraction

The desired compounds are separated from the raw material via the extraction stage. A solvent is an essential factor in extraction. Thus, the matter helps to increase the diffusion and solubility of the component into the solvent, enhancing the extraction yield.

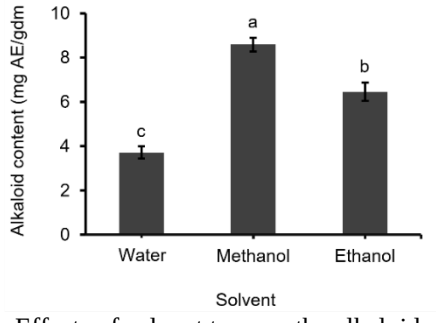

Figure 1. Effects of solvent type on the alkaloid extraction
Note: Different letters represent a statistically significant difference at $p < 0.05$.

A suitable solvent is characterized by optimal extraction and maintenance of the chemical structure stability of the desired compounds [21]. The recovery of phytochemical compounds could be affected by the dielectric constant, the chemical structure of the organic solvent, and the chemical properties of the phytochemical compounds [22]. Therefore, the solvent and its polarity could significantly impact the recovery yield of alkaloid extraction. The results (Figure 1) showed that the methanol solvent resulted in the highest alkaloid content (8.59 ± 0.31 mg AE/gdm) and the water solvent was the lowest (3.72 ± 0.28 mg AE/gdm). The alkaloid content obtained by ethanol solvent was 6.46 ± 0.42 mgAE/gdm, lower than that of methanol solvent. Both free alkaloids and their salts are soluble in methanol and ethanol [8]. The polarity of methanol is higher than that of ethanol, with a lower boiling point, so the ability to dissolve alkaloid salts is better [23]. The ANOVA test results showed a significant difference in alkaloid content for the three solvents ($p < 0.05$), so methanol was chosen as a parameter for the following experiments. Many studies also showed that methanol is the optimal solvent commonly used to extract alkaloids from plants [24, 25].

3.2. Effects of solvent concentration on the alkaloid extraction

Solvent concentration plays a vital role in the extraction of organic compounds. The degree of polarity of the solvent depends on the dielectric constant and the hydrogen bond value. Methanol and water with high polarity, the mixtures of them would have different degrees of polarity, and the solvent with the same polarity as the extracted compound would dissolve that substance better [26].

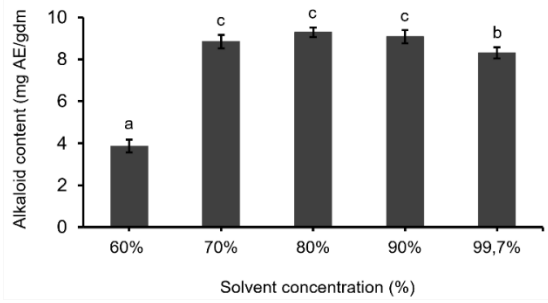

Figure 2. Effects of solvent concentration on alkaloid extraction
Note: Different letters represent a statistically significant difference at p < 0.05.

Figure 2 showed that the obtained alkaloid contents were different under the same conditions with different solvent concentrations. Alkaloid content increased gradually with increasing concentrations from 60% to 70% and peaked at 80% (9.28 ± 0.24 mg AE/gdm). At the concentration of 99.7% methanol, the alkaloid content decreased. The main reason is that the higher the concentration of the methanol, the stronger the penetration rate into the cell, and the easier it is for the solvent to penetrate deep inside the cell and perform the extraction process. The increase in methanol concentration would enhance solvent flow through the sample, which improves the extraction speed. However, the solvent flow rate increased too much, leading to the solute and solvent not being able to bind together, leading to a decrease in extraction efficiency, in which the total alkaloid content also showed this [27]. The results of the ANOVA test showed that there was no significant difference in the concentrations from 70% to 90%. Thus, 70% methanol was selected as the parameter for further experiments ($p<0.05$). The result was consistent with the study of Zhang *et al*. (2007). It was reported that methanol was better than dichloromethane and acetone for alkaloid extraction from the seeds and leaves of *Camptotheca acuminata* [28].

3.3. Effects of material/solvent on alkaloid extraction

The material/solvent ratio significantly influences the extraction process of organic compounds of biological value. A reasonable ratio of solvent and material impacts the recovery yield and the cost.

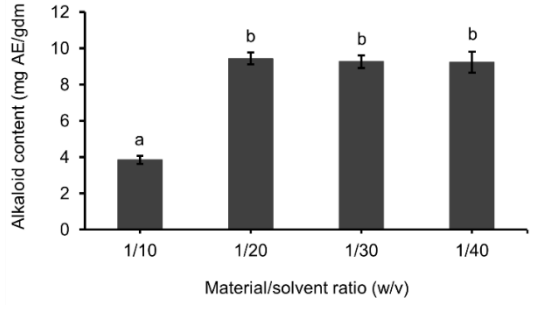

Figure 3. Effects of material/solvent ratio on alkaloid extraction
Note: Different letters represent a statistically significant difference at p<0.05.

Alkaloid content at the ratio of 1/20 (w/v) was the highest (9.43 ± 0.32 mgAE/gdm) compared with the other rates. There was no significant difference in the results at 1/30 w/v and 1/40 w/v with the content at 9.23 ± 0.58 mgAE/gdm. The driving force of mass transfer is the concentration gradient between the solid and the solvent. More solvent could enhance the concentration gradient, resulting in an increase in the solute's diffusion rate into the solvent and, therefore, better recovery yield of the extraction [29]. However, at the equilibrium status,

more amount of solvent did not improve solute content, but it was not economic efficiency. Through analysis of variance, the ratio of 1/20 w/v was the optimal parameter for the subsequent experiments. The result was consistent with the study of Mu *et al*. (2012), extracting four main alkaloids from the leaves of *Catharanthus roseus*. They reported that the extraction efficiency increased with the increase in the material/solvent ratio [30].

3.4. Effects of temperature and time on alkaloid extraction

Alkaloid stability is affected by solvents and oxidation of nitrogen, heat, and light. The temperature affects the efficiency of phytochemical extraction. Higher temperatures lead to better cell disruption and thus increase the solute's solubility in the solvent [31].

The temperature can influence the equilibrium status and mass transfer rate (diffusion coefficient). The higher temperature increases the rate of diffusion of the solute into the solvent and the permeability of the solvent to the raw material, enhancing the extraction efficiency and shorter extraction time [32]. Figure 4 showed that higher temperatures increased the amount of the obtained alkaloid. The highest extraction efficiency (9.95 ± 0.41 mgAE/gdm) was observed at 50 ^{o}C. From 60 ^{o}C, the alkaloid content tended to decrease, and the lowest was 7.79 ± 0.43 mgAE/gdm at 70 ^{o}C. Therefore, similar to many biological compounds, the nitrogenous alkaloid from *V. amygdalina* was unstable because the chemical composition and structure would be changed in cases of increasing temperature. High temperatures could soften plant tissue and help release biological compounds in raw materials more efficiently, combined with an increase in the permeability of cell membranes to help the extraction with high efficiency [33]. The analysis of variance ANOVA and Duncan's test showed that the alkaloid content obtained at 50 ^{o}C had a significant difference from the remaining temperatures, so it should be selected as a parameter for the next experiment ($p<0.05$).

Extraction time is vital in determining the end of the process and obtaining the highest solute content while saving time and improving economic efficiency. The survey was carried out with fixed parameters obtained from previous experiments, combined with an investigation of extraction time levels from 1 to 5 hours.

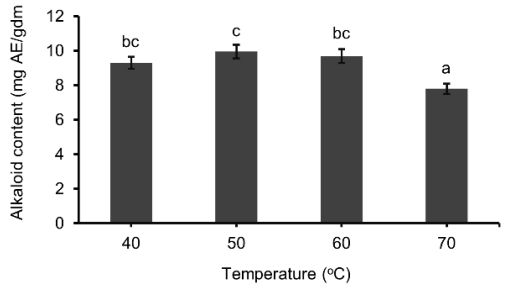

Figure 4. Effects of temperature on alkaloid extraction

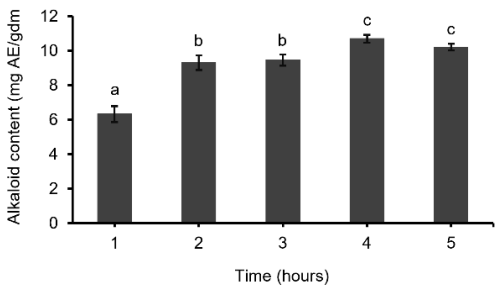

Figure 5. Effects time on alkaloid extraction

Note: Different letters represent a statistically significant difference at $p < 0.05$

Extraction time for obtaining the desired biological compounds was considered until the solute concentration was half-balanced inside and outside the cell. Thus, if the extraction time is not enough, the target compounds will not completely be dissolved into the solvent. Still, if the time is too long, it can lead to changes in composition due to the susceptibility of biological compounds to reduce yield recovery. Besides, the shorter time helps to save a large amount of energy for the extraction process to improve economic efficiency [31]. A similar result was observed in Figure 5. The extraction time increased the obtained alkaloid content from 1 hour to 4 hours. After 4 hours of extraction, the alkaloid content peaked at 10.69 ± 0.24 mgAE/gdm, and continued to decrease for 5 hours. A slight decrease in content was observed, but it is no

significant difference ($p<0.05$). The results agreed with the findings of Koomson *et al.* (2018) that 4 hours is a suitable time for alkaloid extraction from *Solanum torvum* with 6.32 ± 0.12 mg/g and 16.94 ± 2.3 mg/g in mature and immature fruits, respectively [34].

3.5. The reducing power (RP) of alkaloid-rich extracts

The antioxidant capacity of alkaloid-rich extracts from *V. amygdalina* was demonstrated by determining reducing power (RP).

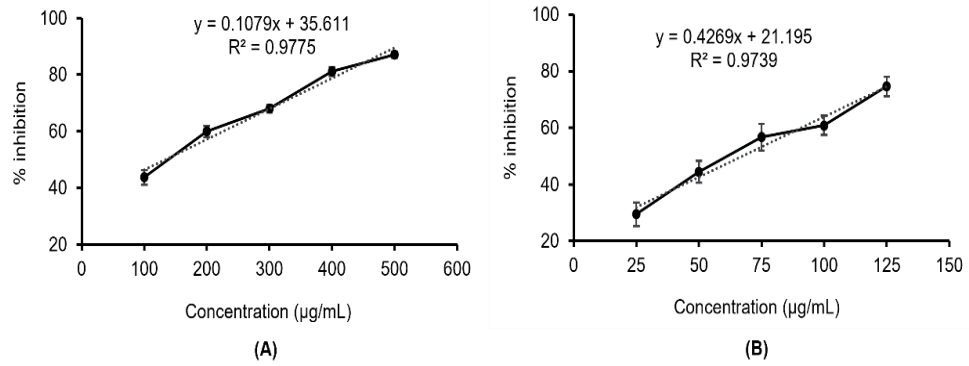

Figure 6. Antioxidant activity of alkaloid-rich extract (A) and acid ascorbic (B) via reducing power essay

The antioxidant capacity of the obtained alkaloid was determined via RP assay. It measured the ability of a compound to reduce ferric ions to ferrous ions. The higher absorbance at 700 nm indicated the better capacity of the compound's reducing power. Accordingly, the color of the test solution changing from yellow to blue of the $KFe[Fe(CN)_6]$ complex results in a higher percentage of iron reduction. Substances have antioxidant properties for a hydrogen atom, reducing the Fe^{3+} ion in the potassium ferricyanide molecule ($K_3[Fe(CN)_6]$) to the Fe^{2+} ion in the potassium ferrocyanide molecule ($K_4[Fe(CN)_6]$), this complex reacts with $FeCl_2$ to form a blue solution and with the increase of the concentration compounds [35]. The results in Figure 6 showed the ability to reduce iron with the rise of alkaloid concentration. The iron reduction capacity increased gradually from 43.73 ± 2.61% to 87.04 ± 1.05% from 100 to 500 µg/mL. The obtained IC_{50} value of the alkaloid extract was 133.36 µg/mL, higher than that of the ascorbic acid control (IC_{50} = 67.47 µg/mL).

The presence of reductones is responsible for reducing capacity, which is involved in preventing chain initiation, binding of metal ions, decomposition of peroxides, and radical scavenging [36]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The alkaloid extract had effective and powerful reducing power using the potassium ferricyanide reduction method compared to the standards. The results on reducing power demonstrate the electron donor properties of alkaloid extract, thereby neutralizing free radicals by forming stable products [37]. Regarding this result, it was reported that the lower the IC_{50} of the sample, the more effective the antioxidant ability. Therefore, it can be inferred that the alkaloid extract from *V. amygdalina* has a high antioxidant potential, which is beneficial in treating diseases caused by oxidative stress. Similarly, there have been many studies evaluating the antioxidant capacity of bitter leaves in many methods. Oluwaseun Ruth Alara *et al.* (2019) evaluated the free radical scavenging ability of the bitter leaf ethanol extract *Vernonia amygdalina* with microwave support, the results showed that IC_{50} = 162.38 ± 2.01 µg/mL for method ABTS and IC_{50} = 286.00 ± 2.20 for DPPH [38].

4. CONCLUSION

The study successfully extracted alkaloids from *V. amygdalina* with high antioxidant capacity. It has been proved that methanol is a suitable solvent for extracting alkaloids. The optimal conditions for the extraction process to obtain alkaloids were determined through the objective function of total alkaloid content (mg AE/g dry matter). The obtained results were methanol 70% v/v, the ratio of raw material/solvent 1/20 w/v, extraction time of 4 hours and temperature of 50 °C. Under these conditions, the alkaloid content obtained was 10.69 ± 0.24 mg AE/gdm. This study is a preliminary assessment of reducing capacity, and alkaloid extracts extracted from *V. amygdalina* showed high antioxidant activity with IC_{50} = 133.36 µg/mL. Besides, further studies about the purification, and determination of the alkaloid characteristics should be done to have the comprehensive properties of alkaloids from *V. amygdalina* with the tendency to apply the obtained products in the food and pharmaceutical industries.

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TÓM TẮT

NGHIÊN CỨU QUÁ TRÌNH TRÍCH LY VÀ ĐÁNH GIÁ KHẢ NĂNG KHÁNG OXY HÓA CỦA ALKALOID TỪ CÂY LÁ ĐẮNG (*Vernonia amygdalina* Del.)

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Nghiên cứu được tiến hành nhằm xác định các điều kiện ảnh hưởng đến hiệu suất trích ly alkaloid từ lá đắng *Vernonia amygdalina* Del. và xác định khả năng kháng oxy hóa của dịch chiết. Các đặc tính của dung môi, tỷ lệ nguyên liệu/dung môi, nhiệt độ và thời gian trích ly đều là các yếu tố trực tiếp ảnh hưởng đến quá trình trích ly, nên đây là các yếu tố cơ bản được lựa chọn để tiến hành nghiên cứu. Các điều kiện khảo sát bao gồm loại dung môi sử dụng (methanol, ethanol và nước), nồng độ dung môi (60, 70, 80, 90, 99,7%, v/v), tỷ lệ nguyên liệu trong dung môi (1/10, 1/20, 1/30, 1/40, w/v), nhiệt độ trích ly (30, 40, 50, 60, 70 ℃) và thời gian trích ly (1, 2, 3, 4, 5 giờ). Hiệu quả quá trình trích ly thể hiện qua hàm lượng alkaloid tổng được sử dụng làm chỉ tiêu đánh giá các thông số khảo sát. Hàm lượng alkaloid tổng được xác định thông qua xây dựng đường chuẩn Atropine bằng phương pháp quang phổ UV - Vis. Nhìn chung, các yếu tố đều ảnh hưởng đáng kể đến hiệu quả trích ly, kết quả nghiên cứu thu được dung môi tối ưu cho quá trình trích ly là methanol 70%, tỷ lệ nguyên liệu/dung môi 1/20 (w/v), nhiệt độ trích ly 50 ℃ trong thời gian 4 giờ. Khảo sát năng lực khử (Reducing Power – RP) để đánh giá sơ bộ khả năng kháng oxy của dịch chiết alkaloid cho thấy tiềm năng kháng oxy cao với IC_{50} = 133,36 µg/mL.

Từ khóa: Alkaloid, chống oxy hóa, trích ly, *Vernonia amygdalina*.