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Phytochemical Profile and Pharmacological Activity of *Vernonia amygdalina* Delile Stem Bark Extracts Using Different Solvent Extraction

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Abstract

BACKGROUND: *Vernonia amygdalina* is a native plant from Africa which is widely distributed to Asia, especially in Indonesia. Parts of *V. amygdalina* such as roots, leaves, and bark are used by the community as traditional medicines such as antidiabetic, antibacterial, and anticancer.

AIM: This study aims to show the effect of solvents in the *V. amygdalina* stem bark extraction process on phytochemical's content and their correlation with pharmacological activities.

METHODS: *V. amygdalina* extract from stem bark in this study was obtained using the maceration method with different solvents. The extracts were investigated for total phenolic content (TPC) and total flavonoids content (TFC) using calorimetry assay. Principal Component Analysis (PCA) was used to grouping the extracts based on Fourier-transform infrared (FTIR) data. Antioxidant activity of the extracts was done using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethyl benzothiazoline sulphonic acid) (ABTS) assay. While, the cytotoxic activity of the extracts was carried out using MTT assay on PANC-1 cell line. The correlation phytochemical content and pharmacology activities of extracts were analyzed using person correlation method.

RESULTS: There were significantly different TPC and total flavonoid content of extracts ($p < 0.05$). The ethyl acetate extract was identified to have the highest TPC, TFC, DPPH, ABTS, and cytotoxic values of 3.61 ± 0.03 mg GAE/g dry powder, 25.12 ± 0.09 mg QE/g dry powder, 27.12 ± 0.65 μg/mL, 67.02 ± 0.23 μg/mL, and 33.83 ± 0.82 μg/mL, respectively ($p < 0.05$). This is supported by PCA analysis which shows that there are differences in extracts based on FTIR data and there is a strong correlation between TPC and TFC values with antioxidant and cytotoxic activities.

CONCLUSIONS: This study report that each extract of V. amygdalina stem bark gives a distinct phytochemical profile (TPC, TFC, and FTIR spectrum) that contributes to the antioxidant activity and cytotoxic activity.

Introduction

Vernonia amygdalina is the scientific name for bitter leaf. It is widely used as a medicinal herb in Africa and Asia [1]. The leaf, root, and stem of *V. amygdalina* have been utilized for their anti-diabetic [2], antioxidant [3], antibacterial [4], anti-cancer [5], anti-inflammatory [6], and antiplasmodial properties [7]. So far, the part of the plant that is often explored and researched is the leaf. The leaf section of the plant has been identified as having the greatest chemical components and nutritional contents [8]. Many interesting active chemicals were revealed during detailed research into the compound purification of *V. amygdalina* extract, including flavonoids, triterpenoids, saponins, tannins, sesquiterpene lactones, alkaloids, terpenes, phenolics, and steroidal glycosides [9], [10].

In an effort to develop herbal medicines, the search for phytochemical profiles and pharmacological activities of plants must be explored more widely, one of which is by utilizing all parts of the plant [11]. The stem bark of a plant becomes a part that is often forgotten whereas the stem bark contains secondary metabolites as well as leaves [12]. Research on the activity of the bark of *V. amygdalina* is rarely done. It was reported that the hydromethanol extract of the stem bark of *V. amygdalina* contains alkaloids, phenols, flavonoids, saponins, and tannins and has antidiarrheal activity in rats induced by castor oil [13]. Meanwhile, the antipyretic, antiplasmodial, and antioxidant activities of the hydroethanolic stem bark extract of *V. amygdalina* were observed [14].

This study will discuss the effect of solvents in the extraction process on the phytochemical profile and pharmacological activity of the stem bark of *V amygdalina*. It has been found that the extraction of

metabolites using various solvent extraction techniques has a significant impact on the number of metabolites extracted [15], [16]. Bioactive component composition and concentration levels must be constant for biological activity to be consistent [17]. Bioactive components that have biological activity are basically different chemical structures and polarities [18]. For example, phenolic compounds and flavonoids are compounds that have antioxidant and cytotoxic activity which are dissolved in polar solvents [19]. Until now there are no articles has been published about *V. amygdalina* stem bark that was extracted using a various solvent to determine the phytochemical profile and pharmacological activity.

Materials and Methods

Materials

V. amygdalina stem barks were obtained from Medical Plant Garden, Faculty of Pharmacy, Universitas Sumatera Utara, Indonesia. The sample was identified in The Indonesian Institute of Sciences (LIPI) (No: 403/IPH.1.02/if.8/III/2021), Bogor, Indonesia. Methanol, ethanol, n-hexane, ethyl acetate, Folin-Ciocalteu (FC) reagent, sodium carbonate, potassium permanganate, aluminum (III) chloride, potassium acetate, copper (II) chloride, potassium ferricyanide ($K_3Fe(CN)_6$), and trichloroacetic acid were obtained from Merck (Darmstadt, Germany). Gallic acid, quercetin, neocuproine, indigo carmine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethyl benzothiazoline sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA) Fetal Bovine Serum (Gibco), Penicillin-streptomycine (Gibco), and fungizone (Gibco).

Sample preparation and extraction

The stem barks of *V. amygdalina* were dried, crushed into a powder, and sieved with a particle size of 100 mesh. Extraction was performed using a procedure described in a previous study with the maceration technique. About 100g of samples in different places were macerated with 1000 mL n-hexane, ethyl acetate, ethanol, and water for 24 h at room temperature, respectively. After continuous stirring of samples for 6 h, samples left for a further 18 h without stirring. The filtrate was collected and concentrated with a rotary evaporator at 50°C. The extraction procedure was done 3 times with each solvent [20].

Determination of total phenolics

The Folin-Ciocalteu technique was used to estimate the total phenolic content (TPC) in each extract of *V. amygdalina* stem barks, following a protocol published in a previous work. Briefly, 100 μL of each extracts (500 μg/ml) were mixed with 7.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu's reagent (1:10 v/v) and mixed with vortex for 1 mine. After mixing, 1.5 mL of 20% aqueous sodium bicarbonate was added, and the mixture was allowed to stand for 90 min within termittent shaking. The absorbance was measured at 775 nm using a spectrophotometer. Total phenolic concentration is expressed as gallic acid equivalent in mg per gram of extract. The methanol solution was use a blank. All assays were carried out in triplicate [21].

Determination of total flavonoids

The amount of total flavonoid contents (TFC) in the extracts were measured using spectrophotometrically as previously reported. Briefly, 2 mL of each extracts in methanol was mixed with 0.10 mL of 10% aluminum chloride ($AlCl_3{\cdot}_6H_2O$), 0.10 mL of sodium acetate ($NaC_2H_3O_2{\cdot}_3H_2O$) (1 M), and 2.80 mL of distilled water. After incubation of 40 min, absorbance was measured at 432 nm using a spectrophotometer. To calculate the concentration of flavonoids, we prepared a calibration curve using quercetin as standard. The flavonoid concentration is expressed as quercetin equivalents in mg per gram of extract. All assays were carried out in triplicate [22].

DPPH radical scavenging activity

The antioxidant activity of the extracts was determined using the DPPH technique, as reported in a prior work. 0.2mM solution of DPPH• in methanol was prepared and 10 0μl of this solution was added to various concentrations of each extract at the concentrations of 50, 100, 200, and 400 μg/ml. After 60 min, absorbance was measured at 516 nm. Quercetin was used as the reference material. All the tests were performed in triplicate and percentage of inhibition was calculated by comparing the absorbance values of the control and test samples [23].

ABTS radical scavenging activity

The antioxidant activity of the extracts was determined using the ABTS method as reported in a previous study. Briefly, the each extracts with various concentrations with 1 mL ABTS radical solution was homogenized and its absorbance was recorded at 734 nm. PBS blanks were run in each assay and all of measurements were done after at least 6 min. Similarly the reaction mixture of standard group was obtained using quercetin, ABTS scavenging ability was expressed as IC_{50} (μg/mL) [24].

Cytotoxic activity

The each extract was submitted for cytotoxic examination using MTT assay method. Briefly, PANC-1 cell line was grown in n DMEM medium containing 10% Fetal Bovine Serum (Gibco), 1% penicillin-streptomycine (Gibco), and fungizone 0.5% (Gibco) in a flask in a humidified atmosphere (5% CO_2) at 37°C. The inoculums seeded at 1 × 10^4 cells/mL at an optimal volume of 0.1 mL per well. After 24 h incubation, the medium was discharged and treated by each extracts. After incubation for 24 h, the cells were incubated with 0.5 mg/mL MTT for 4 h at 37°C. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, SDS 10% as stopper (Sigma) in 0.01N HCl (Merck) was added to dissolve the formazan crystals. The cells were incubated for 24 h in room temperature and protected from light. After incubation, the cells were shaken, and absorbance was measured using microplate reader at λ 595 nm. The data which were absorbed from each well were converted to percentage of viable cells. The equation to determine viability of cells [25]:

$$\%Viability = \frac{Abs\,of\,treatment - Abs\,of\,medium}{Abs\,of\,control\,cells - Abs\,of\,medium} \times 100\%$$

FTIR spectra and classification of V. amygdalina extracts

Using a Shimadzu hand press, 2 mg of *V. amygdalina* stem barks extract was combined with 200 mg of KBr, then homogenized and shaped into a pellet. A personal computer equipped with OPUS software version 4.2 was used to measure the FTIR spectra in the mid-IR region (4000–400 cm^{-1}). The IR spectra of *V. amygdalina* extracts were also utilized to classify them. The Minitab software version 19 (Minitab LLC, State Collage, Pennsylvania, USA) was used to categorize the *V. amygdalina* extracts using principal component analysis (PCA). The IR spectra were preprocessed using standard normal variate before being applied to the PCA [26].

Statistical analysis

The experiments were conducted in triplicate and the results were provided as mean ± standard deviation. A one-way analysis of variance was used to make statistical comparisons, followed by the Tukey HSD test. At the 95% confidence level ($p<0.05$), a significant difference was identified. Pearson correlation and PCA were used to correlate phytochemical content with antioxidant activity of V amygdalina extracts, respectively. SPSS 22 was used for all statistical analyses (IBM, USA).

Results

Phytochemical profile of V. amygdalina stem bark extract

An assessment of the phytochemical profile of the *V. amygdalina* stem bark extract was carried out by determining the TPC, TFC, and FTIR spectra. Based on the test results, it can be seen that the difference in solvent causes differences in the results of TPC and TFC. The results are shown in Table 1.

Table 1: The total phenolics and flavonoids content in the four extract of *Vernonia amygdalina* stem barks (n = 3)

Extract	Total phenolics (mg GAE/g dry powder)	Total flavonoids (mg QE/g dry powder)
n-hexane	0.05 ± 0.04	7.13 ± 0.11
Ethyl acetate	3.61 ± 0.03	25.12 ± 0.09
Ethanol	2.51 ± 0.07	19.11 ± 0.12
Water	0.53 ± 0.05	2.72 ± 0.34

The results of the Tukey HSD test showed that there was a significant difference ($p < 0.05$) between each extract group based on the obtained TPC and TFC values. If it is shown from Table 1, the ethyl acetate extract has the highest TPC and TFC values, namely, 3.61 ± 0.03 mg GAE/g dry powder and 25.12 ± 0.09 mg QE/g dry powder. This is very interesting to discuss considering that ethyl acetate is a solvent with a lower polarity compared to ethanol and water. However, if we look at the results of the FTIR spectra of each extract (Figure 1), it can be seen that the ethyl acetate extract has the same number of peaks as the ethanol extract and different if compared with n-hexane extract.

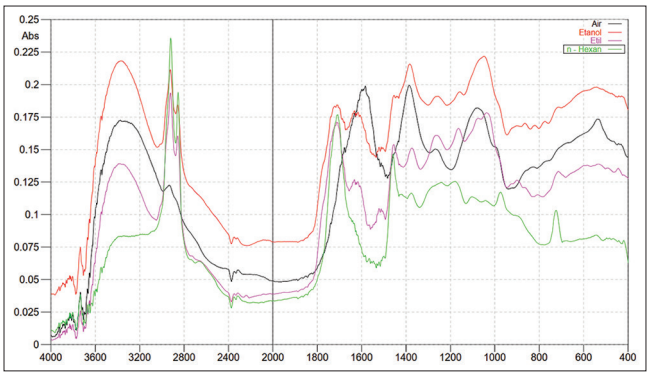

Figure 1: *Representative of FTIR spectrum derived from extract of V. amygdalina stem barks (——water,——ethanol,——ethyl acetate,——n-hexane)*

Classification of *V. amygdalina* stem barks extracts with different solvent extraction was done using FTIR spectra combined with chemometrics. The results of the FTIR spectra of each extract were compared and evaluated. A very interesting difference can be seen in the OH alcohol absorption area, which is 3704-3333 cm^{-1}. In that area, no absorption from the n-hexane extract was seen. The aliphatic C-H uptake area of 2962-2853 cm^{-1} and C=O ketone absorption at 1725-1675 cm^{-1} was not identified in the water extract. The rest of the differences in absorption from each extract can be seen in the fingerprint area, namely 666–1667 cm^{-1} [27]. To facilitate the identification process of the extract's FTIR spectrum, the absorption data were processed by the PCA method using the minitab 19 software, the results of which are shown in Figure 2.

The first two main components (Figure 2) of

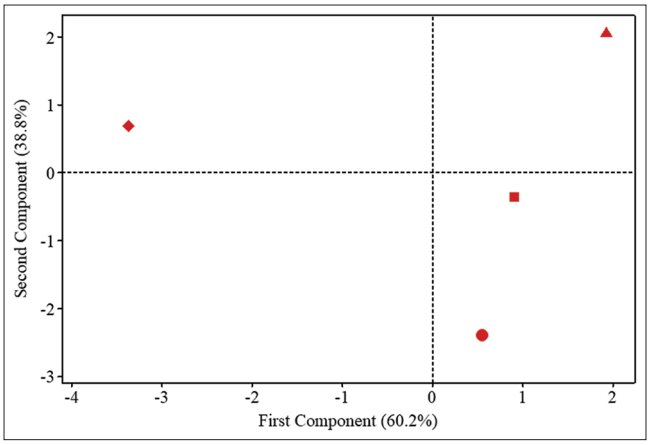

Figure 2: The score plot of PCA derived from extract of V. amygdalina stem barks using variable mid-IR area (4000-400 cm^{-1}) (■= ethanol, ●= ethyl acetate, ▲= n-hexane, ◆= water)

the PCA of the FTIR spectrum data were shown in a two-dimensional plot, accounting for PC1 (60.2%) and PC2 (38.8%) of the 99% total variance. PCA demonstrated a pretty strong differentiating capability between *V. amygdalina* stem barks using four different solvents for the extraction procedure in a dependent manner, according to the PCA score plot. It can be seen that the presence of ethanol extract and ethyl acetate extract because they are in one location. However, water extract and n-hexane extract have differences against other types of extracts.

Antioxidant and cytotoxic activity of V. amygdalina stem bark extracts

Antioxidant activity using DPPH and ABTS methods as well as cytotoxic activity on PANC-1 cells was parameters for the pharmacological effect of *V. amygdalina* extract in this study. The IC_{50} value was determined to describe the extract's ability to inhibit free radicals and the growth of PANC-1 cells. The IC_{50} value of the extract in suppressing free radicals and inhibiting the growth of PANC-1 cells can be seen in Table 2.

Table 2: The IC_{50} of *Vernonia amygdalina* stem barks extract as antioxidant and cytotoxic (n = 3)

Extract	Antioxidant activity		Cytotoxic activity (IC_{50} µg/mL)
	DPPH (IC_{50} µg/mL)	ABTS (IC_{50} µg/mL)	
n-hexane	112.37 ± 0.23	211.13 ± 0.26	158.41 ± 0.81
Ethyl acetate	27.12 ± 0.65	67.02 ± 0.23	33.83 ± 0.82
Ethanol	49.17 ± 0.19	84.09 ± 0.14	118.62 ± 0.81
Water	106.38 ± 0.43	150.36 ± 0.47	206.93 ± 0.42

DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis (3-ethyl benzothiazoline sulphonic acid).

The ethyl acetate extract from *V. amygdalina* showed a reading of the highest of DPPH and ABTS scavenging activity with IC_{50} of 27.12 ± 0.65 µg/mL and 67.02 ± 0.23 µg/mL. The results of the Tukey HSD test showed that there was a significant difference ($p < 0.05$) between the groups. While, the n-hexane extract had the lowest scavenging activity with each IC_{50} value of 112.37 ± 0.23 µg/mL and 211.13 ± 0.26 µg/mL ($p < 0.05$). The ethyl acetate extract also had the best cytotoxic activity on PANC-1 cells compared to other extracts with an IC_{50} value of 33.83 ± 0.82 µg/mL ($p < 0.05$). Meanwhile, water extract had the lowest cytotoxic activity in PANC-1 cells with a value of 206.93 ± 0.42 µg/mL ($p < 0.05$). This is in accordance with the TPC and TFC content of each extract which is shown in Table 1. It is interesting to see whether there is a correlation between the TPC and TFC values of each extract and its pharmacological activity.

Correlation between phytochemical content and pharmacological activity

The correlation between phytochemical contents on the pharmacological activity of four *V. amygdalina* stem barks extracts was carried out using the Pearson correlation. The correlation coefficient (R) between the phytochemical content (total phenolics and flavonoids) in each extract and pharmacological activity was employed as the parameter. The results of this test are shown in Table 3.

Table 3: Pearson correlation phenolic, flavonoids, and pharmacological activity

Extract	Variable	r		
		DPPH	ABTS	Cytotoxic
n-hexane	Phenol	−0.471	−0.702	0.646
	Flavonoid	−0.254	0.999*	0.045
Ethyl acetate	Phenol	0.992	0.997*	−0.994
	Flavonoid	0.992	0.997*	−0.994
Ethanol	Phenol	0.323	0.010	0.265
	Flavonoid	0.548	0.783	−0.923
Water	Phenol	0.881	0.399	0.493
	Flavonoid	0.864	0.335	0.431

*Significant different with $p < 0.05$.

Based on Table 3, it can be seen that the R-value between variables and pharmacological activity. The stronger the correlation between variables, the higher the R-value. It was reported that total phenols and flavonoids had a positive correlation with antioxidant activity using the DPPH method on ethyl acetate extract and ethanol extract with values of 0.992; 0.992; 0.881; and 0.864. Total phenol also has a positive correlation with antioxidant activity using the ABTS method on ethyl acetate extract with the same value of 0.997. However, in n-hexane and ethanol extracts, only total flavonoids had a positive correlation with values of 0.999 and 0.783, respectively. Total phenol and flavonoid in ethyl acetate extract also had a strong correlation with cytotoxic activity with each value of 0.994. While, the total flavonoid ethanol extract had a correlation with its cytotoxic activity with a value of 0.923.

Discussions

The use of bioactive chemicals derived from plants as herbal medicine to improve human health and treat a variety of ailments is gaining popularity [28]. The stem barks of *V. amygdalina* were employed as a natural source of secondary metabolite components

such phenolics and flavonoids in this investigation. There are various procedures involved in extracting bioactive components from the plant, including grinding, milling, homogenization, and extraction [29]. Extraction is the most critical of these stages for recovering and isolating bioactive chemicals from the materials [30]. The extraction method, temperature, extraction duration, phytochemical makeup, and solvent utilized all have a significant impact on extraction efficiency [31]. The solvent is acknowledged as one of the most essential characteristics under the same extraction circumstances, according to the findings of this study [32].

Extraction solvents have an influence on the content of bioactive chemicals, which has a substantial impact on the extract's biological activity [33]. The antioxidant activity of extracts derived from various solvents was investigated in this work utilizing DPPH and ABTS scavenging activity tests. In terms of IC_{50} values for DPPH and ABTS scavenging activity, the ethyl acetate extract was the most powerful of the extracts examined ($p < 0.05$). This could be because this extract contained the highest level of phenolic and flavonoid that be correlated with antioxidant activity (0.997). These chemicals have strong antioxidant properties and so protect the human body from oxidative damage by scavenging a variety of reactive oxygen species such as hydroxyl radicals, peroxyl radicals, hypochlorous acid, peroxynitrite, and superoxide anions [33], [34], [35].

Based on the TPC, TFC of extract and antioxidant activity of extracts, the cytotoxic activity was investigated on PANC-1 cell line. The cytotoxic activity was obtained using MTT assay method. This test was carried out by observing the number of formazan crystals formed as a result of MTT being reduced by living cells [36]. The results presented in Table 2 show the order of the cytotoxic activity of the samples from the largest to the smallest, namely, ethyl acetate > ethanol > n-hexane > water ($p < 0.05$). This activity is based on the IC_{50} value of the extract, the smaller the value, the better the activity [37]. The cytotoxic activity of ethyl acetate extract against PANC-1 cell was in correlation with phytochemical content (−0.994). This is indicating that phytochemical content could play a key role against these cancer cell line due to their ability to remove reactive oxygen species which cause cell damage [38], [39], [40]. According to these results, the varied cytotoxic activity of extracts may be attributed to the difference in the concentration of phenolic and flavonoid compounds in each extracts.

Conclusions

This study reports the extraction of *V. amygdalina* stem barks using different solvent. Among the solvents tested, ethyl acetate was the best solvent for extracting bioactive compounds from *V. amygdalina* stem barks since it resulted in the highest TPC and TFC. The pharmacological activity of the extracts was also investigated. Compared with other extracts, ethyl acetate extract of *V. amygdalina* exhibited the highest antioxidant activity and cytotoxic activity in PANC-1 cell.

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