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Phytochemical, antioxidant and antibacterial activities in different maturity stages of wild grape (*Ampelocissus martinii* Planch.) fruits

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Abstract

This present work investigated phytochemicals, antioxidant and antibacterial activities of methanolic extracts of wild grape (*Ampelocissus martinii* Planch.) fruits in different maturity states. The young stage composed highest total phenolic and total flavonoid while ripe extract had highest total anthocyanin and total saponin. With antioxidant activities, young stage of wild grape were found to have low IC_{50} values (41.50 µg/mL), the concentrations of the extracts that exhibit 50% reduction in 2,2-diphenyl-1-picryl hydrazyl hydrate (DPPH), and highest scavenging of 2,20-azinobis 3-ethylbenzothiazoline-6-sulfonate ($ABTS^{\bullet+}$) radicals (18.96 µg/mL). Moreover, the extracts of young stage have highest reduce metals, Fe (FRAP) (15.76 mM $FeSO_4$/g) and Cu (CUPRAC) (17.13 mgTE/g), thus indicating high antioxidant activities. All tested phytochemicals showed directly correlated to the scavenging activity of DPPH and ABTS radicals and the ferric and cupric reducing ability.

Keywords: Biological activity, extract, flavonoid, phenolic, wild grape

INTRODUCTION

Medicinal plants have been used for traditional treatment of various diseases since long history until now, especially in development country [1]. Natural product which derived mainly from plant has been discovered and reported [2]. The phytochemical compounds obtained from plant composed of different structures which are secondary metabolites such as phenols, flavonoids, quinines, tannins, alkaloids, saponins and sterols [3]. In recent, some important drugs have been derived and developed from natural products including drug cancer [4,5]. Many researchers are interested in

discovery new natural products and study their biological activity. Among the activity of phytochemical, antioxidant and antibacterial activities have been focused. Free radicals are the main causes of oxidative stress. This condition has been proved that is a risk factor of many degenerative diseases such as cancer, heart failure and aging [6-8], chronic inflammatory, diabetes, pressure, cataract, rheumatoid, malaria, Parkinson and Alzheimer [9]. Moreover, oxidative stress also affects on DNA and protein structures included some diseases caused from both biomolecules [10].

Grapes are a commercial crop grown worldwide. It is well know that grape is an excellent source of phytochemicals [11]. Many compounds have been reported including monomeric flavanols, catechin and epicatechin, dimeric, trimeric and polymeric procyanidins, gallic acid, anthocyanins [12]. These compounds consisted of antioxidant [13], antibacterial [14], antimutagenic, antineoplastic, reduce low-density lipoprotein (LDL) oxidation, allergic inflammation, anti-inflammatory, antiarthritic, anti-allergic and anti-cancer [15]. In the central of the North-East of Thailand, Maha Sarakham Province, we collected a wild herb grape (*Ampelocissus martinii* Planch.). It is similar stem, fruit, leaf and maturity stage of growth compared to the commercial grape. In the past, leaf and root of wild grape have been used as medicinal plane for abscess treatment and relieve intestinal pain. However, the phytochemical components in wild grape are very rare available. This work aimed to investigate phytochemicals, antioxidant and antibacterial activities of methanolic extracts of wild grape fruits in different maturity states. The influence of the growth stage on their active ingredient and biological activity were being discussed.

MATERIALS AND METHODS

Reagents and chemicals

2,2′-Azino-bis (3-ethybenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picryl hydrazyl hydrate (DPPH), were purchased from Sigma-Aldrich (Singapore). Aluminium chloride ($AlCl_3$) was purchased from Merck (England). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and Folin-Ciocalteu's reagent were purchased from Carlo Erba Reagents. 2,4,6-Tri (2-pyridyl)-s-triazine ($C_{18}H_{12}N_6$) was purchased from Acros organics. (±)-catechin hydrate ($C_{15}H_{14}O_6$), ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) and gallic acid were purchased from Univar. All other chemicals and reagents of analytical grade were used.

Preparation of wild grape extracts

Fresh fruits of wild grape were collected in August 2015 grouped into young (green), mature (red) and rip (black). Then they were dried in an oven at 100 ˚C for 3 days. The dried fruits were grinded to small pieces. To extract phytochemical, 1 g of powder was mixed with 10 mL of methanol using Soxhlet extractor. All of extracts were concentrated by rotary evaporation.

Determination of total phenolic content

The amount of total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent according to the method of Bonoli et al. [16]. Briefly, 50 mL of crude extract

was mixed with 3 mL of 10% Folin-Ciocalteu reagent (diluted 10 fold with distilled water), then stand at room temperature for 15 min. The mixture was then added with 1.5 mL of 10% (w/v) sodium carbonate solution and left for 15 min. The absorbance of all samples was measured at 750 nm using an UV-Vis spectrophotometer (UV-1610, Shimadzu). The experiment was performed in triplicate. The TPC was analyzed against gallic acid calibration curve and expressed as milligrams of gallic acid equivalents per grams of fresh weight (mg GAE/g).

Determination of total flavonoid content

The total flavonoid content (TFC) was evaluated according to the modified method of Yang et al. [17]. Briefly, the 250 µL of extract was mixed with 1.25 mL of deionized water, 75 µL of 5% sodium nitrite ($NaNO_2$) solution and allowed to stand for 5 min at room temperature. The 150 µL of 10% aluminium chloride ($AlCl_3$) was added to the mixture solution. After reacting for 6 min, 500 µL of 1M sodium hydroxide (NaOH) and 775 µL of distilled water were added to the mixture. The absorbance of all samples was immediately measured at 510 nm. TFC was calculated using the standard curve of (±)-catechin, and expressed as milligrams of catechin equivalents per gram of fresh weight (mg CE/g).

Determination of total anthocyanin content

Total anthocyanin content (TAC) was performed using the method of Boyles and Wrolstad [18]. The extract solution was mixed with 0.025 M KCl buffer pH 1 at 1:2 ratio of extract to buffer. On the other hand, the extract solution was mixed with a sodium acetate buffer pH 4.5 at the same ratio. The absorbance was measured at 510 and 700 nm using UV-Vis spectrophotometer. Total anthocyanin content was calculated by comparison with total monomeric anthocyanin (mg)/kg cyanidin 3-glucoside equivalent (mgC3GE/kg) as followed formula;

$$\text{Total anthocyanin (mg/100g)} = \Delta A \times MW \times Df \times 1000/(\varepsilon \times l)$$

$$\Delta A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$$

Where A_{510} is the absorbance of the extract mixed with KCl buffer, A_{700} is the absorbance of the extract mixed sodium acetate buffer, MW is a molecular weight of cyaniding 3-glucoside, Df is a dilution factor of sample, ε is an absorbtivity of cyaniding 3-glucoside and l is a path length in cm of cuvette.

Determination of total saponin content

The total saponin content (TSC) was assessed as described by Hiai et al. [19]. Briefly, 0.5 mL of extract solution was mixed with 0.5 mL of 8% of vanillin. The mixture solution was mixed with 5 mL concentrated H_2SO_4 (72%) before incubation in a water bath at 60 ˚C for 15 min and then cooled on ice to room temperature. The mixture solution was measured of absorbance at 560 nm. Aescin was used as standard for a calibration curve and the results were expressed as mg of aescin equivalents per gram of dried wild grape (mg Aes/g)

DPPH radical scavenging activity

Free radical scavenging activity was determined using a stable 2,2′-diphenyl-1-picrylhydrazyl (DPPH) following a modified method of Chan et al. [20]. A total of 1.0 mL of crude extract was added to 2.0 mL of 0.1 mM DPPH solution. The mixture solution was incubated at room temperature in a dark room for 30 min. Absorbance of all samples was measured at 517 nm using an UV-Vis spectrophotometer. The percentage of radical scavenging activity as calculated using the following equation;

$$\text{Radical scavenging activity (\%)} = [A_{control} - A_{sample}] / A_{control} \times 100$$

Where, $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the crude extract. BHA dissolved in methanol was also analyzed as control. DPPH radical scavenging activity was expressed as IC_{50} value, which represented the amount of antioxidant in the crude extract necessary to reduce the initial DPPH concentration by 50%. The experiment was performed in triplicates.

ABTS radical scavenging activity

The ABTS assay is performed following the method of Zuleata et al. [21]. Briefly, 2′,2′-azinobis(3-ethylbenzothiazline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) radical cations were prepared by adding potassium persulphate (2.6 mM) to a 7.4 mM aqueous stock solution of $ABTS^{\bullet+}$ in proportion of 1:1 (v/v). The mixture was thoroughly mixed and stands for 16 h prior to use. Butylated hydroxyl anisol (BHA), vitamin C and trolox were used as positive control for comparison. Crude extracts (0.5 mL) were allowed to react with 1 mL ABTS in the dark at room temperature for 5 min then the absorbance was measured at 734 nm using UV-Vis spectrophotometer. The results were expressed as concentration providing 50% scavenging (IC_{50}).

Determination of ferric reducing/antioxidant power assay (FRAP)

The reducing power of the crude extract was detected using a ferric reducing antioxidant power (FRAP) assay described by Benzie and Strain [22] with some modifications. Briefly, the fresh solution of FRAP reagent contained 2.5 mL of 10 mL 2,4,6-Tri (2- pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl with 2.5 mL of mM $FeCl_3$ and 25 mL of 0.3M acetate buffer pH 3.6 was freshly prepared. The 20 µL of crude extract was mixed with 180 µL of FRAP reagent and allowed to stand at 37 ˚C for 4 min. The absorbance of the mixture solution was measured at 593 nm using UV-Vis spectrophotometer. The methanolic solution of know Fe (II) concentration in the range of 50-500 µM ($FeSO_4$) was used as calibration curve. The ferric reducing ability of the crude extracts was expressed as mM of $FeSO_4$ equivalent concentration per gram of fresh weight. BHT and quercitin was used as positive controls. Samples were measured in three replicates.

Cupric reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was explained by Apak et al. [23]. Briefly, 1 mL of $CuCl_2$, 1 mL of neocuproin, 1 mL NH_4Ac pH 7 and 1.1 mL of extract were mixed in a test tube. The mixture solution was incubated at 37˚C for 30 min. The absorbance at 450 nm

was then measured. Trolox was used as standard for calibration curve. The data were indicated as mg of trolox equivalent (TE)/g of crude extract.

STATISTICAL ANALYSIS

All the compounds and parameters reported below were evaluated in triplicate, in each of the samples.

RESULTS AND DISCUSSION

Table 1 indicates that total phenolic content was highest in young (8.19.56 ± 0.35 mg GAE/g), then mature (3.73 ± 0.12 mg GAE/g) and the lowest content was found in ripe stage (1.38 ± 0.12 mg GAE/g). In the similar trend, total flavonoid content was highest in young (17.65 ± 0.69 mg CE/g), then mature (15.15 ± 0.41 mg CE/g) and ripe stage (5.40 ± 0.23 mg CE/g) was the lowest content. In opposite of TPC and TFC, total anthocyanin content was found highest in ripe stage (15.36 ± 0.04 mg C3GE/kg), then mature (12.48 ± 0.02 mg C3GE/kg) and young (2.41 ± 0.01 mg C3GE/kg), respectively. These results were similar trend for total saponin content. The ripe stage was found the highest content of TSC (9.79 ± 0.01 mg Aes/g), then mature (8.18 ± 0.03 mg Aes/g) and young stage (1.46 ± 0.01 mg Aes/g). Thus it can be divided into 2 groups for phytochemical containing in wild grape fruit. First is TPC and TFC which are found most in young stage and second is TAC and TSC which are found most in ripe stage. The obtained results did not surprise due to the maturity stage of plant growth is an important on both type and content of natural products [24]. Moreover, the phytochemical compounds are also influenced by various factors such as solvent used for extraction, structure or chemical characteristics, polarity of phytochemical, condition for study as well as procedure to analyze [25,26].

Table 2 indicates antioxidant activity of wild grape fruit analyzed by different methods. The radical scavenging activity of DPPH and ABTS were expressed by IC_{50} values. It means the concentration of antioxidant that can be inhibited free radical by half of initial number. Therefore, lower IC_{50} value indicates high antioxidant activity. The results revealed that young stage exhibited lower IC_{50} values for DPPH (41.50±0.31 μg/mL) and ABTS (18.96±0.18 μg/mL) radicals, respectively. The mature stage shows moderate IC_{50} values for DPPH (63.08±1.92 μg/mL) and ABTS (28.42±0.94 μg/mL). The highest IC_{50} values were found in ripe stage which was 255.13±8.60 μg/mL and 156.80±0.32 μg/mL for DPPH and ABTS radicals, respectively. In general, phytochemical, especially phenolic compounds are the main substances, responsibility for antioxidation reaction. These were caused from the hydroxyl groups in their structure as well as benzene ring [27]. However, antioxidant activity was varied by type of compounds and natural sources [28]. The reducing power metal antioxidant activity of the wild grape extracts were analyzed by FRAP and CUPRAC assays. The results indicated that mature stage shows higher activity by FRAP than standards, Trolox and vitamin C. With CUPRAC, mature stage has higher reducing power activity than young and ripe. In summary, all maturity stage of wild grape has high activity by CUPRAC.

Table 3 shows correlation (r) between phytochemical contents and antioxidant activity. The result shows minus values for DPPH and ABTS assays, due to IC_{50} was used as data for analysis the correlation. The minus means low IC_{50} value is high antioxidant activity. The correlation between TPC and DPPH was -0.987, while ABTS was -0.983). The correlation of TPC and FRAP assay, and TPC and CUPRAC assay was 1.000 and 0.976, respectively. The correlation between TFC and DPPH was -0.998, while ABTS was -0.997. The correlation of TFC and FRAP assay, and TFC and CUPRAC assay was 0.993 and 0.948, respectively. The correlation between TAC and DPPH was -0.992, while ABTS was -0.988. The correlation of TAC and FRAP assay, and TFC and CUPRAC assay was 0.999 and 0.969, respectively. The correlation between TSC and DPPH was -0.961, while ABTS was -0.970. The correlation of TFC and FRAP assay, and TFC and CUPRAC assay was 0.904 and 0.798, respectively. Results show a positive correlation coefficient between all phytochemical content and antioxidant activity assays. This indicated that phenolic compounds are major contributors to antioxidant activity by free radical scavenging and reducing power antioxidant mechanisms [29]. Antioxidant activity is found to be linearly proportional with phenolic contents [30] and have been studied and reported in vegetables, fruits, spices and medicinal herbs [31,32].

Table 1. Phytochemical contents of wild grape fruit in different maturity stages.

Phytochemicals	**young**	**mature**	**ripe**
Total phenolic content (mg GAE/g)	8.19 ± 0.35	3.73 ± 0.12	1.38 ± 0.12
Total flavonoid content (mg CE/g)	17.65 ± 0.69	15.15 ± 0.41	5.40 ± 0.23
Total anthocyanin content (mg C3GE/kg)	2.41 ± 0.01	12.48 ± 0.02	15.36 ± 0.04
Total saponin content (mg Aes/g)	1.46 ± 0.01	8.18 ± 0.03	9.79 ± 0.01

Table 2. Antioxidant activity of wild grape fruits in different maturity stages comparison to standard Trolox and vitamin C.

Stages	$DPPH^{\cdot}$ IC_{50} (µg/mL)	$ABTS^{+\cdot}$ IC_{50} (µg/mL)	FRAP (mM $FeSO_4$/g)	CUPRAC (mg TE/g)
young	41.50±0.31	18.96±0.18	15.76±0.01	17.13±0.02
mature	63.08±1.92	28.42±0.94	12.00±0.01	29.16±0.02
ripe	255.13±8.60	156.80±0.32	9.54±0.01	12.33±0.01
Trolox	4.04±0.06	4.26±0.08	6.07±0.03	-
Vitamin C	2.63±0.03	2.37±0.04	1.93±0.02	-

Table 3. Correlation value (r) of phytochemical and antioxidant activity of wild grape fruits in different maturity stages.

Phytochemicals	DPPH	ABTS	FRAP	CUPRAC
Total phenolic content	-0.987	-0.983	1.000	0.976
Total flavonoid content	-0.998	-0.997	0.993	0.948
Anthocyanin	-0.992	-0.988	0.999	0.969
Saponin	-0.961	-0.970	0.904	0.798

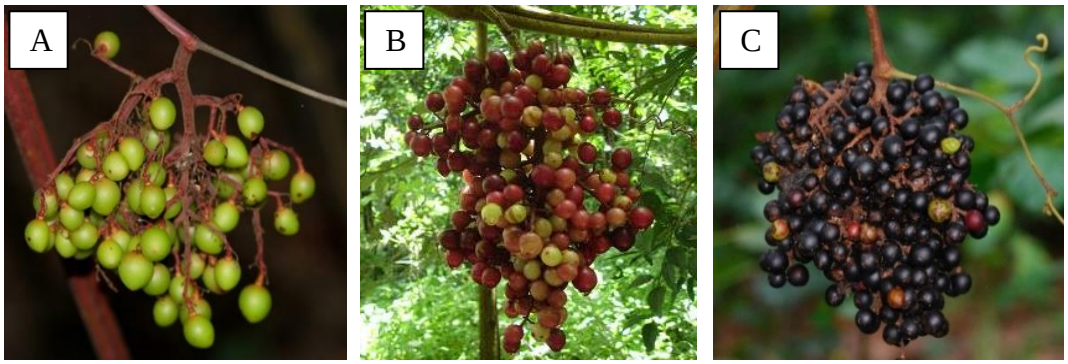

Figure 1. Physical appearances of wild grape fruits in different maturity stages; young (A), mature (B) and ripe (C).

CONCLUSION

The maturity growth stages of wild grape fruit are the main factor on types and contents of phytochemicals. The TPC and TFC were found highest in young stage while TAC and TSC were found highest in ripe stage. The mature stage found moderate contents of all tested phytochemicals. The extracts of young wild grape fruit showed lowest of IC50 values indicating highest free radical scavenging antioxidant activity. For reducing power antioxidant activity, young stage had highest potential by FRAP assay, while mature stage had highest potential for CUPRAC assay. The phenolic compounds in the wild grape fruit extracts were responsible for their antioxidant capacity.

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