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Original research article

Effect of Solvent Extraction on Phytochemical Component and Antioxidant Activity of Vine and Rhizome *Ampelocissus martini*

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ABSTRACT

Som Kung, locally known as wild grape, belongs to the Vitaceae family and its scientific name is *Ampelocissus martini*. Its leaf, root and bark are used in traditional Thai medicine for providing relief of symptoms. In this work we studied the phytochemical and antioxidant activities of the hexane, ethyl acetate and methanol crude extracts of vines and rhizomes of *Ampelocissus martini*. Qualitative analysis of the phytochemical screening of the various extracts revealed the presence of terpenoid, flavonoid, saponin, phenolic acid and alkaloid. Total phenolic and flavonoid contents were investigated by using Folin-Ciocalteu and colorimetric aluminum chloride assays, respectively. The results showed that the methanolic extract of vines and rhizomes gave significantly higher total phenolic and flavonoid contents than the ethyl acetate and hexane extracts. In addition, the methanolic and ethyl acetate extracts of both parts had higher antioxidant activities than their hexane extracts. Positive correlation coefficients were observed (r = 0.987) between total phenolic and flavonoid contents and also (r = 0.998) between DPPH and ABTS. The present study provides evidence that solvent extracts of *A. martini* contain important bioactive compounds, especially the methanol extract, which produced a number of phytochemical compounds. Further study will isolate and identify the active compounds of vines and rhizomes from this solvent

Keywords: Phytochemical; Antioxidant; *Ampelocissus martini*; DPPH, ABTS

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1. Introduction

The therapeutic components of plants have long been used to treat human diseases [1]. Most medicines used by western doctors are derived from medicinal plants. Medicinal herbs contain numerous chemical compounds or bioactive ingredients such as terpenoid, alkaloid, phenolic compound, saponin and flavonoid [2]. These phytochemical or bioactive compounds are formed during the plant's normal metabolic process and they are used to protect the plant for survival adaptation. Free radicals are important ions implicated directly in damage to living systems, especially degenerative diseases, heart diseases, stroke and high blood pressure. Antioxidants reduce oxidative stress which could affect and damage biological molecules [3]. Synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), which have been used to prevent oxidation, have been found to cause internal bleeding in rats [4-5], leading to the use of natural antioxidants such as bioactive flavonoid. In addition, phenolic compounds are good natural antioxidants found in a variety of plants such as tea [6].

Wild grape (*Ampelocissus martini* Planch) belongs to the VITACEAE (VITIDACEAE) family which is generally found in Thailand. It is a folk herb ingredient and has been used for a long time. Previous research reported that fruit extracts of *A. martini* have good antibacterial and antioxidant activities [7-10]. However, some activities of another part like the vine and rhizome of *A. martini* have not yet been reported. In the present study, phytochemical screening and total phenolic as well as flavonoid contents and antioxidant activity of *A. martini* extracts were investigated. To the best of our knowledge, this is the first study to extract bioactive compounds from vines and rhizomes of *A. martini* with different solvents and to study the correlation of the phenolic acid and flavonoid compositions obtained with the antioxidant actions observed in DPPH and ABTS assays.

2. Materials and Methods

2.1 Plant collection and successive extraction

Fresh vines and rhizomes of *A. martini* were collected from Tha Sae district, Chumphon province, Thailand. The voucher specimen (S. Aiamyang 1) is deposited at Herbarium, Department of Biology, Faculty of Science, Chiang Mai University. Both parts (Fig. 1) were washed under tap water, cut into small pieces and dried for one week. The maceration was carried out using one thousand and two hundred grams of vine and rhizome of *A. martini* extracted for a week in sequentially polar organic solvents. These solvents were, in order of increasing polarity, hexane, ethyl acetate and methanol. The resulting extracts were filtered with Whatman No. 1 filter paper and the filtrates concentrated by rotary evaporator in a vacuum at 45°C to give dry residues which were kept in a refrigerator until use. The yields of vine extracts from hexane, ethyl acetate and methanol were 0.10%, 0.60% and 4.50%, respectively. For rhizome extracts, the yields were 0.25%, 0.55% and 24.10% from hexane, ethyl acetate and methanol, respective. All extracts were then analyzed for the determination of phytochemical, total phenol and flavonoid content as well as antioxidant activity.

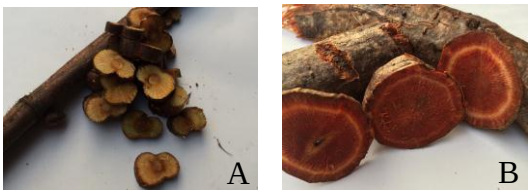

Fig. 1. Fresh vine (A) and fresh rhizome (B) of *A. martini*.

2.2 Standard and reagents

Gallic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2,2′-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid diammo nium salts) (ABTS), butylated hydroxy toluene (BHT), butylated hydroxyanisole (BHA) and Ascorbic acid (Vit.C) were purchased from Sigma-Aldrich. Folin-Ciocalteu reagent and potassium persulfate were purchased from Lobal Chemie. All the chemicals and reagents used in the study were of analytical grade.

2.3 Phytochemical screening

The phytochemical constituents like anthraquinone, terpenoid, flavonoid, saponin, phenolic compounds and alkaloids were investigated by using the individual phytochemical tests regarding methods in our previous work [11] as follows:

Anthraquinone

Each vine and rhizome sample weighing 0.2 g was shaken with 10% of H_2SO_4 and heated for 5 mins. The filtrate extracts were extracted using 5 mL of chloroform for 3 times. The extracts were added to the 3% ammonia solution. A rosy pink color in the ammonia layer indicated that anthraquinone was detected.

Terpenoid

Each vine and rhizome sample weighing 0.2 g was extracted using 5 mL of petrolium-ether for 2-3 times. Chloroform 2 mL was added into the extract. Then 15 mL of concentrated H_2SO_4 was added. A brown-ring between two layers was formed indicating the presence of terpenoid.

Flavonoid

Each vine and rhizome sample weighing 0.2 g was shaken with 95% ethanol. Magnesium (2-3 small pieces) was put into the mixture and filtered. Then 2-3 drops of concentrated HCl was added. Yellowish-orange precipitate appeared, indicating the presence of flavonoid.

Saponin

To each leaf and bark sample weighing 0.2 g., 5 mL of water was added, warmed and filtered. Then 2-3 mL of water was added to the filtrate. The appearance of foam indicated the presence of Saponin.

Phenolic compounds

Each vine and rhizome sample weighing 0.2 g was shaken with water and warmed. The samples were filtered and a few drops of 1% $FeCl_3$ solution was added. The brownish-green or blackish-blue precipitate was an indicator of tannin detection.

Alkaloid

Each vine and rhizome sample weighing 0.2 g was shaken with 2% of H_2SO_4 for 15 mL and warmed for 2 minutes. The samples were filtered and 2-3 drops of Dragendroff's reagent were added into the filtrates. The formation of brownish-orange precipitate indicated the presence of alkaloids.

2.4 Total phenolic content determination

The total phenolic content (TPC) of the vine and rhizome crude extracts was determined using the Folin-Ciocalteu method [12] using gallic acid as the standard. Each sample was prepared by dissolving 10 mg of each dried extract in 10 mL methanol to obtain 6 sample solutions. A concentration of 1 mg/mL and 0.2 mL of each sample was pipetted into a vial containing 2.5 mL of H_2O. Folin-Ciocalteu reagent (0.2 mL) was then added, mixed well and left for one minute. Finally, 2 ml of Na_2CO_3 solution (7%) was added and the mixture was incubated for 60 min in the dark at room temperature. The absorbance was recorded at 765 nm using a UV-vis spectrophotometer. The total phenolic content for each sample was calculated alongside the standard curve of gallic acid at concentration 50-450 μg/ml and expressed in terms of gallic acid equivalents (GAE) per gram of crude extract.

2.5 Total flavonoid content determination

The total flavonoid content (TFC) of the vine and rhizome crude extracts was determined using the Aluminium Chloride method modified by the method of Zhishen et al [13] using rutin as the standard. Two hundred microliters of 1 mg per milliliter concentration of each sample was placed in a centrifuge tube and 0.5 mL of $NaNO_2$ (5%) was added. The reaction mixture was left to stand for 6 min at room temperature. Then, 0.2 mL of $AlCl_3$ was added. After 5 min, 1 M of the NaOH 0.5 mL was added and the total was made up to 1.5 mL with distilled water. The solution was mixed well again and the absorbance was measured against a blank at 510 nm with UV-Visible spectrophotometer. The total flavonoid content for each sample was calibrated alongside the standard curve of rutin at concentrations of 50-550 μg/ml and expressed in terms of rutin equivalents (RU) per gram of crude extract.

2.6 Antioxidant activity

2.6.1 DPPH free radical scavenging assay

The free radical scavenging activity of extracts was determined using the DPPH method as described by Seethalaxmi et al [14]. The reaction mixture consisted of 1 mL of each test sample and standard mixed with 1mL of 0.15 mM methanolic DPPH solution. The mixture was vortexed and incubated at room temperature in the dark for 30 min. The remaining DPPH was measured for absorbance at 517 nm using a spectrophotometer. The data were presented as the average of triplicate analyses. Methanol was used as a blank. BHT (Butylated hydroxytoluene), BHA (butylated hydroxyanisole) and Vit.C (Ascorbic acid) were used as positive controls. The IC_{50} value of each concentration was determined by plotting the inhibition percentage of DPPH free radicals. The inhibition of DPPH free radicals as a percentage was calculated as % Inhibition defined as $(1- (A_{sample} - A_{sample\ blank})/A_{control})) \times 100$, where A_{sample} is the absorbance of the test sample with DPPH solution, $A_{sample\ blank}$ is the absorbance of the test sample only, and $A_{control}$ is the absorbance of DPPH solution. The antioxidant activity of the test sample was expressed as the effective concentration of the extract (mg/mL) required for scavenging the free radicals. All measurements were performed in triplicate and expressed as the average values. IC_{50} was calculated by plotting inhibition percentages against sample concentrations. BHT, BHA and Vit C were used as standards.

2.6.2 ABTS free radical scavenging assay

This decoloration assay was used to determine free radical by using 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonate radical cation ($ABTS^+$) as described by Re et al [15]. ABTS radical cation ($ABTS^+$) was prepared by mixing 50 ml (7 mM) of ABTS with 880 μL (140 mM) of $K_2S_2O_8$ (potassium persulfate) and keeping it away from light for 16 hours at room temperature. It was then diluted with methanol to give an absorbance of 0.700 ± 0.025 units at 734 nm using a spectrophotometer. Then 0.1 mL of sample extract was added to 0.9 mL of diluted $ABTS^+$ solution. The reaction mixture was shaken and left for 6 min at room temperature. After incubation, absorbance was measured at 734 nm. The free radical scavenging assay was carried out in triplicate. The ABTS radical scavenging capacity was calculated as % Inhibition define as $[(A_{control} - A_{sample})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the extract without $ABTS^+$ solution, A_{sample} is the absorbance of the extract with $ABTS^+$ solution.

2.7 Statistical analysis

Data were repeated as mean ± SD for triplicate determinations. Duncan's multiple range test (DMRT) was used to determine

the correlation between means. In addition, Pearson Correlation was used to study the correlation between the total phenol, flavonoid content and antioxidant activity (DPPH and ABTS).

3. Results and Discussion

3.1 Phytochemical screening

In the present study, six crude extract of *A. martini* vine and rhizome were successively macerated with hexane, ethyl acetate and methanol. The results of different extracts have been tested for anthraquinone, terpenoid, flavonoid, saponin, phenolic compounds and alkaloid as presented in Table 1. We found only terpenoids and alkaloid in hexane extract of vine and rhizome. However, all the phytochemical substances except anthrquinone were found in ethyl acetate and methanol extracts. These types of phytochemical compounds are known to have medicinal importance [16-18].

3.2 Total phenolic and flavonoid contents of the extracts

Antioxidant activity, especially of plant and herb extracts, is often determined by the total phenolic and flavonoid contents, and these contents of the vine and rhizome extracts of *A. martini* are presented in Table 2. The total phenolic content of all extracts was determined by the Folin-Ciocalteu method and the data shown in Fig. 2. The total phenols of *A. martini* extracts were calculated according to the equation as gallic acid equivalent (GAE mg/g crude extract). The total phenolic contents show values between 0.04 to 4.22 mg GAE/g crude in the various extracts. The methanolic extracts had higher contents of phenolics (1.96 and 4.22 mg GAE/ g of crude extract) than hexane (0.04 mg GAE/ g of crude extract) and ethyl acetate extracts (0.65 and 1.42 mg GAE/ g of crude extract). It has been hypothesized that the phenolic compounds present in vine and rhizome extracts of *A. martini* may play an important role in their biological activity [19]. The total flavonoid contents of *A. martini* extracts were investigated by colorimetric aluminum chloride assays and calculated according to the equation as rutin equivalents (RU mg/g crude extract). Results for the total flavonoid contents show values between 1.73 to 8.08 mg RU/g crude in the various extracts. As with phenolic contents, the methanolic extracts contained higher contents of flavonoids (4.00 and 8.08 mg RU/ g of crude extract), compared to hexane and ethyl acetate extracts. The difference in the polarity of the extracted solvent could be the reason for the variation in the polyphenol and flavonoid contents of the extracts. Methanol has the highest polarity followed by ethyl acetate and hexane, respectively. Results from above data showed that phenolics and flavonoids have a similar polarity with methanol which gives a rich source of bioactive constituent [20]. Correlation analysis was performed on the polyphenol content analysis method for the vine and rhizome extracts. The result showed TPC and TFC correlation was 0.987 which is significantly correlated as shown in Fig. 3. Similar correlations were also found by other researchers [12]. The high total phenol and flavonoid contents of vine and rhizome of *A. martini* may play an important role in their antioxidant activity [21].

Table 1. Phytochemical constituent of the extracts of *A. martini*.

Partes used	Phytoconstituents	*A. martini*		
		Hexane extract	Ethyl acetate	Methanol extract
Vine	Anthraquinone	-	-	-
	Terpenoid	+	+	+
	Flavonoid	-	+	+
	Saponin	-	+	+
	Phenolic compounds	-	+	+
	Alkaloid	+	+	+
Rhizome	Anthraquinone	-	-	-
	Terpenoid	+	+	+
	Flavonoid	-	+	+
	Saponin	-	+	+
	Phenolic compounds	-	+	+
	Alkaloid	+	+	+

Note: "+" means presence and "-" means absence

Table 2. Total phenolic and total flavonoid contents wild grape extracts.

Plant	Used Parts	Solvents	Total phenolic content (mg GAE)/ g CE	Total flavonoid content (mg RU/ g CE)
A.martini	Vine	Hexane	0.04 ± 0.00^{a}	1.91 ± 0.26^{a}
		Ethyl acetate	0.65 ± 0.03^{b}	2.61 ± 0.16^{b}
		Methanol	1.96 ± 0.08^{d}	4.00 ± 0.10^{d}
	Rhizome	Hexane	0.04 ± 0.01^{a}	1.73 ± 0.24^{a}
		Ethyl acetate	1.42 ± 0.09^{c}	3.11 ± 0.13^{c}
		Methanol	4.22 ± 0.28^{e}	8.08 ± 0.23^{e}

Note: data presented as mean ± SD from analysis of three samples, in triplicate; superscripts a – e indicate the difference compared with the others (in the same column) is significant at 0.05 level TPC and TFC from higher to lowest values.

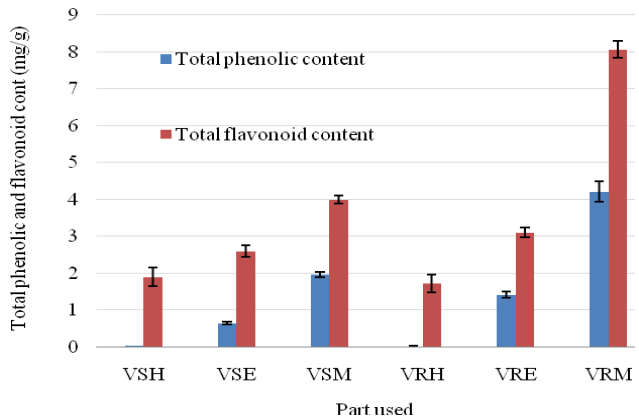

Fig. 2. Total phenolic and flavonoid contents of vine and rhizome extract of *A. martini*. VSH, VSE, VSM are vine of *A. martini* extracted by hexane, ethyl acetate and methanol, respectively. VRH, VRE, VRM are rhizome of *A. martini* extracted by hexane, ethyl acetate and methanol, respectively.

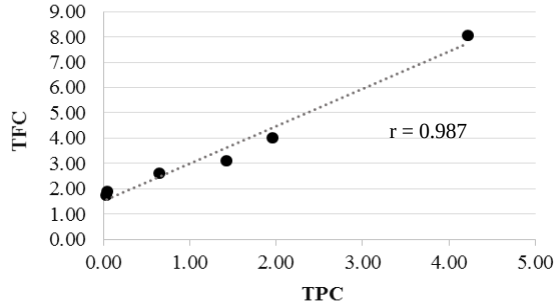

Fig.3. Correlation plot between total phenolic content (TPC) and total flavonoid (TFC).

3.3 DPPH and ABTS free radical scavenging activity

The antioxidant capacities were studied with 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis-(3-ethylbenzo thiazoline-6-sulfonic acid diammonium salts (ABTS) methods. The DPPH method is used to measure free radical scavenging activity by testing the oxidation reaction of crude extracts when reacted with DPPH, which is a stable purple free radical at room temperature. When DPPH accepts electrons or hydrogen free radicals, it changes from purple to the yellow color of DPPH:H. This ability of the DPPH free radical to undertake reduction by an antioxidant is measured in terms of the reduction in its absorbance at 517 nm. In our work, the extracted vine and rhizome of *A. martini* show the difference of DPPH scavenging activity in a range from 16.19% to 96.13% in different solvent. The results of these methods are shown in Table 3. The methanolic extracts of vine and rhizome of *A. martini* showed the highest antioxidant activity with IC_{50} 11.77 ± 0.14 and 4.84 ± 0.42 μg/mL, respectively, followed by ethyl acetate and hexane. As a result of methanol solvent, the phytochemical constituents were extracted in high yield and give more antioxidant activity. Generally, phenolic compounds are major components in plants which play an important role in antioxidants. It may be due to hydroxyl groups existing in the phenolic compounds' chemical structure providing the necessary component as a radical scavenger [22].

Additionally, the $ABTS^+$ method is also a good tool to determine antioxidant activity. The decolorization of $ABTS^+$ radical indicates the capacity of an antioxidant species to provide electrons or hydrogen atoms to inactivate the radical species. In this study, the samples exhibited a decrease in absorption with moderate scavenging activity, observed ranging from 4.53% to 99.01% in vines and rhizomes. The results from our study suggest good agreement between the DPPH and $ABTS^+$ assays, the methanolic extracts of vines (6.02 ± 0.10 μg/mL) and rhizomes (3.97 ± 0.61 μg/mL) presented better antioxidant activity than ethyl acetate and hexane extracts for $ABTS^+$ method (Table 3). In comparison, it appeared that antioxidant activities of the positive control, BHT (IC_{50} = 10.40 ± 0.00 and 10.73 ± 0.12 μg/mL for DPPH and ABTS, respectively) were higher than those of methanolic rhizome extracts but similar to BHA (IC_{50} = 3.44 ± 0.00 and 2.41 ± 0.09 μg/mL for DPPH and ABTS, respectively). However, the use of synthetic antioxidants may be harmful to humans when given in excessive amounts or may be accumulated within the body over time. As a result, various diseases such as liver cancer and high blood pressure could occur.

3.4 Relationship between antioxidant activity of TPC and TFC

Correlation between amount of phenolic and flavonoid of medicinal plants on antioxidant activity has been previously reported by different authors [23-24]. The results are presented in Table 4. A good correlation between TPC and TFC in *A. martini* was observed (r = 0.987). In addition, TPC and TFC of both parts were also significantly positively correlated with DPPH and ABTS activities. Such positive correlation of phenolic and flavonoid with antioxidant activity was observed in Liu's research [25]. Moreover, it was reported that phenolic and flavonoid could be related to other antioxidant compounds contained in the vine and rhizome like animo acid and protein which can also react with Folin-Ciocalteu reagent. To increase the antioxidant effect in extracts of *A. martini*, it seems important to analyze and purify their phenolic and flavonoid compounds in further studies.

Table 3. Antioxidant activity of wild grape vine and rhizome extracts by DPPH and ABTS assays.

Plant	Used Parts	Solvents	IC_{50} (μg/mL)	
			DPPH	ABTS
A.martini	Vine	Hexane	> 500	> 500
		Ethyl acetate	108.76 ± 2.12	42.47 ± 1.71
		Methanol	11.77 ± 0.14	6.02 ± 0.10
	Rhizome	Hexane	> 500	> 500
		Ethyl acetate	63.93 ± 0.33	24.02 ± 4.19
		Methanol	4.84 ± 0.43	3.97 ± 0.61
	BHT		10.40 ± 0.00	10.73 ± 0.12
	BHA		3.44 ± 0.00	2.41 ± 0.09
	Vit. C		1.41 ± 0.00	2.66 ± 0.10

Note: data presented as mean ± SD from analysis of three samples, in triplicate

Table 4. Regression coefficients (r2 value) for antioxidant activity analyzed by different methods and the relative influence of antioxidant components.

Methods of analysis of antioxidant activity	Vine		Rhizome	
	TPC	TFC	TPC	TFC
IC_{50} (μg/mL) of DPPH method	-0.782*	-0.701*	-0.751*	-0.759*
IC_{50} (μg/mL) of ABTS method	-0.771*	-0.688*	-0.746*	-0.752*

Note: Data were statistically analyzed using Pearson correlation coefficient test. *Indicates a significant difference at the level of $P < 0.05$

4. Conclusion

The result of the present study concluded that the appropriate solvent in extracting bioactive compounds from *A. martini* is methanol. A rich source of bioactive compounds such as terpenoid, flavonoid, saponin, phenolic acid and alkaloid were observed in methanolic extracts and it also showed high antioxidant activity. In comparison to synthetic antioxidant (BHT, BHA, Vit.C), the antioxidant activity of methanolic extracts from *A. martini* rhizome was similar to synthetic compounds. Therefore, *A. martini* may be a good source of natural antioxidant which decreases the risk of disease derived from free radicals or oxidative stress in body. In addition, *A. martini* may be used

for several applications in medicine, and in the pharmaceutical and food industries.

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