ABSTRACT: The research objectives were to quantify the amount of proanthocyanidins and determine the antioxidant property of grape seeds, cinsaut variety, cultivated in Nakhon Ratchasima province. The defatted grape seeds powder was extracted by 2 methods. The first method was soxhlet extraction with ethanol, and the product hereby was a precipitate of crude proanthocyanidins 0.223%. The second method was maceration with ethylacetate and water (90:10). The extract was concentrated under vacuum and then redissolved in ethylacetate. The crude proanthocyanidins were precipitated out 0.216%, when petroleum ether was added into this crude extract solution. The antioxidant activities of these two crude proanthocyanidins were compared with those of L-ascorbic acid and of trolox by spectrophotometry using Ferric Reducing/Antioxidant Power assay. The antioxidant activities per milligram of L-ascorbic acid, trolox and crude proanthocyanidins by soxhlet and maceration were equal to 14.01, 10.50, 2.98 and 3.22 μmole of Fe²⁺, respectively. The total amounts of flavonols in crude proanthocyanidins from soxhlet extraction and maceration were 48.47% and 46.28%, respectively, calculated in catechin equivalent by vanillin–HCl assay.

Key words: grape seeds, proanthocyanidins, Ferric Reducing/Antioxidant Power assay, vanillin–HCl assay

INTRODUCTION

Grape seed extracts are increasingly popular as a dietary supplement. It has been suggested that they demonstrate cytotoxicity towards human breast, lung and gastric adenocarcinoma cells. They may enhance the growth and viability of normal human gastric mucosal cells (1). They provide protection against oxidative stress and free radical–mediated tissue injury (1), inhibit oxidation of human low–density lipoproteins (LDL) in vitro (2), improve cardiac recovery during reperfusion after ischemia (3), and act as antimutagenic as well as antiviral (4) and antimicrobial agents (5). Grape seed extract contains high concentrations of oligomeric proanthocya-
nidins (OPCs), a class of polyphenolic biflavonoids. OPCs are proanthocyanidins containing two or more monomers of ((+)-catechin and (−)-epicatechin), proanthocyanidins polymer and also tannins. The (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-O-gallate, dimer (B₁, B₂, B₃, B₄, B₅, B₆, B₇), trimer C₁, gallate esters of dimers (B₁, B₂, B₃, B₄, B₅), procyanidin B₆ (and etc.) are the major constituents of grape seed OPCs(6). OPCs are water-soluble and have powerful antioxidant properties, but there is a wide variability in the compositions and biological values of the different kinds of grape seeds. The amounts and types of OPCs present in a particular grape seed extract are various, since they are greatly influenced by the extraction process, the sources of the materials and the variety of seeds.

Nowadays, the One Tambon One Product (OTOP) policy is outstanding and is supported in all provinces in Thailand. One product that is very popular is grape wine. To support a greater demand for the grapes used in the production of wine, areas devoted to the cultivation of grape are expanding. Grape seed is a by-product of wine making process. It simply discarded by the vineyards. The aim of this study is : 1) to determine the antioxidant capacity of grape seed extract from of the Vitis vinifera Lin. variety cinsaut, which is cultivated in the Pakchong, Nakhon Ratchasima area, 2) to evaluate the potential of grape seed, from Thai vineyard to the dietary supplement industry.

This study uses Ferric Reducing Antioxidant Power assay (FRAP assay) as an in vitro analytical tool to quantify the antioxidant property of proanthocyanidins in crude proanthocyanidins extract. The FRAP assay utilizes a reducing mechanism. It depends on the reduction of a ferric tripyridyltriazine [Fe³⁺/TPTZ] complex to a ferrous tripyridyltriazine [Fe²⁺/TPTZ] complex (blue colour), by antioxidants(7, 8). FRAP assay is an accurate method for measuring antioxidant activity in phenolics and L-ascorbic acid. The antioxidant capacities of L-ascorbic acid and of trolox, (a water-soluble analog of α-tocopherol), are compared with those of crude proanthocyanidins.

The amounts of total flavonols were determined by vanillin–HCl assay(9) using catechin as a standard. The condensation between ring A of flavonol and vanillin gave bright-red adduct as shown in figure 1.

![Figure 1 The reaction of vanillin–HCl assay](image)

**MATERIALS AND METHODS**

**Plant**

Grape seeds (Vitis vinifera Lin. variety cinsaut) received from vineyard in Nakhon Ratchasima province.

**Chemicals**

Bidistilled water, 2,4,6-tri-pyridyl-s-triazine (TPTZ), and vanillin (99%) from Fluka
Chemie GmbH (Switzerland). FeSO$_4 \times 7$H$_2$O, and sodium acetate from Asia Pacific Specially Chemicals Limited (Seven Hills, Australia). FeCl$_3 \times 6$H$_2$O from Carlo Erba Reagenti. Trolox ((+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, 97%, and catechin from Aldrich. L-Ascorbic acid, and methanol (HPLC grade) from Fisher Scientific UK Limited (Loughborough, UK). Glacial acetic acid, acetone (AR grade), concentrated hydrochloric acid, formic acid (AR grade) and toluene (AR grade) from Merck (Darmstadt, Germany). Redistilled hexane, redistilled ethylacetate, and redistilled petroleum ether are commercial grade.

**Apparatus**
Agilent 8453E UV-Visible Spectroscopy System

**Methods**

**Thin Layer Chromatography**
Stationary phase Silica gel 60 F$_{254}$
Aluminium sheet 20 × 20 cm (Merck)
Mobile phase Toluene : Me$_2$CO : HCO$_2$H (3 : 6 : 1)
Spray reagent 10% w/v Vanillin in conc. HCl

**Ferric Reducing/Antioxidant Power assay (FRAP assay)**

**Preparation of crude proanthocyanidins.**

**Soxhlet**
Eight hundred grams of grape seeds were washed, dried at 45°C and then ground. Grape seed powders were divided into 4 portions (200g per each portion) and quadruplicated as the steps followed. They were defatted with hexane for 2 days by soxhlet. Defatted powder was soxhleted 3 days with 95% ethanol. The ethanol extract was collected. The extracts were evaporated under vacuum by rotary evaporator until the volume was reduced by half. The proanthocyanidins were precipitated after the extracts were left at room temperature for 3 days. The precipitant was collected as crude proanthocyanidins by centrifuge. Crude proanthocyanidins were dried and weighed.

**Maceration**
Eight hundred gram of grape seeds were washed, dried at 45°C and then ground. Grape seed powders were divided into 4 portions (200g per each portion) and made quadruplicated as the steps followed. They were defatted with hexane for 2 days by soxhlet. Defatted powder was macerated 8 times with ethylacetate : water (90:10), each maceration was frequently shaken overnight.$^{(10)}$ The extracts were filtered and evaporated under vacuum by rotary evaporator. Dried extracts were weighed as crude grape seeds extracts. The crude grape seeds extracts were redissolved with a little amount of ethylacetate and then petroleum ether were added. The precipitant was collected as crude proanthocyanidins by centrifuge. Crude proanthocyanidins were dried and weighed.

**Preparation of FRAP reagent.**
This solution was dissolved in bidistilled water.

FRAP reagent was prepared by mixing 30 mL of 0.3 mol/L sodium acetate buffer, pH 3.6, 3 mL of 0.01 mol/L TPTZ in 0.04 mol/L HCl and 3 mL of 0.02 mol/L FeCl$_3 \times 6$H$_2$O.
Standard solutions of FeSO$_4$.7H$_2$O were prepared in concentration 0.1, 0.5, 1, 5, 10, 15, 20 and 25 μmol/10 mL in 50% methanol.

Crude proanthocyanidins solutions were prepared in concentration 0.1, 0.5, 1, 5 and 10 mg/10 mL in methanol.

L-ascorbic acid solutions were prepared in concentration 0.1, 0.5, 1, 5 and 10 mg/10 mL in methanol.

Trolox solutions were prepared in concentration 0.1, 0.5, 1, 5 and 10 mg/10 mL in methanol.

**Calibration curves of Fe$^{2+}$ (FRAP assay).**

FeSO$_4$.7H$_2$O was used as standard substance for preparing calibration curves in concentration 0.1, 0.5, 1, 5, 10, 15, 20 and 25 μmol/10 mL in 50% methanol. The experiments were done in hexaplicate in each concentration.

For calibration, mixing 3 mL FRAP reagent, 300 μL bidistilled water and 100 μL FeSO$_4$.7H$_2$O of each concentration of standard solutions. Absorbance was monitored at 595 nm for 30 mins. Blank was prepared by mixing 3 mL FRAP reagent, 300 μL bidistilled water and 100 μL 50% methanol.

**Calculation of antioxidant activity.**

Calibration curve was plotted between concentrations of FeSO$_4$ and the average of absorbances of each concentration at 30 mins. The coefficient of determination ($r^2$) was calculated from the graph.

**Measurement of samples (crude proanthocyanidins, L-ascorbic acid and trolox).**

All experiments were done in quadruplicate in each concentration.

Mixing 3 mL FRAP reagent, 300 μL bidistilled water and 100 μL in each sample concentration. Absorbance was monitored at 595 nm at 2, 4, 10, 20, 30 mins.

**Preparation of Blank–FRAP reagent.**

Blank–FRAP reagent was prepared by mixing 33 mL of 0.3 mol/L sodium acetate buffer, pH 3.6 and 3 mL of 0.01 mol/L TPTZ in 0.04 mol/L HCl.

Blank was prepared by mixing 3 mL blank–FRAP reagent, 300 μL bidistilled water and 100 μL sample.

**Determination of the amount of total flavonols by vanillin–HCl assay.**

Preparation of vanillin reagents.

1% vanillin in MeOH
4% concentrated HCl in MeOH
8% concentrated HCl in MeOH

**Standard catechin solution** in MeOH (concentration 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.6, 1.0, and 2.0 mg/mL)

**Sample solution of soxhlet and maceration** of crude proanthocyanidins in MeOH, concentration 0.4, 0.8, 1.2, 1.6, and 2.0 mg/mL, respectively.
Calibration curve of catechin (vanillin–HCl assay).

1. The working vanillin reagent must be freshly prepared from mixing equal volumes of 1% vanillin solution and 8% HCl solution together.

2. Mixing 5 mL of working vanillin reagent with 1 mL of standard catechin solution at 1.0 min intervals to one set of standard concentration. Each concentration of standard catechin solution was done in hexaplicate.

3. The mixing solutions were left at room temperature for exactly 20.0 mins before the absorbances were read at 500 nm.

4. Blank of each concentration was prepared by mixing 5 mL 4% HCl with 1 mL of standard catechin solution. The absorbance at 500 nm was then set 0 against these blanks, after it was left at room temperature for exactly 20.0 min.

Measurement of the amount of total flavonol in crude proanthocyanidins extract.

1. Mixing 5 mL of working vanillin reagent with 1 mL of crude proanthocyanidins solution at 1.0 min interval to one set of concentration. Each concentration of crude proanthocyanidins solution was done in quadruplicate.

2. The mixing solutions were left at room temperature for exactly 20.0 min, and then the absorbances were read at 500 nm.

3. Blank of each concentration was prepared by mixing 5 mL of 4% HCl with 1 mL of proanthocyanidin solution. The absorbance at 500 nm was then set 0 against these blanks, after it was left at room temperature for exactly 20.0 min.

RESULTS

The percentage of oil in grape seed powder after defatting by hexane was 13.50%. The percentage of crude proanthocyanidins by soxhlet and maceration were 0.223% and 0.216%, respectively. The identification of proanthocyanidins was accomplished by comparing with the retardation factor ($R_f$) of standards (+) catechin ($R_f = 0.74$) on TLC and then vanillin–HCl solution was sprayed on chromatogram (figure 2).

Figure 2 The TLC chromatogram of crude proanthocyanidins and catechin after vanillin sprayed, A = crude proanthocyanidin from soxhlet, B = Standard catechin, C = crude proanthocyanidin from maceration.
**FRAP assay**

Figure 3 showed that the absorbance capacity of [Fe²⁺/TPTZ] complex was time independent. This meant that Fe²⁺ bound with TPTZ giving a stable blue complex. The absorbance of L-ascorbic acid, trolox and crude proanthocyanidins from soxhlet and maceration were time dependent (see figure 4). They required a period of time for reducing Fe³⁺ to Fe²⁺. L-ascorbic acid and trolox required around 10 mins to reach their full reducing capacities. For crude proanthocyanidins, they needed around 30 mins for reducing Fe³⁺.

![Figure 3](image1.png)

**Figure 3** The absorptivity of Fe²⁺ at 595 nm in various concentration (µmol/100 µL) (n=6) for 30 mins.

![Figure 4](image2.png)

**Figure 4** The absorptivity at 595 nm of Fe²⁺ (0.1 µmol/100 µL), L-ascorbic acid (0.1 mg/100 µL), trolox (0.1 mg/100 µL), crude proanthocyanidins (soxhlet) (0.05 mg/100 µL) and crude proanthocyanidins (maceration) (0.05 mg/100 µL) for 30 mins.
The FRAP assay evaluated the antioxidant activities of polyphenols on the basis of the reduction of Fe$^{3+}$/TPTZ complex to Fe$^{2+}$/TPTZ complex. The antioxidant power of crude proanthocyanidins was measured by the increase in blue colour intensity. The higher amount of Fe$^{2+}$/TPTZ complex increased, the higher intensity in blue colour appeared. Figure 5 showed the calibration curve of antioxidant capacity at 30 mins of [Fe$^{2+}$/TPTZ] complex using Fe$^{2+}$ as standard substance. The curve showed linearity with equation $y = 6.654x$ ($r^2 = 0.9964$).

\[ y = 6.654x \]

![Figure 5](image)

**Figure 5** The calibration curves of antioxidant capacity plotted between amount of Fe$^{2+}$ ($\mu$mol/100 $\mu$L) and absorbance (595 nm) at 30 mins.

The antioxidant activities per milligram of L-ascorbic acid, trolox and crude proanthocyanidins from soxhlet and maceration were 14.01, 10.50, 2.98 and 3.22 $\mu$moles of Fe$^{2+}$, respectively. On the other hand, the amount of antioxidant of crude proanthocyanidins from soxhlet 4.70 and 3.52 mg were equal to 1 mg of L-ascorbic acid and trolox respectively, and the amount of antioxidant of crude proanthocyanidins from maceration 4.35 and 3.26 mg were equal to 1 mg of L-ascorbic acid and trolox, respectively.

The vanillin–HCl assay

The amounts of total flavonols in crude proanthocyanidins were calculated from vanillin–HCl assay. The calibration curve was plotted between concentrations of standard catechin (mg/mL) and absorbance at 500 nm as shown in figure 6. The curve showed linear relationship ($y = 0.554x + 0.0208, r^2 = 0.9985$). From table 1, the amounts of total flavonols in crude proanthocyanidins from soxhlet (48.47%) and maceration (46.28%) were calculated in catechin equivalent.
Figure 6  The correlation curves between concentration of standard catechin (mg/mL) and absorbance (500 nm).

Table 1  The absorbance at 500 nm. \(n = 4\) of crude proanthocyanidin (mg/mL) derived from soxhlet and maceration methods by vanillin–HCl assay.

<table>
<thead>
<tr>
<th>Extraction Methods (concentration)</th>
<th>absorbance (500 nm.)</th>
<th>average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soxhlet (1.6 mg/mL)</td>
<td>Sample A</td>
<td>0.4783</td>
</tr>
<tr>
<td></td>
<td>Sample C</td>
<td>0.4347</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td>0.4504</td>
</tr>
<tr>
<td>Maceration (2 mg/mL)</td>
<td>0.5740</td>
<td>0.5146</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td>0.5336</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The analysis of proanthocyanidin is difficult because its complete extraction is hard to achieve. The amount of proanthocyanidin in grape seed varies depending on the cultivation, method of extraction, and type of extraction solvent. The heated extraction method (soxhlet) makes proanthocyanidin polymerisation during extraction. This phenomenon is proved by proanthocyanidin solubility and TLC chromatogram. The extract from soxhlet has low solubility in ethanol and its TLC chromatogram shows only dense red spot at the base and thin red spot at the top. The crude proanthocyanidin from maceration has high degree of polymerisation of proanthocyanidin comparing to those from soxhlet as shown on TLC chromatogram.

\[
y = 0.554x + 0.0208
\]
\[
R^2 = 0.9985
\]
The antioxidant activities of crude proanthocyanidin by soxhlet and maceration by means of FRAP assay were about 3–5 times lower than L-ascorbic acid and trolox. From this experiment, the mechanism of crude proanthocyanidin for inhibiting radicals was reduction.

The dimer, trimer, tetramer, ..., and polymer of proanthocyanidin are not equally sensitive to vanillin–HCl reaction. The red colour development is reduced unless the polymer is completely hydrolysed by HCl\(^{11}\). So, these can lead to underestimate of proanthocyanidin concentration. Furthermore, vanillin–HCl assay can not differentiate between monomer, dimer, trimer,... and polymer. Therefore, the results of these calculations are the amount of total flavonols (proanthocyanidin + monomer). However, until now, there is no other method with better specificity and accuracy for determining the exact amount of proanthocyanidins. Jayaprakasha and his group evaluated the amount of total flavanols from grape seed (\textit{Vitis vinifera}) extract (ethylacetate : water, 9:1) by vanillin–HCl method using catechin as standard\(^{12}\). They found that the amount of total flavonols is 43 ± 3.34% per 100 g extract. This amount was close to the amount of total flavonols estimated in Thai grape seed by our group.

**CONCLUSION**

The suitable method for extracting proanthocyanidin is maceration. Because it does not cause polymerisation. Crude proanthocyanidins received from maceration dissolves easily in ethanol, so it is suitable for product preparation. Crude proanthocyanidins extracted from Thai grape seeds by soxhlet and maceration had antioxidant properties equal to 2.98 and 3.22 \(\mu\)mole of Fe\(^{2+}\), respectively. The total amount of flavonals in crude proanthocyanidins from soxhlet and maceration that derived from Thai grape seed were 48.47% and 46.28% respectively, calculated in catechin equivalent by vanillin–HCl assay. The grape seeds, cinsaut variety, cultivated in Nakhon Ratchasima, gave antioxidant activities and good yields of crude proanthocyanidins. So they are good sources of crude proanthocyanidins that could be developed and used as dietary supplement.

**ACKNOWLEDGEMENT**

Dr. Tanya Tapen (vineyard owner in Nakhon Ratchasima) and Dr. Pornsak Srimornksak (Department of Pharmaceutical Technology, (faculty of Pharmacy, Silpakorn University) are acknowledged for giving grape seeds.

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การหาทุกข์ต้านอนุมูลอิสระของโปรanthocyanidin ของเมล็ดองุ่นที่ปลูกในประเทศไทย กับวิตามินซี และโทรออกซ์ โดยวิธี FRAP.

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บทคัดย่อ: วัตถุประสงค์ของการวิจัยเพื่อที่จะวิเคราะห์ปริมาณโปรanthocyanidin และอนุมูลอิสระของเมล็ดองุ่นสายพันธุ์ cinsaut ที่ปลูกที่จังหวัดนครราชสีมา มองผ่านการตัดเมล็ดองุ่นขนาด 2 วิธีคือ วิธีที่ 1 สัดส่วนตัวของกลีโคไซด์โดยใช้สูงน้ำอับในด้วิทละลาย จะได้ค่าผลของโปรanthocyanidinประมาณ 0.233% และวิธีที่ 2 หนักยาน เอื้อติวิตอลิตติ : น้ำ (90:10) น้ำสารกลีโคไซด์ที่ได้มาทำให้เข้มข้นภายใต้สูญญาภาพ จากนั้นเอื้อติวิตอลิตติแล้วทดสอบโปรanthocyanidin ขนาดได้รับผลการวิจัย 0.216% ด้วยการเดินปีเตอร์เทลลิ่มอิธ์ เวิร์มเบิร์ดตันเชื้อต้านอนุมูลอิสระของโปรanthocyanidin ขนาดได้รับผลการวิจัย 0.223% นักที่ 1 วิธีกับแลดแอคซิบิล และโทรออกซ์ โดยนักวิจัยได้ตัดวิธี Ferric Reducing/Antioxidant Power พบว่าทุกข์ต้านอนุมูลอิสระในขนาด 1 มิลลิกรัม ของแลดแอคซิบิล โทรออกซ์ และโปรanthocyanidin ขนาดจากวิธีตัดกลีโคไซด์และการหมักที่จะเพิ่มทำให้ปริมาณ FeSO4 14.01, 10.50, 2.98 และ 3.22 ในโคลน ตามลำดับ ปริมาณตัวเอนซิมรวมในโปรanthocyanidin ขนาดจากวิธีตัดกลีโคไซด์ และการหมักเมื่อมีค่าคงในรูปแสดงที่นั้น คิดเป็น 48.47% แต่และ 46.28% ตามลำดับ เมื่อวิเคราะห์ด้วยวิธีวิวัฒนีน-กรดเกลือ