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Short Communication

Condensed tannins in mangosteen pericarps determined from ultra – performance liquid chromatography – mass spectrometry

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Abstract

The objective of the present work was to determine the types and concentrations of condensed tannins in mangosteen pericarps. Three common solvents, methanol, ethanol and acetone, were employed to extract tannins from the mangosteen pericarps. The tannin extracts were depolymerized using 2-mercaptoethanol as a nucleophile. Ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) was used to identify the tannins structurally and to determine their concentrations. UPLC chromatograms showed five different tannin monomers: catechin, epicatechin, catechin mercapto, catechin gallate mercapto and epigallocatechin gallate. Catechin mercapto was the major monomer (~32 to 60 mg/g of extract). The average degree of polymerization indicated that the condensed tannins in the pericarps were mainly dimers.
and trimers. The experimental results showed that methanol was the most efficient extraction solvent in the present work and that UPLC-MS was a powerful technique for evaluating the condensed tannins in mangosteen pericarps in terms of qualitative and quantitative analysis.

**Key words:** condensed tannin, depolymerization, solvent extraction, mangosteen pericarps, UPLC-MS

### 1. Introduction

Mangosteen (*Garcinia mangostana* Linn.) is a tropical fruit tree cultivated in Thailand, Malaysia, Indonesia, the Philippines, Vietnam, Singapore and Myanmar (Naczk, Towsend, Zadernowski, & Shahidi, 2011). The mangosteen fruit comprises 65% pericarp, 31% fresh mangosteen and 4% cap (Chaovanalikit et al., 2012). In Thai traditional medicine, the pericarps have been used for the treatment of skin infections, wounds and diarrhea (Mahabusarakam & Wiriyachitra, 1987). The major chemical components in the pericarp were phenolic compounds, which included xanthones (Al-Massarani et al., 2013; Mahabusarakam & Wiriyachitra, 1987), phenolic acids (Chaovanalikit et al., 2012; Cheok, Chin, Yusof, & Law, 2012), condensed tannins (Fu, Loo, Chia, & Huang, 2007; Zhou, Lin, Wei, & Tam, 2011) and anthocyanins (Chaovanalikit et al., 2012; Mai & Tan, 2013).

Solvent extraction is the most common method for the isolation of phenolic compounds in the mangosteen pericarp and the different solvents used have included benzene (Mahabusarakam & Wiriyachitra, 1987), methanol (Cheok et al., 2012; Jung, Su, Keller, Mehta, & Kinghorn, 2006; Kosem, Han, & Moongkarndi, 2007;
Suksamrarn, Suwannapoch, Rattananukul, Aroonlerk, & Suksamrarn, 2002; Zadernowski, Czaplicki, & Naczk, 2009, ethanol (Al-Massarani et al., 2013; Moosophin, Wethaisong, Seeratchakot, & Kokluecha, 2010; Pothitirat, Chomnawang, Supabphol, & Gritsanapan, 2009; Pothitirat & Gritsanapan, 2009), acetone (Chaovanalikit et al., 2012; Fu et al., 2007; Naczk et al., 2011; Zhou et al., 2011), and ethyl acetate (Chaivisuthangkura et al., 2009). Tannins in mangosteen pericarps were extracted with methanol (Zadernowski et al., 2009), ethanol (Moosophin et al., 2010; Pothitirat et al., 2009) and acetone (Fu et al., 2007; Naczk et al., 2011; Zhou et al., 2011) and although water has been used to extract phenolic compounds in mangosteen pericarps the work of Cheok et al. (2012) ranked the solvation power of solvents on phenolic compounds in mangosteen pericarps in the following order: methanol > acetone = ethanol > distilled water. For this reason, water was not used in the present study. They stated that solvation represented the interaction between a solvent and a molecule or an ion dissolved in that solvent.

The chromatographic techniques used for qualitative and quantitative analysis of plant phenolics have included HPLC (Chen, Fu, Qin, & Huang, 2009; Fu et al., 2007; Pothitirat et al., 2009), high speed counter current chromatography (HSCCC) (Cao, Wang, Pei, & Sun, 2009; Krishnan & Maru, 2006; Yanagida et al., 2006), supercritical fluid chromatography (SFC) (Karnangerpour, Khorassani, Taylor, McNair, & Chorida, 2002; Khoddami, Wilkes, & Roberts, 2013), thin-layer chromatography (TLC) (Rastija & Medić-Šarić, 2009) and gas chromatography (GC) (Zadernowski et al., 2009; Zafra et al., 2006). Mass spectrometry (MS) has also been used to investigate the chemical structures of polyphenols (Fulcrand et al., 2008; Monagas, Quintanilla-López, Gómez-Cordovés, Bartolomé, & Lebrón-Aguilar, 2010). Zhou et al. (2011) characterized
depolymerized tannin by UV-VIS spectrum, HPLC-MS and matrix-assisted laser
desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Fu et al. (2007) characterized the structure of an extract of mangosteen pericarps
by means of nuclear magnetic resonance spectroscopy ($^{13}$C- and $^1$H-NMR), UV-visible
spectrophotometry and high performance liquid chromatography-mass spectrometry
(HPLC-MS). Afzelechin/epiafzelechin, catechin/epicatechin and gallocatechin/
egigallocatechin were present after depolymerization with benzylmercaptan. However,
the concentrations of all the tannins was not been reported. Using mercaptoacetic acid
as a nucleophile, Chen et al. (2009) studied a one-pot extraction/depolymerization in
methanol of condensed tannins in mangosteen pericarps. The obtained major product
was 4β-(carboxymethyl) sulphanyl(-)epicatechin methyl ester. Zhou et al. (2011)
extracted phenolic compounds from mangosteen pericarps using eight different
solvents, and benzylmercaptan as a nucleophile for depolymerization of the tannin.
Three tannin monomers were released as both terminal and extension units:
(epi)catechin, (epi)afzelechin and (epi)gallocatechin.

Recently, ultra-performance liquid chromatography-mass spectrometry (UPLC-
MS) has been employed to characterize the complex structures and determine the
concentrations of natural polymers such as procyanidins and proanthocyanidins (Aouf,
Guernevé, Caillol, & Fulcrand, 2013; Benyahya et al., 2014; Roumeas, Aouf, Dubreucq,
& Fulcrand, 2013). It is a very effective technique due to its very high sensitivity and
selectivity.

As described above, although the common solvents used for the extraction of
tannins from mangosteen pericarps were methanol, ethanol and acetone, the extraction
efficiency of each solvent was not reported. Additionally, most of the cited works have
employed UV-Visible spectrophotometry to determine the concentration of the extracted tannins but none of them has identified the tannins structure. Therefore, the objectives of this work were to compare the efficiency of these solvents for extracting tannins in mangosteen pericarps and to identify the structures and determine the concentrations of the obtained tannins in the extracts using UPLC-MS.

2. Materials and methods

2.1 Materials

Mangosteens (Garcinia mangostana Linn.) were purchased from a fruit shop in Hat Yai, Songkhla, Thailand. The fruit had been cultivated in the South of Thailand. All chemicals were AR grade including methanol (Sigma-Aldrich), ethanol (Sigma-Aldrich), acetone (AnalaR NORMAPUR®, VWR chemicals), trifluoroacetic acid (Sigma-Aldrich), n-hexane (AnalaR NORMAPUR®, VWR chemicals), hydrochloric acid (Riedel-de Haën, 37%) and 2-mercaptoethanol (Sigma-Aldrich).

2.2 Experimental

2.2.1 Extraction of phenolic compounds from mangosteen pericarps powder

Mangosteen pericarps were dried in an oven at 40°C overnight and ground in liquid nitrogen. The pericarps powder was dried in an oven at 60°C for 24 h and kept in a desiccator prior to use. Phenolic compounds were extracted from the pericarps powder using 3 methods: A, B and C.

In method A, 6 g of mangosteen pericarps powder was extracted for 1 h at room temperature (25°C) with aqueous 80%(v/v) methanol at a ratio of 1:10 (weight of mangosteen pericarps powder/ volume of methanol) (Zadernowski et al., 2009). After
extraction, the mixture was centrifuged at 3000 rpm at 4°C for 5 min and filtered through a filter paper to separate the pericarps powder from the solution. Methanol in the solution part was removed in a rotary evaporator at 35°C, and the solution was freeze-dried at -40°C for 2 days to obtain a dry red-brown solid powder (17.77%w/w). The purified extract was kept in the freezer before use. The remaining solid part (the pericarps powder) was extracted again twice with the same process.

In method B, 6 g of the mangosteen pericarps powder was extracted for 1 h at 60°C with a mixture (at a ratio of 1:10 w/v) of 40 mL of ethanol (40%v/v aqueous solution), 1.5 mL of HCl (1.5%v/v aqueous solution) and 58.5 mL of H₂O (Mai & Tan, 2013). After extraction, the mixture was separated, evaporated and freeze-dried similarly to method A. After drying, a red-brown solid powder was obtained (21.31%w/w), and the remaining solid part was also further extracted twice, as in method A.

The extraction by method C was developed by our group and consisted of two steps. Firstly, lipids in 6 g mangosteen pericarps powder were removed with n-hexane (at the ratio 1:10 w/v) at 25°C for 1 h. The mixture was then centrifuged at 3000 rpm at 4°C for 5 min and filtered through filter paper. The lipids removal process was repeated twice. The pericarps powder was then extracted for 1 h at room temperature (25°C) with 60 mL from a mixture of 70 mL of acetone (70%v/v aqueous solution), 0.05 mL of trifluoroacetic acid and 29.95 mL of H₂O. After extraction, the pericarps powder was separated from the extraction solution and the solvent removed following methods A and B. A dry red-brown solid powder was obtained (18.58%w/w) and, as in the other two methods, three successive extractions were carried out.
2.2.2 Depolymerization of phenolic compounds in mangosteen pericarps

A depolymerization solution was prepared from 38.7 mL of methanol, 333 µl of 37% HCl and 1 mL of 2-mercaptoethanol. Twenty milligrams of the extracted powders were reacted in 1 mL of the depolymerization solution for 2 h at 40°C. The mixture was centrifuged and the supernatant, which was the depolymerized tannins extract in solution, was analyzed by UPLC-MS to identify the tannins structure and to determine their concentration (Roumeas et al., 2013).

2.2.3 UPLC-MS Analysis

The UPLC-MS apparatus consisted of a Waters® Acquity UPLC coupled with a diode array detector (UV detector) and a Brucker Daltonics® Ion trap mass spectrometer. Two microliters (µL) of standard and depolymerized extract solutions were injected via the auto sampler into a Nucleosil® 120-3 C18 encapp ed Machery-Nagel® column (100 mm x 2.1 mm, 5 µm particle size). The mobile phase consisted of two solvents: solvent A, water/formic acid (99:1 v/v) and solvent B, acetonitrile/water/formic acid (80:19:1 v/v/v). Phenolic compound solutions were eluted under the conditions described by Benyahya et al. (2014). The mass spectrometer was equipped with an electrospray ionization (ESI) source and was operated in positive ion mode. The analysis was carried out as follows: drying gas flow of 12 L/min, drying gas temperature of 200°C, nebulizer pressure of 44 psi, and capillary voltages of 5500 V. The mass spectra were recorded in the range of m/z 70-1500. The tannins extract solution was prepared by dissolving 20 mg of tannins extract in 2 mL of methanol (10 mg/mL). To find a range of signals suitable for determining the molar relative response
factor (MRRF), standard solutions were prepared following the method of Benyahya et al. (2014).

### 2.2.4 Analysis of tannin monomer concentration and degree of polymerization

The mechanism of the depolymerization of the tannins is proposed in Figure 1. After depolymerization, the condensed tannins are transformed into extension and terminal subunits. The extension subunits are linked to a nucleophilic reagent, whereas the terminal subunits are released as neutral monomers. The extension and terminal subunits were analyzed by UPLC-MS. A quantitative analysis, based on the UPLC chromatograms, was carried out in accordance with Aouf et al. (2014) and Benyahya et al. (2014) to determine the monomer content of the tannins. The area of a spectral peak in a UV chromatogram is proportional to the amount of the substance that was detected by the LC instrument. The quantity of each tannin monomer can be calculated according to equation (1) and (2):

\[
C_x \ (\text{mmol} / \text{L}) = \frac{A_x \ (\text{mg} / \text{L})}{\text{MRRF}_x \ (\text{g} / \text{mol})} \quad (1)
\]

\[
\text{Tannins content (mg/g)} = \frac{A_x \ (\text{mg} / \text{L})}{\text{Concentration of sample (mg/mL)}} \quad (2),
\]

where \(C_x\) was the molar concentration of each tannin monomer (mmol/L), \(A_x\) was the area of the spectral peak of each tannin monomer (mg/L) present in the mangosteen tannin extract, and MRRF\(_x\) was the molar relative response factor of each phenolic standard. MRRF\(_x\) was determined by analyzing the standards of known concentration with UPLC-MS and was calculated by dividing the area of the spectral peak by the molar concentration of the standard, after Benyahya et al. (2014).
The molar concentration of each tannin monomer (from equation 1) was used to calculate the degree of polymerization (DP) according to equation (3) (Vernhet et al., 2011):

\[
DP = \frac{\text{molar amount of (extension units + terminal units)}}{\text{molar amount of terminal units}}
\]

(3).

3. Results and discussion

3.1 Analysis of mangosteen tannin extract before and after depolymerization

Dry red-brown solid powders were obtained from all the extraction methods (Figure 2). The tannin extract from method A was characterized by UPLC-MS (Figure 3, method A before). Broad and overlapped peaks were found, similar to the UPLC spectrum of condensed tannin from grape seed before depolymerization (Roumeas et al., 2013). It is not possible to elucidate the chemical structure of tannins, if they are not first depolymerized.

The tannin polymers can be converted into monomers by using the right nucleophiles in the depolymerization reaction. The nucleophilic agents used have included many sulphur compounds, such as benzyl mercaptan (Fu et al., 2007; Zhou et al., 2011), mercaptoacetic acid (Chen et al., 2009; Vernhet et al., 2011) and 2-mercaptoethanol (Roumeas et al., 2013). In the present work, 2-mercaptomethanol was selected to depolymerize the mangosteen tannin extracts because this reagent has some advantages compared to other nucleophilic reagents. Firstly, 2-mercaptoethanol is less odorous than benzyl mercaptan and mercaptoacetic acid. Mercaptoacetic acid in alcohol may induce esterification as a side reaction: 2-mercaptoethanol does not. Moreover, mercaptoacetic acid may polymerize at room temperature to give thioester (Roumeas et al., 2013). Roumeas et al. (2013) studied the effect of methanol, ethanol and water on
the depolymerization of tannins. According to their results, methanol was the best solvent because it produced the highest yield of depolymerized tannins. Therefore, we used methanol as a solvent and 2-mercaptoethanol as a reagent for the acid depolymerization of the extracted mangosteen tannins.

Chromatograms at 280 nm of the depolymerized extracts A, B and C exhibit five peaks attributed to the monomeric units derived from the pericarp tannins. These peaks correspond to (1) catechin (C), (2) epicatechin (EC), (3) catechin mercapto (C-SCH₂CH₂OH), (4) catechin gallate mercapto (CG-SCH₂CH₂OH) and (5) epigallocatechin gallate (EGCG) (Figure 3). These monomeric units could be confirmed by the retention time of standard condensed tannin from UPLC analysis, and mass number from MS analysis. In addition, the mass number of each monomeric unit was in agreement with the mass number of the same monomeric units in Benyahya et al. (2014). These monomeric units were also present in mangosteen pericarps in the work of Fu et al. (2007). The retention times and mass numbers are listed in Table 1.

The mangosteen tannin extract was depolymerized with 2-mercaptoethanol under mild acidic conditions to release thiolated monomers as extension subunits and monomeric flavan-3-ol as terminal subunits. The thiolated monomers, containing the thiol group of 2-mercaptoethanol (78.13 g/mol), were catechin mercapto (m/z 367) and catechin gallate mercapto (m/z 519). The terminal subunits were released as free monomers of condensed tannin and included catechin, epicatechin and epigallocatechin gallate. The chemical structures of the tannin monomers in the mangosteen tannin extract are shown in Figure 1.

Depolymerization of condensed tannins in mangosteen pericarps was also used to determine the degree of polymerization (DP) of the condensed tannins but this
technique was unable to provide polydispersity (Vernhet et al., 2011). DP may be defined as the average number of monomeric units per molecule in an oligomeric or polymeric condensed tannin. Gu et al. (2002) defined the DP of condensed tannins in the following way: DP 1 = monomers, DP 2-10 = oligomers and DP >10 = polymers. In previous works, the DP of condensed tannin in mangosteen pericarps was in the range of 1.91 to 16.80 (Chen et al., 2009; Fu et al., 2007; Zhou et al., 2011). In the present work, the DP of the condensed tannin from extraction methods A, B and C was 3.56, 3.28 and 2.81, respectively. This indicated that the condensed tannins in our mangosteen pericarps were mainly dimers and trimers.

The concentration of tannin monomers was determined, by means of UV chromatograms, as milligram of monomer per gram of extract (Benyahya et al., 2014; Roumeas et al., 2013). The area of the spectral peak and MRRF obtained from the UV chromatogram of each tannins monomer (Table 2) were used to calculate the molar concentration ($C_x$) and the tannin monomer content. The content of each tannin monomer and total condensed tannin content from extraction methods A, B and C are shown in Table 3. Catechin mercapto was the major product (32-60 mg/g) and, based on the total content, comprised > 60% of all tannins extracted by each extraction method. Epicatechin was the second most abundant product (16-19%) of method A and B, whereas epigallocatechin gallate was the second most abundant (19%) of method C. The concentration of each tannin monomer depended on the extraction method. For example, the tannin monomers least produced by method A, B, and C respectively, were epigallocatechin gallate, catechin gallate mercapto, and catechin. Among the three methods, method A produced the highest yield (87.82 mg/g). This indicated that method A should be the most effective method for tannins extraction. The present result is
similar to that of Cheok et al. (2012) who determined that methanol was the best solvent for the extraction of phenolic compounds from mangosteen pericarps, due, they suggested, to a solubility parameter. The efficiency of the solvents in the present study could be ranked in the following order: methanol > ethanol > acetone. In order to verify complete extraction, the remaining mangosteen pericarps were powdered, depolymerized in the same way as the extracts, and analyzed by UPLC-MS. No condensed tannin was detected, indicating complete extraction of the condensed tannins in the mangosteen pericarps.

4. Conclusion

The present work comprised a systematic evaluation of the types and concentrations of tannin monomers extracted from mangosteen pericarps by 3 methods: method A used – methanol and water; method B used – ethanol, water and acid; and method C used – acetone, water and acid. The extracted tannins were depolymerized with 2-mercaptoethanol. UPLC-MS was applied to determine the structure of the condensed tannins, as well as tannin content. All extraction methods produced similar results: five tannin monomers and xanthes. The five tannin monomers in the depolymerized extracted tannins were identified as catechin (C), epicatechin (E), catechin mercapto (CM), catechin gallate mercapto (CGM) and epigallocatechin gallate (EGCG). CM was the major product (~32 to 60 mg/g, > 60%) in all extracts. E was the second major product in the extracts from method A (~17 mg/g, 16%) and method B (~12 mg/g, 19%), whereas EGCG was the second major product from method C (~10 mg/g, 19%). The determined degree of polymerization (DP) indicated that the
condensed tannins in the pericarps were mostly dimers and trimers. Finally, methanol was the most efficient extraction solvent.

Acknowledgments

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References


Figure 1 Schematic diagram of depolymerization mechanism of tannin: (1) catechin, (2) epicatechin, (3) catechin mercapto, (4) catechin gallate mercapto and (5) epigallocatechin gallate.

Figure 2 Characteristics of extracted powders derived from 3 extraction methods: (a) method A, (b) B, and (c) C.
Figure 3 UPLC chromatograms of the extract before depolymerized (method A only) and after depolymerization (method A, B and C): (1) catechin, (2) epicatechin, (3) catechin mercapto, (4) catechin gallate mercapto and (5) epigallocatechin gallate.
Table 1 Retention time and mass number of phenolic compounds in the mangosteen pericarp analyzed by UPLC-MS.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Phenolic compound</th>
<th>Retention time (min)</th>
<th>(M+H)⁺ m/z</th>
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<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>3.0</td>
<td>291</td>
</tr>
<tr>
<td>2</td>
<td>EC</td>
<td>3.5</td>
<td>291</td>
</tr>
<tr>
<td>3</td>
<td>C-SCH₂CH₂OH</td>
<td>4.0</td>
<td>367</td>
</tr>
<tr>
<td>4</td>
<td>CG-SCH₂CH₂OH</td>
<td>4.5</td>
<td>519</td>
</tr>
<tr>
<td>5</td>
<td>EGCG</td>
<td>5.3</td>
<td>459</td>
</tr>
</tbody>
</table>

(1) catechin (C), (2) epicatechin (EC), (3) catechin mercapto (C-SCH₂CH₂OH), (4) catechin gallate mercapto (CG-SCH₂CH₂OH) and (5) epigallocatechin gallate (EGCG)

Table 2 The spectral peak area ($A_x$), the molar relative response factor (MRRF$_x$) and the molar concentration ($C_x$) of tannin monomers from UPLC chromatograms of phenolic compounds in mangosteen pericarps extracted by method A, B and C.

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>Method A</th>
<th>Method B</th>
<th>Method C</th>
<th>MRRF$_x$ (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_x$ (mg/L)</td>
<td>$C_x$ (mmol/L)</td>
<td>$A_x$ (mg/L)</td>
<td>$C_x$ (mmol/L)</td>
</tr>
<tr>
<td>C</td>
<td>46.54</td>
<td>0.16</td>
<td>32.56</td>
<td>0.11</td>
</tr>
<tr>
<td>EC</td>
<td>174.15</td>
<td>0.60</td>
<td>121.40</td>
<td>0.42</td>
</tr>
<tr>
<td>C-SCH₂CH₂OH</td>
<td>620.86</td>
<td>2.14</td>
<td>489.78</td>
<td>1.69</td>
</tr>
<tr>
<td>CG-SCH₂CH₂OH</td>
<td>41.40</td>
<td>0.09</td>
<td>26.96</td>
<td>0.06</td>
</tr>
<tr>
<td>EGCG</td>
<td>33.31</td>
<td>0.11</td>
<td>68.17</td>
<td>0.24</td>
</tr>
</tbody>
</table>

(1) catechin (C), (2) epicatechin (EC), (3) catechin mercapto (C-SCH₂CH₂OH), (4) catechin gallate mercapto (CG-SCH₂CH₂OH) and (5) epigallocatechin gallate (EGCG)

Table 3 Condensed tannin monomer content of mangosteen pericarps extracted by method A, B and C.

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>Condensed tannin monomer content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Method A</td>
</tr>
<tr>
<td>C</td>
<td>4.46</td>
</tr>
<tr>
<td>EC</td>
<td>16.69</td>
</tr>
<tr>
<td>C-SCH₂CH₂OH</td>
<td>59.50</td>
</tr>
<tr>
<td>CG-SCH₂CH₂OH</td>
<td>3.97</td>
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<td>EGCG</td>
<td>3.19</td>
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<td>Total content</td>
<td>87.82</td>
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(1) catechin (C), (2) epicatechin (EC), (3) catechin mercapto (C-SCH₂CH₂OH), (4) catechin gallate mercapto (CG-SCH₂CH₂OH) and (5) epigallocatechin gallate (EGCG)