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Inhibitory Effects of *Vernonia amygdalina* Leaf Extracts on Free Radical Scavenging, Tyrosinase, and Amylase Activities

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ABSTRACT: Cytotoxicity and instability are the limitations when using bioactive compounds in cosmetic and pharmacology products. This study assesses *Vernonia amygdalina* leaf extracts for their antityrosinase, antiamylase, and antioxidant activities. Group A extracts were obtained using an aqueous solvent system [70% (v/v) of methanol (BTL70ME), ethanol (BTL70ET), and acetone (BTL70AC)]. Group B extracts were obtained using organic solvents of varying polarities. The results displayed that all extracts exhibited antityrosinase, antiamylase, and antioxidant activities *in vitro*. The most potent antityrosinase activity was observed in BTL70AC, with a half-maximal inhibitory concentration (IC_{50}) value of 20 μg/mL. BTL_Ethyl acetate and BTL70AC showed potential antiamylase activity. BTL_Isopropanol and BTL_Ethanol exhibited potential antioxidant activity, with IC_{50} values of 4.0 μg/mL. The total phenolic content of BTL70ME, BTL70ET, and BTL70AC was 72.29±14.14, 65.98±11.91, and 69.37±7.72 mg gallic acid/g extract, respectively. The total flavonoid content was 53.04±5.22, 44.35±13.17, and 61.74±13.17 mg quercetin/g extract, respectively. Group A extracts contained polyphenols, flavonoids, saponins, terpenoids, steroids, and cardiac glycosides. These biological properties can potentially be attributed to the types and quantities of phytochemicals present. Bioactive compounds in the extracts may exert synergistic effects *in vitro* by interfering with the conformational changes of tyrosinase during substrate binding. Both groups of extracts have the potential to suppress biomolecule degradation, promote antiaging and antimelasma effects, and their phytochemicals can help lower blood glucose levels in diabetes.

Keywords: alpha-amylase, antioxidant, antityrosinase, inhibitors, phytochemicals

INTRODUCTION

Wrinkles, melasma, freckles, and spots are common skin issues, particularly in aging individuals. These problems are influenced by two main factors: intrinsic factors, such as hormones and oxidative damage, and extrinsic factors, such as pollution, alcohol consumption, smoking, and sunlight exposure (Brincat, 2000; Makrantonaki et al., 2006). These factors contribute to the generation of free radicals in skin cells. Sunlight, a natural energy source of ultraviolet (UV) radiation, plays a crucial role in this process. UV radiation consists of UVA, UVB, and UVC rays, which induce the production of reactive oxygen species (ROS) and stimulate melanin synthesis. ROS can cause oxidative stress and damage various biomolecules, especially the plasma membrane of cells, leading to the loss of skin strength and flexibility and the formation of wrinkles.

Additionally, ROS can enhance the activity of enzymes such as collagenase, elastase, and hyaluronidase, which contribute to skin aging and wrinkle formation. Melasma, a skin condition characterized by pigmentation on the epidermis, is influenced by tyrosinase, an enzyme that controls melanocyte function (Fisher et al., 2002; Maity et al., 2011).

The cosmetic industry uses various whitening agents, including hydroquinone, butylated hydroxytoluene, kojic acid, ascorbic acid, and alpha-arbutin. However, hydroquinone, a skin-lightening compound, has been associated with mutagenic effects, adverse reactions, and skin irritation when used in high doses (Sarkar et al., 2012); hence, strict regulations govern its usage. Kojic acid inhibits tyrosinase but poses challenges owing to its cytotoxicity and instability. Ascorbic acid shows potential as

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an antityrosinase compound but is prone to decomposition under high temperatures and UV radiation (Bae et al., 2013). The use of pure compounds as cosmetic ingredients increases product value but comes with higher costs. To address these issues, the cosmetic industry incorporates extracts from plants, animals, and marine organisms into their products.

In addition to avoiding external factors that contribute to skin issues, two primary goals are targeted: preventing free radical generation through antioxidants and inhibiting enzymes that cause cellular degradation, including tyrosinase, elastase, hyaluronidase, and collagenase. Natural extracts containing potent antiwrinkle properties are key components of antiaging products. For example, red grape (*Vitis vinifera*) seed extracts contain various active compounds, including proanthocyanidins, resveratrol, phenolic acids, flavonoids, tannins, anthocyanins, carotenoids, and terpenes, which can neutralize free radicals and inhibit metalloproteinase in human skin cells (Chainani-Wu, 2003; Baxter, 2008; Sumiyoshi and Kimura, 2009; Cronin and Draelos, 2010; Maity et al., 2011; Bae et al., 2013; Garg, 2017). Marigold (*Tagetes erecta*) extracts contain β-amyrin, provitamin A, and syringic acids, which act as active inhibitors of metalloproteinase, elastase, and hyaluronidase (Maity et al., 2011). Turmeric (*Curcuma longa* L.) extracts contain curcuminoids, such as curcumin, dimethoxy curcumin, and bisdemethoxycurcumin, which inhibit metalloproteinase-2, elastase, hyaluronidase, and lipid oxidation (Chainani-Wu, 2003; Baxter, 2008; Sumiyoshi and Kimura, 2009; Usunobun and Ngozi, 2016).

Vernonia amygdalina, a plant belonging to the *Asteraceae* family and *Vernonia* genus (Alara et al., 2017), is native to tropical regions of Africa and Asia. It is known by different local names, such as bitter leaf, Omjunso, and Ikaru in English-speaking countries, Tanzania, and China, respectively. In Thailand, it is referred to as Nan Fui Chao or Nan Chao Wei. This shrubby plant can reach heights of 2∼10 m (6.6∼16.4 feet), with egg-shaped leaves measuring up to 20 cm (7.09 inches) in length that have a coarse texture and a bitter taste. *V. amygdalina* extracts contain alkaloids, tannins, flavonoids, saponins, triterpenoids, steroids, cardiac glycosides, and reducing sugars (Usunobun and Ngozi, 2016; Iwo et al., 2017). Previous studies have reported the potential biological properties of *V. amygdalina* leaf extracts, including antimicrobial, antifungal, antimalarial, antioxidant, antidiabetic, antifertility, and antithrombotic activities (Alara et al., 2017).

The present study focuses on studying the biological activities of *V. amygdalina* leaf extracts, particularly their antityrosinase, antioxidant, and antiamylase activities. The extraction process involves two groups: group A and group B. In group A, three aqueous solvents, namely 70% (v/v) acetone, 70% (v/v) ethanol, and 70% (v/v) methanol, were used. In group B, hexane, ethyl acetate, isopropanol, and ethanol were used to extract phytochemicals from *V. amygdalina* leaves, sorted by the polarity of each organic solvent. The antityrosinase, antioxidant, and antiamylase activities of the *V. amygdalina* leaf extracts were assessed and compared within and between these two groups.

MATERIALS AND METHODS

Extraction of *V. amygdalina* leaves

The extraction method was adapted from the method of Ekaluo et al. (2015). *V. amygdalina* leaves (Fig. 1) were obtained from Nah-Peau village, Nah-Peau subdistrict, Sahvi district, Choumporne province, Thailand, and stored in ice-filled containers. The leaves were washed three times with tap water and dried in an oven at 60°C for 48 h. Subsequently, the dried leaves were machine-minced into powder.

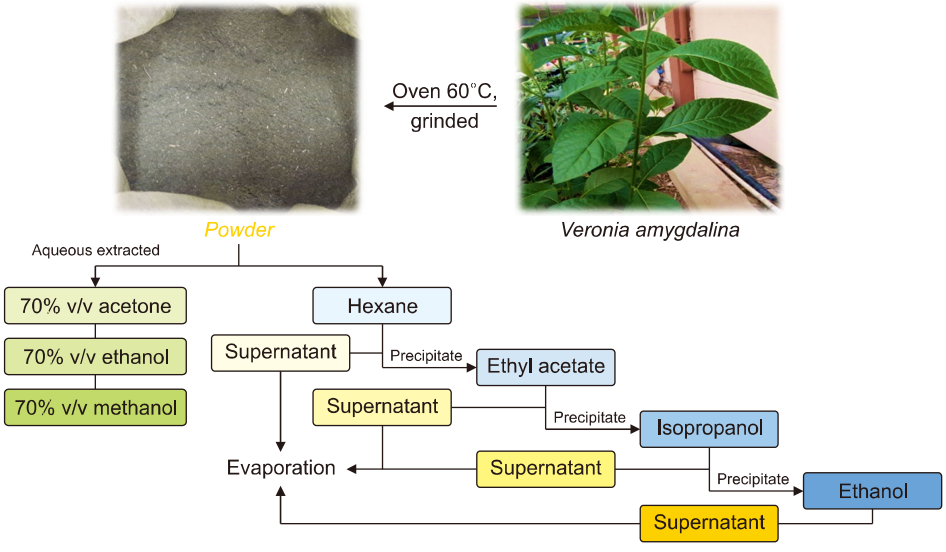

Fig. 1. Schematic diagram depicting the process of *Vernonia amygdalina* leaf extraction.

Two groups of extraction solvents were prepared. In group A, 70% (v/v) methanol (BTL70ME), 70% (v/v) ethanol (BTL70ET), and 70% (v/v) acetone (BTL70AC) were used to extract *V. amygdalina* leaf powder. In group B, hexane (BTL_Hexane), ethyl acetate (BTL_Ethyl acetate), isopropanol (BTL_Isopropanol), and ethanol (BTL_Ethanol) were used as the solvent systems for extraction. In both groups, 20 g of dry *V. amygdalina* leaf powder was placed in bottles with caps, along with 200 mL of the respective solvent systems. In group B, the extraction solvents were used sequentially, starting with hexane, followed by ethyl acetate, isopropanol, and ethanol. Fig. 1 illustrates the steps of the extraction process.

The mixture in each bottle were stirred at 22 *g* using a magnetic stirrer for three consecutive days and then incubated overnight at 4°C. The mixtures were decanted through no. 4 filter paper, and the clear portions were further filtered using 0.45-μm-pore filter paper. The resulting clear supernatant of the extracts was concentrated using a vacuum evaporator. In group A, after removal of the organic solvents, the aqueous extracts were lyophilized using a freeze dryer. All extracts from each group were weighed and stored in a desiccator box at −20°C. The extracts were assigned ID names, as presented in Table 1.

Determination of tyrosinase inhibition property using a modified dopachrome method

The tyrosinase inhibitory effect of the extracts was assessed following the procedure outlined by Patathananone et al. (2019). Each extract powder was dissolved in 5% (v/v) dimethyl sulfoxide (DMSO). Subsequently, 10 μL of various concentrations of each extract were mixed with 100 μL of a 250 unit/mL tyrosinase solution [250 unit/mL tyrosinase in 20 mM phosphate buffer (pH 6.8)] in a 96-well plate. Next, 70 μL of deionized (DI) water was gently added to each well. The mixtures were incubated at 37°C for 10 min, after which 20 μL of 20 mM L-Dopa (HiMedia Laboratories Pvt. Ltd.) was added. The plate was shaken and further incubated at 37°C for 20 min. UV-visible (UV-Vis) absorption measurements were performed at 495 nm to determine the percentage of tyrosinase inhibition.

Antiamylase activity assay

α-Amylase is an enzyme that breaks down starch by hydrolyzing α-(1-4)-glycosidic bonds. The resulting products include short-chain oligosaccharides, trisaccharides, maltose, and glucose. The level of product formation is influenced by several factors, such as enzyme concentration, substrate concentration, pH, and temperature. Inhibition of α-amylase activity is important for controlling sugar production during starch digestion, especially for individuals with hyperglycemia or diabetes. The assessment of reducing sugar products resulting from amylase activity was conducted using a modified version of the method described by Prasathkumar et al. (2021). Reducing sugars react with 3,5-dinitrosalicylic acid (DNS) at 100°C, producing 3-amino-5-nitrosalicylic acid, which exhibits a red-brown color. The absorbance value at a wavelength of 540 nm was measured to quantify the amount of reduced sugar, using the glucose standard curve for the DNS method.

In the experiment, 40 μL of 0.1% (w/v) α-amylase (HiMedia Laboratories Pvt. Ltd.) in 50 mM phosphate buffer at pH 6.8 was mixed with 20 μL of 20 mg/mL of each sample and incubated at 37°C for 10 min. A cuvette containing 1.8 mL of 1.0% w/v starch solution was prepared, and the optical density at a wavelength of 600 nm was measured using a UV-Vis spectrophotometer (UV-Vis-1200, MAPADA). The incubated α-amylase was then added to the starch solution, rapidly mixed, and the optical density at 600 nm (OD_{600} nm) was recorded every 30 s for 5 min. The reactions were halted through incubation at 100°C for 10 min, followed by a 10 min cooling period in water. Subsequently, 2 mL of 0.01 M DNS solution was added to each reaction, mixed, and boiled at 100°C for 10 min. The absorbance values of each reaction were measured at 540 nm. The quantity of reducing sugar products under each condition was determined using the standard curve equation: $y=0.4023x+0.0379$ ($R^2=0.9989$).

Table 1. The IC_{50} values of the extracted samples for antityrosinase and antioxidant activities

Sample groups	Extracted solvent	ID name	IC_{50} value (μg/mL)	
			Antityrosinase activity	Antioxidant activity
Group A	70% (v/v) methanol	BTL70ME	25	16
	70% (v/v) ethanol	BTL70ET	125	37
	70% (v/v) acetone	BTL70AC	20	20
Group B	Hexane	BTL_Hexane	>1,000	>160
	Ethyl acetate	BTL_Ethyl acetate	1,000	9
	Isopropanol	BTL_Isopropanol	720	4
	Ethanol	BTL_Ethanol	360	4
Control		Ascorbic acid	11	2

IC_{50}, half-maximal inhibitory concentration.

Determination of antioxidant properties using the DPPH assay

The antioxidant activity of the extracts was assessed and calculated using a modified version of the method reported by Patathananone et al. (2019). The reaction took place in a 96-well plate, where 10 μL of the extracted samples at different concentrations were added to 190 μL of 0.101 M DPPH solution (0.101 mM DPPH in methanol). The mixture was gently mixed, and incubated at 37°C for 30, 45, and 60 min. Ascorbic acid and 5% (v/v) DMSO served as the positive and negative controls, respectively. The absorbance values were measured at 515 nm (Piao et al., 2002; Theansungnoen et al., 2014; Ekaluo et al., 2015) using a microplate reader (EZ-Read 2000, Biochrom).

Determination of total phenolic contents using Folin-Ciocâlteu reagent

The total phenolic content in the extracts was analyzed using a modified experiment based on the method of Dewanto et al. (2002). Gallic acid, dissolved in methanol, was used as the standard agent. Standard gallic acid solutions were prepared at initial concentrations of 0.2, 0.4, 0.6, 0.8, and 1 μg/mL. For the reaction, 3.5 mL of 2.0% (w/v) sodium carbonate solution was mixed with 100 μL of different concentrations of the extracts (BTL70ME, BTL70ET, and BTL70AC), as well as the gallic acid solutions, in test tubes. Subsequently, 400 μL of 10-fold diluted Folin-Ciocâlteu solution was added to each tube. The mixtures were incubated in the dark at room temperature for 30 min. UV-Vis measurements were taken at 750 nm, using a modified version of the method described by Dewanto et al. (2002), to create a standard curve. Sample measurements were taken under the same conditions to determine total phenolic content.

Determination of total flavonoid content using aluminum chloride reagent

To assess the total flavonoid content in the group A extracts, standard quercetin solutions were prepared at different concentrations (20, 40, 60, 80, and 100 μg/mL). A typical mixture was created in a 10 mL volumetric flask by sequentially adding 1.0 mL of the sample solution, 4 mL of distilled water, and 0.3 mL of 5% (w/v) of sodium nitrite solution. The mixture was left at room temperature for 5 min, after which 0.3 mL of 10% (w/v) aluminum chloride solution was added. Following a 6 min incubation period, 0.2 mL of 1 M sodium hydroxide (NaOH) was added, and the total volume of the mixture was adjusted to 10 mL using distilled water. UV-Vis measurements were taken at 510 nm, using a modified version of the method described by Zhishen et al. (1999).

Preliminary analysis of bioactive compounds

The methods for phytochemical screening in the extracts were mini modified according to the report of Usunobun and Ngozi (2016).

***Analysis of coumarins*:** The leaf extract powder was dissolved in 70% (v/v) methanol. The clear supernatant was transferred to a test tube containing DI water. Filter paper soaked in 10% (w/v) NaOH solution was placed on top of the test tube and heated in boiling water for 5 min. Subsequently, the filter paper was exposed to UV light with a 365 nm wavelength. If coumarins were present, green, or blue luminescence could be readily observed.

***Analysis of saponins*:** First, 200 μL of the sample supernatant was mixed with 2.5 mL of DI water in a test tube. Subsequently, the mixture was incubated at 100°C in a water bath for 5 min. The presence of saponins was indicated by the observation of bubbles in the test tube after vigorous shaking.

***Analysis of tannins*:** Tannin content in each extract was determined using concentrated ferric chloride (Conc. $FeCl_3$). For tannin determination, 200 μL of the extracted sample was mixed with 300 μL of DI water in a test tube. The sample mixture was incubated in a 50°C water bath for 5 min. The clear supernatant was collected, and a few drops of Conc. $FeCl_3$ were added to the remaining liquid. The presence of tannins was indicated by the color change to dark green or dark blue.

***Analysis of terpenoids*:** First, the extracted samples were diluted with 1 mL of dichloromethane. The dissolved sample was then filtered to remove impurities. The supernatant was tested using 0.5 mL of concentrated sulfuric acid (Conc. H_2SO_4). The formation of a brownish ring between the layers of the mixture and Conc. H_2SO_4 indicated the presence of terpenoids.

***Analysis of steroids*:** The presence of steroids was screened in both groups of extracts. Initially, the extracted samples were diluted with 1 mL of dichloromethane and mixed. The precipitant was then filtered out, and 0.5 mL of glacial acetic acid was gradually added to the remaining liquid part. After thorough mixing, five drops of Conc. H_2SO_4 were added. The appearance of a blue or bluish-green color in the mixture indicated the presence of steroids.

***Analysis of cardiac glycosides*:** The presence of cardiac glycosides in the extracts was determined according to the method of Usunobun and Ngozi (2016). The powder of the extracts was dissolved and diluted with 1 mL of dichloromethane and mixed. The precipitated powder was filtered out, and only the clear supernatant was collected. One or two drops of Conc. $FeCl_3$ were then added and mixed. Subsequently, glacial acetic acid (5 drops) was added, and while mixing, 0.5 mL of Conc. H_2SO_4 was gradually added. The formation of a brownish ring between the layers of the mixture and Conc. H_2SO_4 in-

dicated the presence of cardiac glycosides.

Circular dichroism (CD) spectroscopy analysis

The conformational changes of tyrosinase were determined using CD spectroscopy (Jasco J-815 CD Spectrometer, Analytical Lab Science Co., Ltd.). Four solutions were prepared to analyze the CD spectra: (1) 5 μg/mL tyrosinase (E) in 50 mM phosphate buffer (pH 6.8), (2) 125 unit/mL tyrosinase+5 mM L-Dopa (E+S), (3) 5 μg/mL tyrosinase+0.7 mg/mL BTL70ME, and (4) 5 μg/mL tyrosinase+0.7 mg/mL BTL70ME+5 mM L-Dopa (E+I+S). The spectra were analyzed in the 176~260 nm range for all test conditions. A phosphate buffer was used as the buffer blank.

Statistical analysis

One-way ANOVA was used for the statistical analysis (IBM SPSS Statistics version 29, IBM Corp.), as previously explained by Patathananone et al. (2019). Each biological characteristic's proportion (means±SD) was used to represent the data. Statistics were computed within the same extracted group and compared to equivalent concentrations for the results depicted in Fig. 2 and 4. At a *P*-value<0.05, differences between groups were considered statistically significant.

RESULTS

Tyrosinase-inhibiting properties of extracts

The tyrosinase-inhibiting properties of *V. amygdalina* leaf extracts for both groups A and B are presented in Fig. 2. In group A, all extracts exhibited antityrosinase activity *in vitro*. The half-maximal inhibitory concentration (IC_{50}) values of each extract are shown in Table 1. The IC_{50} values of BTL70AC, BTL70ME, and BTL70ET were approximately 20, 25, and 125 μg/mL, respectively. Ascorbic acid, used as a positive control, exhibited an IC_{50} value of 11 μg/mL. Therefore, BTL70AC exhibits higher antityrosinase activity compared with BTL70ME and BTL70ET but lower activity compared with ascorbic acid. Furthermore, the group B extracts (BTL_Hexane, BTL_Ethyl acetate, BTL_Isopropanol, and BTL_Ethanol) also exhibited inhibition of the tyrosinase enzyme. The IC_{50} values of each extract in group B were >300 μg/mL (Table 1). Therefore, the extraction process used in group A may be more effective in dissolving bioactive compounds that exhibit antityrosinase activity compared with that used in group B.

Effect of *V. amygdalina* leaf extracts on α-amylase

The results of the antiamylase activity experiment are presented in Fig. 3. The rate of change in OD_{600} nm for 50 mM phosphate buffer (pH 6.8), BTL_Hexane, BTL_Ethyl acetate, BTL_Isopropanol, BTL_Ethanol, BTL70AC, BTL 70ME, and BTL70ET was 5.2, 2.7, 4.3, 4.5, 2.7, 3.8, 4.7, and 3.8×10^{-3} unit/s, respectively, and the amount of reducing sugar products under these conditions were 1.41±0.003, 0.94±0.056, 0.46±0.090, 0.57±0.040, 0.85±0.090, 0.43±0.020, 0.58±0.060, and 0.79±0.090 mM, respectively. The number of products observed under treated conditions was lower compared with the control, indicating a decrease in amylase activity. These findings suggest that the bioactive compounds present in *V. amygdalina* leaf extracts exhibit *in vitro* antiamylase activity.

Antioxidant properties of extracts

Antioxidants can decrease oxidative stress and oxidative damage. Primary antioxidants show the mechanism of action as electron donors. This reaction is the beginning step to inhibiting the chain reaction of dangerous free radicals. The antioxidant screening ability of both ex-

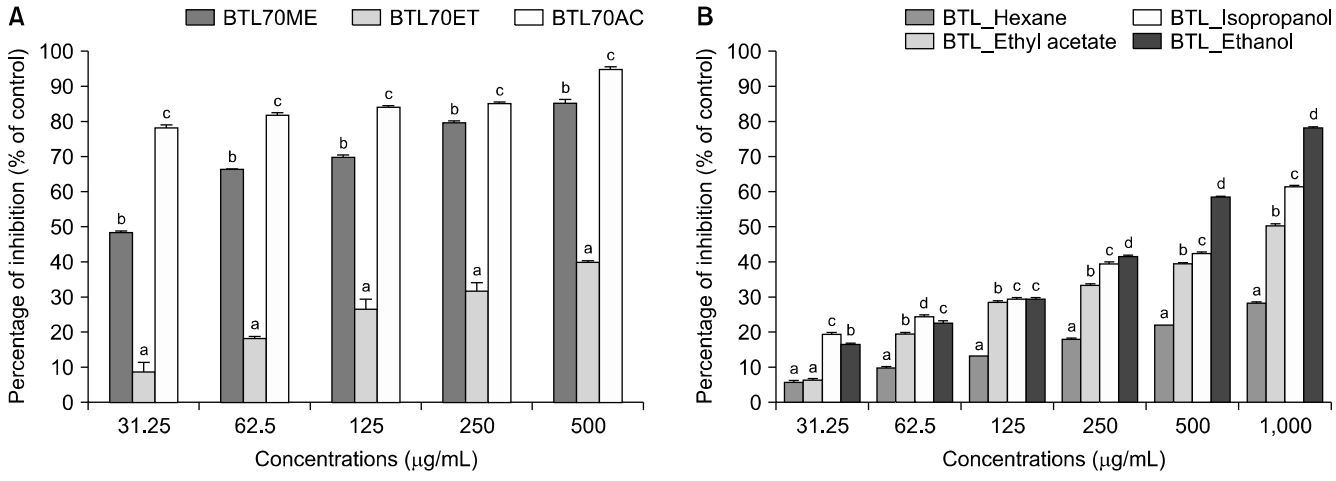

Fig. 2. Antityrosinase activity of the extracts in both groups. (A) Group A and (B) group B. The data are presented as means±SD (n=3). The statistical information was analyzed using one-way ANOVA. When the *P*<0.05, the letters (a-d) showed statistically significant differences within the same concentration.

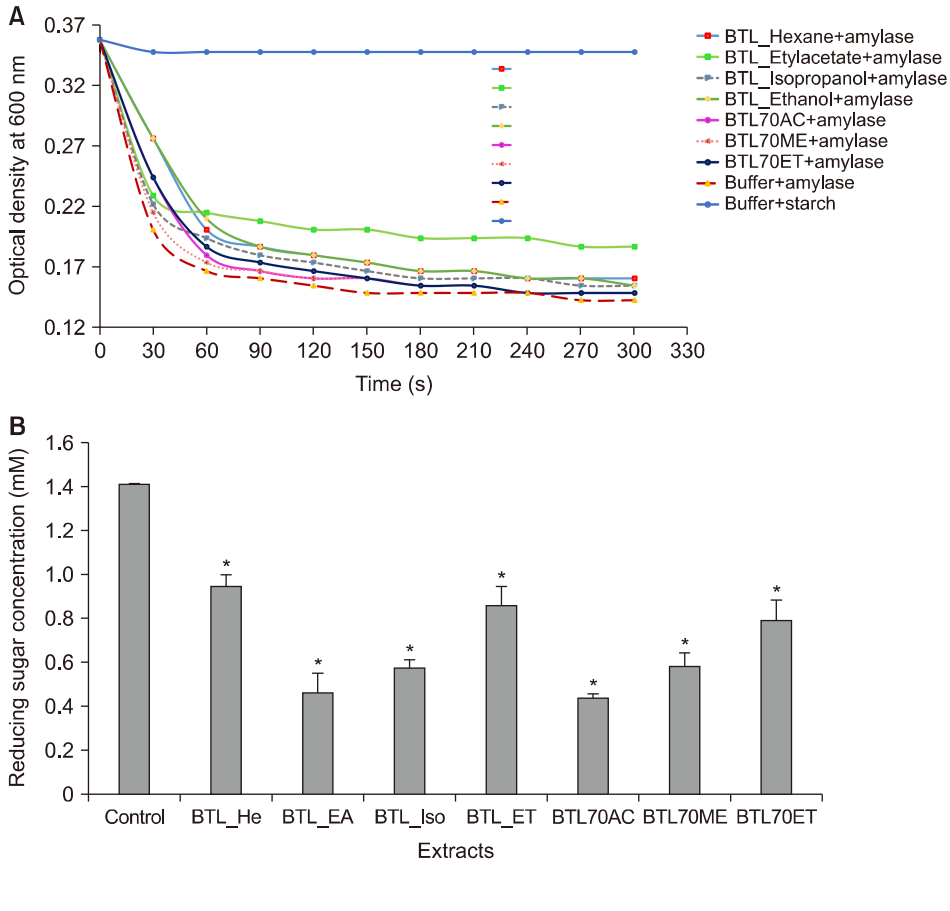

Fig. 3. Results of antiamylase activity tests. (A) Decrease in optical density at 600 nm of starch solution after incubation with buffer, α-amylase, and the group A and B extracts for 5 min. (B) Quantification of reducing sugar products corresponding to α-amylase activity under each condition. Using one-way ANOVA, the absorbance values of the control and all test groups were compared. *Statistical significance was represented as a $P<0.05$. BTL_He, BTL_Hexane; BTL_EA, BTL_Ethyl acetate; BTL_Iso, BTL_Isopropanol; BTL_ET, BTL_Ethanol.

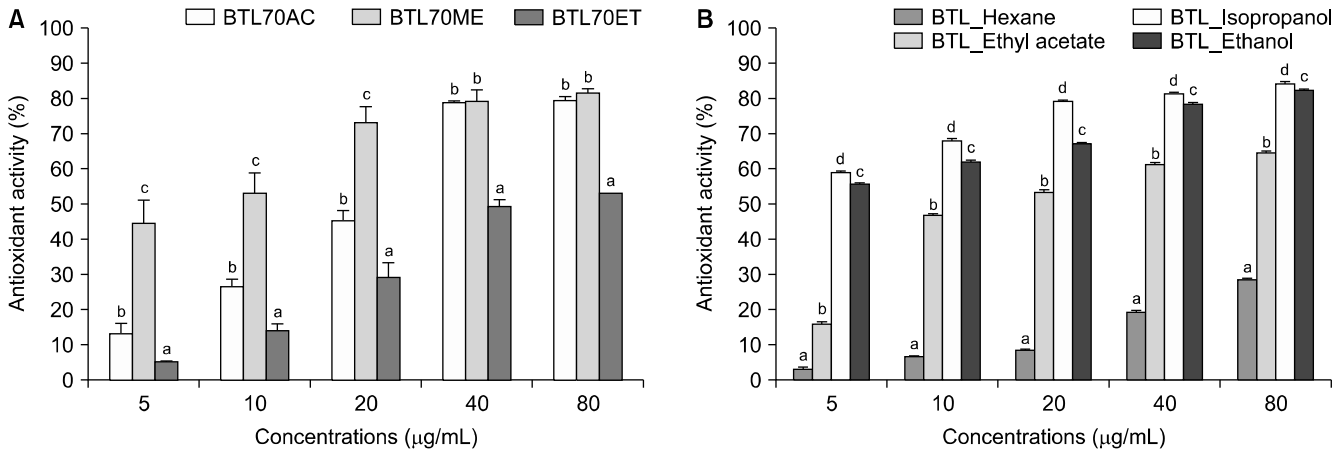

Fig. 4. The antioxidant potential of both extracted groups: (A) group A and (B) group B. The data are presented as means±SD. The letters (a-d) indicated significance different statistically within the same concentration at $P<0.05$.

tracted groups was performed by DPPH assay. The phytochemicals display antioxidant properties by donating the electrons to DPPH (•) radicals, and then the purple color is decreased or changed to a yellow color solution. The colorimetric reaction is determined using the spectrophotometric technique. The results are presented in Fig. 4. The extracts of both groups represented antioxidant activity as dose-dependent. The IC_{50} values of BTL70ME, BTL70ET, and BTL70AC were 16, 37, and 20 μg/mL, respectively. These results differ from those of the group B extracts. The IC_{50} value of BTL_Hexane was >160 μg/mL, whereas the IC_{50} values of BTL_Ethyl acetate, BTL_Isopropanol, and BTL_Ethanol were 9, 4, and 4 μg/mL, respectively. The IC_{50} values of all extracts are summarized in Table 1. Overall, the data suggest that the group A extraction process should be used to obtain bioactive agents with high antioxidant efficacy.

Total phenolic content of group A extracts

The group A extracts exhibited antityrosinase and antioxidant properties. Previous studies have highlighted the crucial role of phenolic content in plant extracts for expressing durable biological properties. Therefore, the total phenolic content of the group A extracts was de-

Table 2. Total phenolic and flavonoid contents of the group A extracts

ID name	Total phenolic contents (mg gallic/g extract)	Total flavonoid contents (mg quercetin/g extract)
BTL70ME	72.29±14.14	53.04±5.22
BTL70ET	65.98±11.91	44.35±13.17
BTL70AC	69.37±7.72	61.74±13.17

termined using Folin-Ciocâlteu reagent with gallic acid as the standard. To calculate the total phenolic content in each sample, the following equation was used: $y=0.0157x-0.0068$ ($R^2=0.9984$). The summarized total phenolic content results are presented in Table 2. BTL 70ME exhibited higher total phenolic content compared with BTL70AC and BTL70ET, although the differences were not statistically significant.

Total flavonoid contents of group A extracts

Flavonoids are a group of phytochemicals known to possess biological activities and efficacy. In this study, the total flavonoid content of the group A extracts was analyzed using quercetin as the standard. The standard equation $y=0.0023x+0.0002$ ($R^2=0.9943$) was used to determine the total flavonoid content in each sample, and the results are presented in Table 2. BTL70AC exhibited higher total flavonoid content compared with BTL70ME and BTL70ET. Therefore, flavonoids may play an essential role in inhibiting tyrosinase and reducing the number of free radicals in BTL70AC extract.

Preliminary analysis of bioactive compounds

A variety of experiments were conducted to determine the types of phytochemicals present in group A and group B extracts, and the results are presented in Table 3. The group A extracts contained a similar range of phytochemicals, although coumarins, and cardiac glycosides were not detected in BTL70AC. These findings suggest that these specific phytochemical types contribute to potential antityrosinase and antioxidant activities. It is possible that certain phytochemicals in group A exhibit a synergistic effect that enhances their biological properties. In contrast, the extraction process used for group B separates different types of phytochemicals, which may reduce their potential biological activities. These results support the hypothesis that bioactive compounds in the group A extracts function together synergistically.

CD spectroscopy of tyrosinase

The secondary structure of the tyrosinase enzyme [125 unit/mL tyrosinase in 50 mM phosphate buffer (pH 6.8)] was analyzed using CD spectroscopy, and the results are summarized in Table 4. The percentages of helix, beta-sheet, turn, and other structures in the tyrosinase enzyme were determined to be 11.79, 30.75, 14.97, and 42.45%, respectively. After incubating the enzyme with the substrate (L-Dopa), the percentage of helix structure increased, which is consistent with the induced fit hypothesis. However, the percentages of helix and β-sheet structures in tyrosinase treated with BTL70ME differed from the current conditions (tyrosinase+L-Dopa). The presence of phytochemicals may interfere with the binding ability be-

Table 3. Preliminary phytochemical compounds analysis of *Vernonia amygdalina* leaf extracts in groups A and B

Bioactive compounds	Preliminary analysis						
	BTL70AC	BTL70ET	BTL70ME	BTL_He	BTL_EA	BTL_Iso	BTL_ET
Phenols/phenolics	+	+	+	−	−	+	+
Flavonoids	+	+	+	−	−	+	+
Coumarins	−	+	+	−	−	−	−
Saponins	+	+	+	+	−	+	+
Tannins	−	−	−	−	−	+	−
Terpenoids	+	+	+	−	+	+	+
Steroids	+	+	+	+	+	+	+
Cardiac glycosides	−	+	+	−	−	+	−

"+" indicates a positive test result.
BTL_He, BTL_Hexane; BTL_EA, BTL_Ethyl acetate; BTL_Iso, BTL_Isopropanol; BTL_ET, BTL_Ethanol.

Table 4. Percentage of tyrosinase secondary structures measured using circular dichroism spectroscopy

Conditions	Percentage of secondary structures (%)			
	Helix	Beta sheet	Turn	Others
125.0 unit/mL tyrosinase enzyme in 50 mM phosphate buffer (pH 6.8)	11.79	30.75	14.97	42.45
125.0 unit/mL tyrosinase enzyme+5.0 mM L-Dopa	12.02	30.47	14.97	42.49
125.0 unit/mL tyrosinase enzyme+0.7 mg/mL BTL70ME	11.40	31.25	14.93	42.33
125.0 unit/mL tyrosinase enzyme+0.7 mg/mL BTL70ME+5 mM L-Dopa	11.25	31.38	14.93	42.39

tween tyrosinase and the substrate, leading to a decrease in catalytic properties.

DISCUSSION

Cell degeneration results from intrinsic and extrinsic factors that induce oxidation reactions, generating destructive free radicals within cells. Enzymes can also contribute to cell degeneration by damaging protein structures. Skin pigmentation enzymes, such as those associated with melasma, freckles, spots, deep and shallow wrinkles, and specific diseases, are particularly relevant. To address these issues, two primary goals have emerged: (1) inhibiting related enzymes using inhibitors from different sources, especially natural sources such as plants (Brincat, 2000; Fisher, 2002; Makrantonaki et al., 2006); (2) countering the destructive effects of free radicals through the use of antioxidants. Numerous studies have focused on bioactive compounds, with particular emphasis on plant-derived sources. *V. amygdalina* leaf extracts have shown promise in terms of their biological properties, making them potential candidates for use in cosmetics and healthcare products.

In the present study, *V. amygdalina* leaf extracts were obtained using two solvent systems: (1) an aqueous system (group A), and (2) an order polarity solvent system (group B). The extracts exhibited antityrosinase, antiamylase, and antioxidant activities *in vitro*.

Tyrosinase, a key enzyme involved in melanin synthesis, is influenced by both intrinsic and extrinsic factors. Its activity is closely linked to hyperpigmentation (Kim et al., 2002), melasma, and disorders such as Parkinson's disease. Therefore, tyrosinase inhibitors have been investigated to develop solutions for these issues. In the present study, *V. amygdalina* leaf extracts from both groups exhibited potential inhibition of tyrosinase activity. Group A extracts showed a lower IC_{50} value for antityrosinase activity compared with group B. Among the extracts, BTL70AC exhibited the lowest IC_{50} value, although it was 1.8-fold higher than that of ascorbic acid, and contained phenolics, flavonoids, saponins, terpenoids, and steroids. However, the BTL_Hexane extract exhibited a higher IC_{50} value for antityrosinase activity higher than other extracts. In this extract, steroids were detected, as reported by Bestari et al. (2017), as well as saponins, but phenols/phenolics, flavonoids, terpenes/terpenoids, coumarins, and cardiac glycosides were not detected. Previous studies have highlighted the strong tyrosinase inhibitory properties of polyphenolics and flavonoids (Kim and Uyama, 2005). Therefore, the presence of phenolics, and flavonoids, among other bioactive substances, may contribute to the antityrosinase properties of these extracts.

Tyrosinase enzyme inhibitors can be classified into two types based on their characteristics. The first type consists of specific tyrosinase inactivators known as suicide substrates. These inhibitors form covalent bonds with the tyrosinase enzyme, leading to denaturation of the enzyme and dysfunction. The second type includes specific tyrosinase inhibitors that bind to the enzyme structure, reducing its activity (Bae et al., 2013). Tyrosinase is a copper (Cu)-containing enzyme. For example, mushroom tyrosinase (EC. 1.14.18.1) is a conjugated enzyme with an active site containing two Cu atoms. Tyrosinase-Cu^{2+} exhibits catalytic properties (a holoenzyme), whereas tyrosinase lacking Cu^{2+} exhibits a nonenzymatic function (an apoenzyme). Thus, the presence, and location of Cu ions affect the enzyme's activity. Three isoforms of Cu-tyrosinase complexes exist: oxy-tyrosinase, met-tyrosinase, and deoxytyrosinase. Oxytyrosinase, with two Cu^{2+} ions at the active site, can catalyze both monophenol, and orthodiphenol substrates. Met-tyrosinase, which also binds Cu^{2+}, can only activate orthodiphenol substrates. Deoxytyrosinase lacks the Cu^{2+} ion and cannot catalyze any substrate (Silavi et al., 2012; Ramsden and Riley, 2014). Therefore, conformational changes to enzyme structures, especially from the oxy-tyrosinase isoform to the met-tyrosinase or deoxytyrosinase isoforms, may decrease enzyme activity. Chelation is one mechanism by which bioactive compounds inhibit tyrosinase (Silavi et al., 2012; Bae et al., 2013). Polyphenolic compounds containing orthodihydroxyl phenol in their molecules can bind to transition metals, displacing, or removing them from the active site. This structural change in tyrosinase leads to its inactivation, hindering the catalytic reaction. Therefore, the ability of BTL70ME and BTL70AC crude extracts to inhibit tyrosinase may be attributed to the presence of polyphenolic/phenolic compounds.

Polyphenolic compounds have also been reported to induce conformational changes in proteins. The CD spectra of tyrosinase incubated with *V. amygdalina* leaf crude extract revealed no significant changes in helix, β-sheet, or tertiary structures in phosphate buffer alone, indicating that *V. amygdalina* leaf extract does not affect enzyme denaturation. However, differences in helix and β-sheet percentages were observed upon addition of L-Dopa, indicating induced fit. The treated tyrosinase's conformation is stabilized by phytochemicals that may bind to the substrate. Flavonoids, which contain α-ketone or 3-hydroxy structures, similar to the hydroxyl group in L-Dopa, can compete with L-Dopa at the active site of tyrosinase, reducing L-Dopa oxidation (Chang, 2009; Ebanks et al., 2009).

Controlling blood glucose levels remains a significant concern for diabetic patients. α-Amylase plays a crucial role in hydrolyzing the glycosidic linkage in starch, directly impacting glucose levels. Hence, suppressing α-amylase

activity is a key objective in managing blood glucose levels. *V. amygdalina* leaf extract has been reported to have an antidiabetic effect *in vivo* (Asante et al., 2016), with the aqueous extract exhibiting a significant decrease in blood glucose levels in rats (Oguwike et al., 2013). In the present study, extracts from both groups exhibited antiamylase activity *in vitro*, significantly surpassing the negative control. These results indicate that the phytochemicals in the extracts inhibit amylase activity, leading to lower levels of reducing sugars compared with the control. BTL_Ethyl acetate and BTL70AC extracts exhibited lower quantities of reducing sugar products compared with other extracts, and they contained terpenoids, and steroids. These phytochemical types align with those reported in previous studies (Bestari et al., 2017). Terpenes, a class of phytochemicals, have garnered attention in diabetic therapy owing to their potential in inhibiting α-glucosidase and α-amylase and reducing hyperglycemia and blood glucose levels (Panigrahy et al., 2021). Therefore, the efficacy of antiamylase activity in these extracts may be associated with terpenes and terpenoids. However, the bitter taste of *V. amygdalina* leaf extracts limits their application as a functional or dietary supplement (Oguwike et al., 2013).

Free radicals play a significant role in biomolecule degradation, contributing to the incidence of noncommunicable diseases (Abegunde et al., 2007; Dudonné et al., 2009; Terzic and Waldman, 2011). Consequently, antioxidants are essential agents for protecting biomolecules from high levels of free radicals. In the present study, *V. amygdalina* leaf extracts from both groups exhibited effective DPPH radical scavenging activity that was dose-dependent, consistent with previous studies (Erasto et al., 2007). However, extracts obtained using DI exhibited a relatively weak antioxidant potential, representing <20% antioxidant activity (data not shown), which also aligns with previous findings (Erasto et al., 2007). These results suggest that water alone is not an appropriate solvent for extracting antioxidant agents from *V. amygdalina* leaves. In previous studies, the antioxidant properties of scavenging DPPH radicals have been associated with flavonoids (Yao et al., 2004; Erasto et al., 2007; Patathananone et al., 2019). The separated crude extract of *V. amygdalina* leaf contains three flavones: luteolin, luteolin 7-O-β-glucuronoside, and luteolin 7-O-β-glucoside (Alara et al., 2017). Luteolin exhibits higher antioxidant activity than the synthetic compound butylated hydroxytoluene. In the present study, BTL_Isopropanol and BTL_Ethanol extracts exhibited lower IC_{50} values compared with other extracts and contained detectable flavonoids. Thus, the antioxidant activity of *V. amygdalina* leaf extracts may be associated with these flavones. However, BTL_Hexane and BTL_Ethyl acetate showed antioxidant ability without representative flavonoids, suggesting the involvement of terpenoids and steroids in scavenging DPPH radicals.

Based on the abovementioned results, *V. amygdalina* leaf extract shows potential for development and application in cosmetics, alternative pharmaceutical products, and dietary supplements. The extracted substances can be applied to both humans and animals health.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: SP. Analysis and interpretation: SP, WK. Data collection: SP. Writing the article: all authors. Critical revision of the article: SP. Final approval of the article: all authors. Statistical analysis: WK. Overall responsibility: SP.

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