JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Oligomeric Proanthocyanidins from Mangosteen Pericarps

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Oligomeric proanthocyanidins were extracted from mangosteen pericarps and fractionated by a Sephadex LH-20 column to give 0.66% yield (dry matter). ^{13}C and ^{1}H NMR signals showed the presence of predominantly procyanidins together with a few prodelphinidin units along with small amounts of stereoisomers of afzelechin/epiafzelechin, catechin/epicatechin, and gallocatechin/epigallocatechin. Depolymerization with benzylmercaptan resulted in epicatechin thioether as the major product, and the mean degree of polymerization was determined to be 6.6. The electron spray ionization–mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectra revealed the dominant B type oligomers with mainly epicatechin units and with a small amount of A type oligomers. The isolated proanthocyanidins are potent peroxyl radical scavengers as evidenced by the high oxygen radical scavenging capacity at 1.7×10^4 μmol TE/g, much higher than that of pine bark and grape seed extracts.

KEYWORDS: mangosteen; *Garcinia mangostana*; pericarps; proanthocyanidins

INTRODUCTION

Mangosteen (*Garcinia mangostana* Linn) is a tropical fruit belonging to the Guttiferae family (*1*). It is commonly cultivated in Thailand, Malaysia, and Indonesia. Because of its popularity, mangosteen is considered "the queen of the tropical fruit" in South East Asia. The edible portion of mangosteen is milky white, whereas the pericarp is dark red and composes about two-thirds of the whole fruit weight as agricultural waste. The pericarps are rich in bioactive compounds with potential applications as therapeutic agents or as functional food additives. In fact, the nonedible pericarps have been used for treating diarrhea, wounds, and skin infections in traditional Thai medicine (*2*). Mangosteen pericarps are rich in anthocyanins and xanthones. The anthocyanins in the pericarps are reported as primarily cyanidin-3-sophoroside with smaller amounts of cyanidin-3-glucoside (*3*). The high amounts of anthcyanidins in the pericarp indicate that there are proanthocyanidins as well; yet, they have not yet been characterized.

Proanthocyanins are potent free radical scavengers and are believed to be contributors to the health benefits of fruits and vegetables (*4, 5*). Proanthocyanidins from grape seeds have been shown to protect against UV light-induced carcinogenesis, prevent immune suppression, increase interleukin (IL)-12, and decrease IL-10 (*6*). Apple procyanidins have demonstrated synergistic effects with lysosomotropic compounds in improving the anticarcinogenic properties targeting human colon cancer-derived metastatic cells (*7*). Because of the complex structural diversity and related physiochemical properties, proanthocyanidins were considered to be the final frontier of flavonoid research (*8*), and unexplored proanthocyanidins from mangosteen may be potential resources for novel bioactive compounds. This study was designed to separate and characterize proanthocyanidins in mangosteen pericarps and to investigate their peroxyl radical scavenging capacity.

MATERIALS AND METHODS

Instruments. ^{1}H and ^{13}C NMR spectra were recorded in deuterated methanol with a Bruker AC300 spectrometer (Karlsruhe, Germany) operating at 300 and 75 MHz, respectively. The electrospray ionization mass spectra (ESI-MS) were obtained from a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA) equipped with an ESI source. The heated capillary and voltage were maintained at 250 °C and 4.5 kV, respectively. The full-scan mass spectra from *m*/*z* 50 to 2000 were recorded. The mangosteen pericarp proanthocyanidins were dissolved in methanol, and the solution was introduced into the ion spray source with a syringe (100 μL). Liquid chromatography/mass spectrometry (LC/MS) spectra were acquired using Finnigan/MAT LCQ ion trap mass spectrometer equipped with a TSP 4000 high-performance liquid chromatography (HPLC) system, which includes an UV6000LP photodiode array detector, P4000 quaternary pump, and AS3000 autosampler. The heated capillary and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was operated at 80 psi for the sheath gas flow rate and 20 psi for the auxiliary gas flow rate. The full scan mass spectra from *m*/*z* 50 to 2000 were acquired in both positive and negative ion modes with a scan speed of 1 scan/s. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were collected on an Applied Biosystems Voyager-DE STR mass spectrometer (Foster City, CA) equipped with delayed extraction and a N_2 laser set at 337 nm. The length of one laser pulse was 3 ns. The measurements were carried out using the following conditions: positive

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10.1021/jf071166n CCC: $37.00
Published on Web 08/23/2007

polarity, linear flight path with 21 kV acceleration voltage, and 100 pulses per spectrum. The samples were dissolved in methanol (4 mg/mL). Sodium chloride and 2,5-dihydroxybenzoic acid as the matrix were used to enhance ion formation (*9*). An aqueous solution of sodium chloride (1.0 μL, 0.1 M) was added to the sample solution (1.0 mL) followed by addition of an equal volume of methanol solution of 2,5-dihydroxybenzoic acid (10 mg/mL). The resulting solution (1.0 μL) was evaporated and introduced into the spectrophotometer. UV/vis spectra were recorded using a Shimadzu UK1601 spectrophotometer (Kyoto, Japan) fitted with a quartz cell. An oxygen radical absorbance capacity (ORAC) assay was carried out on Bio Tek Synergy HT microplate fluorescence reader (Winooski, VT).

Reagents. All solvents used were of reagent grade unless otherwise specified. Ripe mangosteens with dark brown pericarps (cultivated in Malaysia) were purchased from a local market. Grape seed extracts (95% proanthocyanidins) and pine bark extracts (90% proanthocyanidins) were purchased from Chengdu SanHerb Plant Extract Co. Ltd. (Chengdu, Sichuan, China). Sephadex LH-20 was purchased from GE Healthcare BioSciences AB (Uppsala, Sweden). Benzyl mercaptan, fluorescein disodium salt, and Trolox were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Extraction and Purification of Oligomeric Proanthocyanidins from Mangosteen Pericarps. The mangosteen pericarps (2.0 kg, fresh) were ground and Soxhlet defatted with hexane (3 × 1500 mL). The remaining solids were subsequently extracted by a mixture of acetone/water (7:3, 3 × 4000 mL) for 4 h. The mixture was filtered, and the filtrate was pooled. The acetone in the filtrate was evaporated to yield a slurry, which was centrifuged at 3000*g* for 15 min. The supernatant was collected and liquid–liquid extracted with dichloromethane (3 × 500 mL) to further remove xanthones and other lipophilic compounds. The water phase was collected and concentrated to 60 mL. The crude proanthocyanidin fraction (20 mL) was filtered through a Sartorius Minisart 45 μm porosity filter (Epsom, United Kingdom) and then loaded on a Sephadex LH-20 column containing 50 g of LH-20 equilibrated with MeOH/water (1:1) for 4 h. The column was washed with MeOH/water (1:1) until the eluent turned colorless. The adsorbed proanthocyanidins were then eluted with aqueous acetone (70%, 500 mL). The acetone was removed on a rotary evaporator at 40 °C, and the resulting residue was freeze-dried to give a light brown powder (4.2 g overall yield). The moisture content in mangosteen was determined to be 68.3%, and thus, the yield of the oligomeric proanthocyanidins (OPCs) was 0.66% of dry matter. The "purity" measured by UV/vis colorimetric methods analysis showed that the extract contains over 99% (wt) epicatechin (standard) equivalents.

Thiolysis of the Procyanidins for HPLC Analysis. This was done by following a reported method (*10*). In a small glass vial, a mangosteen proanthocyanidins solution (50 μL, 2.0 mg/mL in methanol) was mixed together with methanol acidified with concentrated HCl (50 μL, 3.3%, v/v) and 100 μL of benzyl mercaptan (5% v/v in methanol). The vial was sealed with an inert Teflon cap. The reaction was carried out at 40 °C for 30 min and then kept at room temperature for 10 h; then, the reaction mixtures were kept in the freezer (−20 °C) until 10 μL was injected directly for reverse-phase HPLC analysis. The thiolysis media were further analyzed using LC/MS with a Shimadzu 250 mm × 4.6 mm i.d., 5 μm C18 column (Kyoto, Japan). The binary mobile phases consisted of A (2% acetic acid in water, v/v) and B (methanol), which were delivered in a linear gradient of B from 15 to 80% (v/v) in 45 min. The flow rate was set at 1.0 mL/min.

Antioxidant Capacity Analysis. $ORAC_{FL}$ assays were carried out on a Synergy HT fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 525 nm (Biotek Instruments Inc.). The temperature of the incubator was set at 37 °C. The procedures were based on the modified $ORAC_{FL}$ method (*11*), and the data are expressed as micromoles of Trolox equivalents per gram (μmol TE/g).

RESULTS AND DISCUSSION

Typical solvent extraction and fractionation on Sephadex LH-20 gave 4.2 g of OPC mixtures from 2.0 kg of fresh mangosteen pericarps, corresponding to 0.21% yield-based fresh weight.

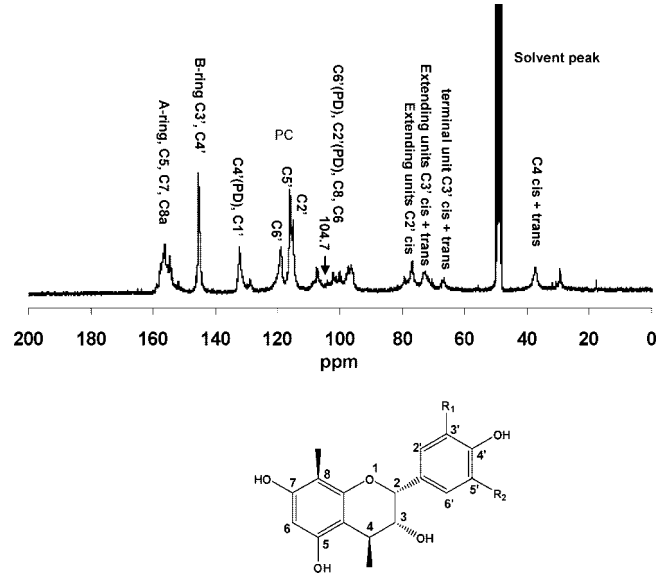

Figure 1. ^{13}C NMR spectrum of proanthocyanidins from mangosteen pericarps; solvent, CD_3OD; room temperature; and 75 MHz. Identity of the structures: R_1 = H, R_2 = H, epiafzelechin; R_1 = H, R_2 = OH, epicatechin; and R_1 = OH, R_2 = OH, epigallocatechin.

Taking into account the moisture in the mangosteen pericarps (68.3%), the proanthocyanidins content in mangosteen is 0.66% and rather low in comparison with that in cocoa and grape seed, which contain over 10% weight in dry matter (*12, 13*). Besides OPCs, xanthones and anthocyanins are the major secondary metabolites in the pericarps obtained in large quantity. Xanthones are lipid soluble and mainly retained in the hexane solution and diethyl ether extraction. We estimated that xanthones are composed of about 6.6% of the dry pericarps, about 10 times higher than that of OPCs. The mangosteen pericarp tastes very astringent due to the presence of OPCs.

The UV/vis spectrum of the OPCs shows high similarity to catechin, indicating that the major monomeric unit is catechin or its diastereomer epicatechin. Using catechin as a reference standard, the catechin content of the OPCs is estimated to be 1300 mg catechin equivalent per gram sample; the higher catechin equivalence based on UV/vis absorbance is likely due to the higher absorbance coefficiency of the OPCs. The absorption maxima of OPCs at ~280 nm overlap well with that of catechin, indicative of a homogeneous procyanidin structure (*14*). The UV/vis method can not be used for quantification purposes; instead, it serves as a quick estimate of the OPC contents in a sample.

The ^{13}C NMR spectrum (**Figure 1**) of the mangosteen OPC in CD_3OD shows characteristic ^{13}C peaks consistent with that of condensed tannins with dominant procyanidin units with a minor amount of prodelphinidin. The structural diversity of the linkage (A and B type) and stereoisomer of catechin and epicatechin units is apparent from the spectrum. Specifically, C5, C7, and C8a carbons of procyanidins appear at 160 to 150 ppm. Peaks at 145.3 and 145.5 belong to C3′ and C4′ of procyanidin units. A small amount of prodelphinidin is also detected as its C4′ peak and appears at 131 ppm, overlapping with the chemical shifts of C1′. The cluster of peaks between 110 and 90 ppm is assigned to C8, C6, C6′, and C2′ of procyanidins. The region between 70 and 90 ppm is sensitive to the stereochemistry of the C ring. The ratio of the 2,3-*cis* to 2,3-*trans* isomers could be determined through the distinct differences in their respective C2 chemical shifts. C2 gives a resonance at 75.3 ppm for the *cis* form and 79 ppm for the

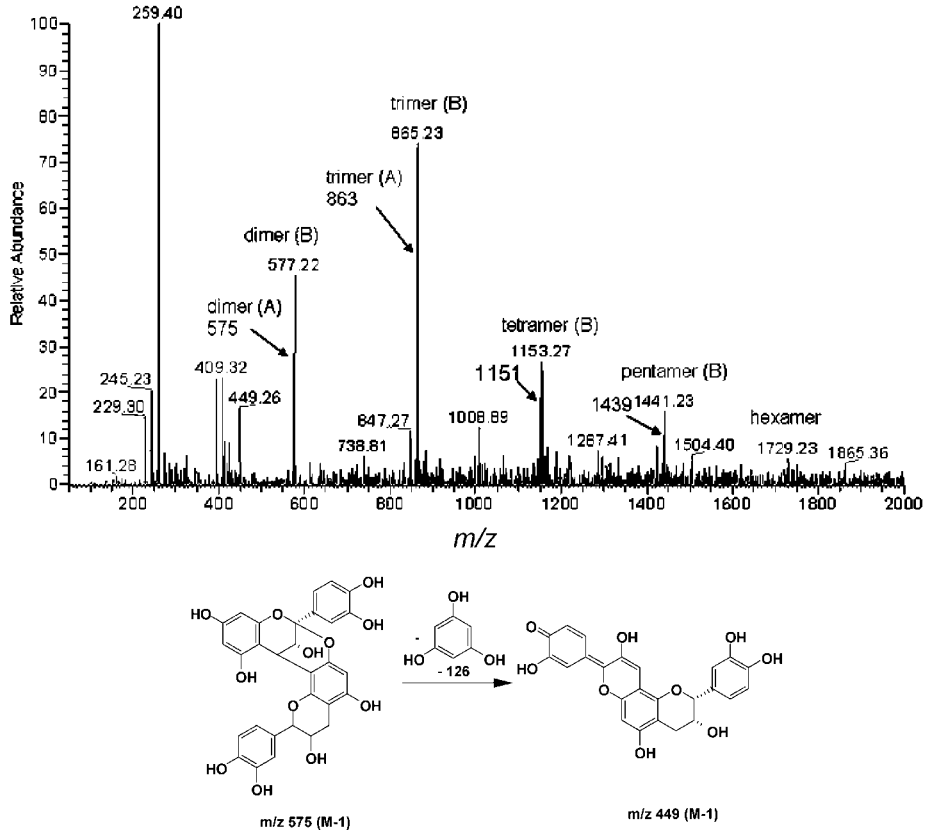

Figure 2. ESI/MS spectra of mangosteen pericarps proanthocyanidins recorded in the negative ion mode and possible fragmentation pathway. ESI conditions: 250 °C and 4.5 kV, full scan *m*/*z* 50–2000, and negative ionization mode. Solvent, methanol.

trans form. From the peak areas, it is estimated that the *cis* isomer is dominant. C3 of both *cis* and *trans* isomers occurs at ~71.4 ppm. A sharp line at 64 ppm is due to C3 of the terminal unit. The C4 atoms of the extension units showed a broad peak at 36 ppm, while the terminal C4 exhibits multiple lines at 29 and 27 ppm (*15*). There are also some A type linkages indicated from the signals at 151–152 ppm due to C5 and C7 of the A ring involved in the double linkage. The chemical shift of the ketal carbon (C2) formed as a result of this additional bond observed at 104.7 ppm provided further support for A type linkage albeit in trace quantities.

Although the ^{13}C NMR spectrum reveals complex structural characteristics of the mangosteen OPCs, quantitative data regarding the degree of polymerization (DP) cannot be reliably obtained. Further characterization was achieved by ESI-MS spectrometry. Analysis was performed in the negative ion mode as proanthocyanidin molecules are better detected than in the positive ion mode due to the acidity of the phenolic protons. **Figure 2** depicts the ESI-MS spectra of the proanthocyanidins. Among the observed signals, a first series of abundant ions separated by 288 Da were observed from *m*/*z* 577 to 1729, corresponding to the molecular masses of procyanidins with DPs of 2–6; this is inconsistent with the ^{13}C NMR spectrum, which reveals that the dominant species is procyandins. Peaks 449 can be accounted for from fragmentation of procyanidin dimer (*m*/*z* 575) after heterocyclic ring fission as shown. Other less intense signals were also observed in the higher *m*/*z* values. Mass spectra also provided evidence for the doubly charged ions $[M - 2H]^{2-}$ of odd polymerization degree. The peak at *m*/*z* 1009 was 144 mass units higher than trimer ion (*m*/*z* 865) and 144 lower than tetramer (*m*/*z* 1153). Hence, it can be attributed to the doubly charged species of heptameric procyanidins.

Signals corresponding to trimeric (*m*/*z* 847) and pentameric (*m*/*z* 1425) oligomers with only one afzelechin/epiafzelechin unit (−16 Da) were also observed but were less abundant. Mass spectra also show a series of compounds that were two units less than dimeric (*m*/*z* 577), trimeric (*m*/*z* 865), tetrameric (*m*/*z* 1153), pentameric (*m*/*z* 1441), and hexameric (*m*/*z* 1729). These masses might represent a series of compounds in which an A type interflavan ether linkage occurs (4β-8, 2β-O-7) between adjacent flavan-3-ol subunits because two hydrogen atoms (Δ2 amu) are lost in the formation of this interflavan bond. It agreed with the NMR results. However, the limited range imposed by the quadruple analyzer as well as the possible presence of multiple-charged ions for the larger molecules, inducing peak dispersion and frequent overlapping, resulted in an increased difficulty of interpretation of the signals when using ESI-MS to higher DP proanthocyanidins. Singly charged ions of higher molecular weight proanthocyanidins are often observed with a weak intensity and are difficult to detect with good precision in ESI-MS (*16*).

A complementary spectroscopic technique to limit the production of multiply charged species is MALDI-TOF MS, allowing the analysis of polymers and revealing information about their chain lengths due to the production of only a singly charged molecular ion for each parent molecule and allowing for the detection of high mass with precision. **Figure 3** shows the MALDI-TOF mass spectra of the mangosteen OPCs, recorded in the positive reflectron ion mode. The major peak assignments are listed in **Table 1**. A series of polyflavan-3-ols extending from the dimer (*m*/*z* 785) to DP13 (*m*/*z* 3785) were observed. In addition, there were many peaks with mass signals having a difference of 16 Da more than homoprocyanidins, corresponding to the addition of *n* gallocatechin/epigallocatechin units. Therefore, MALDI-TOF MS indicated the coexistence of procyanidin polymers and polymers with different ratios of procyandins and prodelphinidins.

To further investigate if the OPCs are composed of epicatechin and catechin, depolymerization through thiolysis reac-

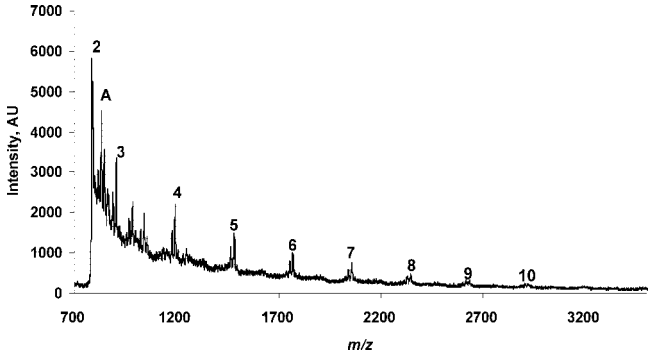

Figure 3. MALDI-TOF mass spectrum of mangosteen proanthocyanidins. MALDI-TOF mass spectra were collected on a Voyager-DE STR mass spectrometer (Applied Biosystems) equipped with delayed extraction and a N_2 laser set at 337 nm. The length of one laser pulse was 3 ns. The measurements were carried out using the following conditions: positive polarity, linear flight path, 21 kV acceleration voltage, and 100 pulses per spectrum. The samples were dissolved in methanol (4 mg/mL). Sodium chloride and 2,5-dihydroxybenzoic acid as the matrix were used to enhance ion formation.

tion was carried out by following standard conditions using benzyl mercaptan. The reaction mixture was analyzed by HPLC as shown in **Figure 4**. The major product observed was the expected epicatechin 4-benzylsulfide along with a significant amount of epicatechin and a much smaller peak for catechin. This result suggests that that there are significant amounts of epicatechin extension units in mangosteen OPCs. The mean DP of mangosteen OPCs was calculated to be 6.6 by comparing the peak areas based on the following equation.

$$\mathrm{DP} = 1 + \frac{\text{area under the curve of benzyl thioether derivative of catechins}}{\text{area under the curve of catechin and epicatechin}} \quad (1)$$

Consistent with this data, the ^{1}H NMR peak integration of terminal C(4)H_2 and extending unit C4(H) gave a mean DP of 7.0. Because there are A type proanthocyanidins that do not react with benzyl mercaptan, the mean DP determined by thiolysis is lower than that determined by ^{1}H NMR. Chemically, most C–C bonds are not easily cleaved and the facile cleavage of interflavanol C–C bonds of B type OPCs by nucleophiles such as thiol reagents can lead to catechin derivatives with diverse functional groups (*17*). The bioactivity of these novel compounds is largely unexplored and has great potential as antioxidants and other therapeutic agents.

The peroxyl radical scavenging capacity of the isolated mangosteen OPCs was determined using the ORAC assay. The kinetic curves from the ORAC assay showed a dose-dependent relationship, with a clear lag phase comparable to Trolox standard. The net area under the curve had an excellent linear relationship ($R^2 > 0.990$) with the concentration of OPCs. The ORAC value was 1.7×10^4 μmol TE/g, which is 4.25 times more than equal weight of pure Trolox (ORAC value of 4000). Therefore, the mangosteen OPCs are excellent peroxyl radical scavengers. For comparison purposes, the antioxidant capacity of the commercially available pine bark proanthocyanidins has an ORAC value of 7.5×10^3 μmol TE/g and that of grape seed proanthocyanidins has a value of 1.0×10^4 μmol TE/g. The higher ORAC values of the OPCs from mangosteen pericarps may be due to the compositional difference and the difference of DPs. The OPCs from mangosteen pericarps contain more higher order oligomers with predominantly epicatechins as the monomeric unit, whereas the pine bark extracts and the grape seed extracts contain mainly lower order (<5) oligomers (data not shown). In addition, grape seed extracts contain complex extension units including gallated catechins. It should be pointed out that ORAC values measured chemically have no implication at all for the potential nutritional values of the OPCs because it speaks nothing about the bioavailability and actual functions when the OPCs is consumed. For evaluation of the nutritional values of OPCs, evidence will be needed to demonstrate if OPCs can indeed reduce oxidative stress biomarkers.

There are exceeding numbers of methods in measuring radical scavenging capacity, and the pros and cons of the methods have been extensively reviewed and debated (*18*). The uniqueness of the ORAC assay is that it quantifies the peroxyl radical scavenging capacity and validated protocols and sound mechanistic studies. In biological systems, there are many types of reactive oxygen species including peroxyl radical, hydroxyl radical, peroxynitrite, and superoxide. To comprehensively quantify the scope of the radical scavenging activity, different methods are needed to give a complete picture of the "total" antioxidant capacity of oligomeric proanthocyanidins.

Table 1. Observed Masses of Mangosteen Oligomeric Proanthocyanidins by MALDI-TOF MS

m/z	polymer	basic unit	interflavan bond
789	dimer	gallocatechin + gallocatechin gallate	B type
817	dimer	epicatechin gallate + chlorogenic acid	1 A type
833	dimer	gallocatechin gallate + chlorogenic acid	1 A type
849	trimer	catchin + two gallocatechin	2 A type
865	trimer	three gallocatechin	2 A type
889	trimer	epicatechin trimer	B type
904	trimer	two epicatechin + gallocatechin	B type
920	trimer	one epicatechin + two gallocatechin	B type
984	trimer	one epicatechin + one catechin gallate + two afzelechin	1 A type
1040	trimer	two epicatechin + catechin gallate	B type
1056	trimer	two epicatechin + gallocatechin gallate	B type
1134	tetramer	two epicatechin + two afzelechin	3 A type
1328	tetramer	three epicatechin + catechin gallate	B type
1465	pentamer	five epicatechin	B type
1481	pentamer	four epicatechin + gallocatechin	B type
1753	hexamer	six epicatechin	B type
2041	heptamer	seven epicatechin	B type
2165	heptamer	six epicatechin + catechin gallate	B type
2345	octamer	eight epicatechin	B type
2633	nonamer	eight epicatechin + gallocatechin	B type
2905	decamer	ten epicatechin	B type

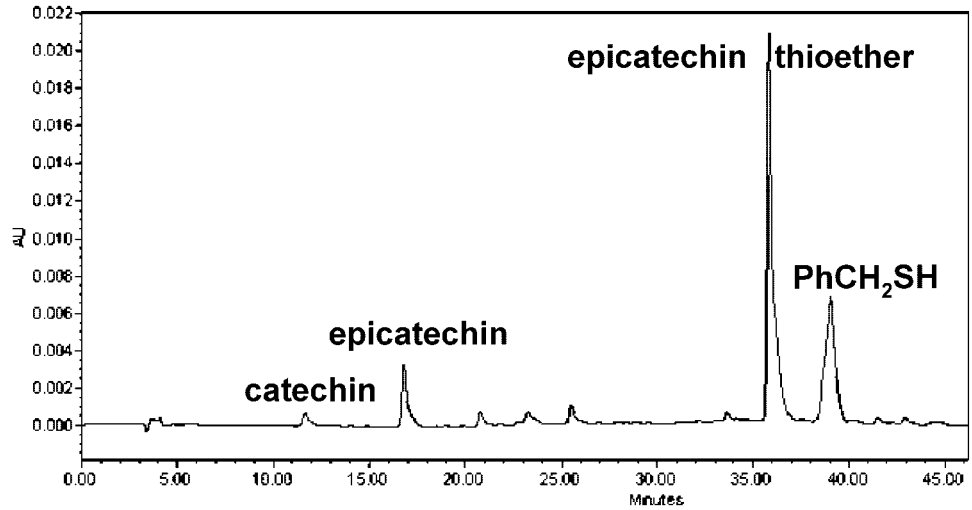

Figure 4. HPLC chromatogram of thiolytic products of OPCs by benzyl mercaptan. Column: 250 mm × 4.6 mm i.d., 5 μm C18 column (Shimadzu); detector set at 280 nm.

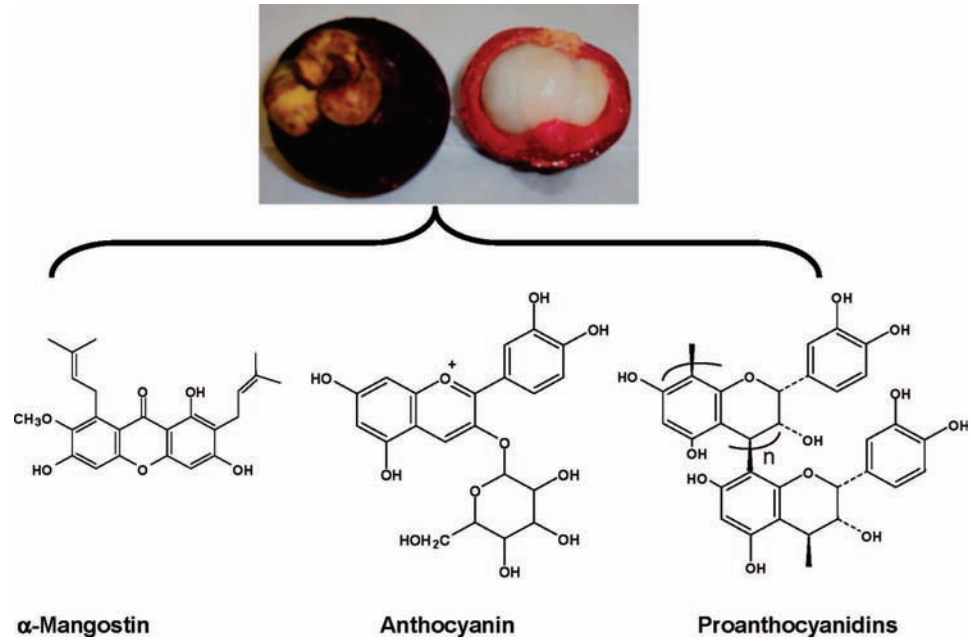

Figure 5. Major bioactive secondary metabolites of mangosteen pericarps.

OPCs isolated from different plants show various DPs and characteristics of monomeric units. Besides the mangosteen OPCs, epicatechin is also the dominant monomer of OPCs isolated from cocoa, whereas OPCs in apple contain mixtures of epicatechin and catechin units (*19*). Certain pine park OPCs, on the other hand, contain dominantly B type oligomers of catechin with only trace amounts of epicatechin (*20*), as do the OPCs from green pear, blueberry, and sorghum (*10*). The OPC database collected by Prior and Gu revealed that, in general, there are more epicatechin- than catechin-containing OPCs in fruits and vegetables, but they have no apparent correlation with the types of the plants (*12*).

In summary, we have shown that mangosteen pericarps are a good source of oligomeric proanthocyanidins dominant with B type linkages. In perspective, mangosteen pericarps are rich in bioactive secondary metabolites including OPCs, xanthones, and anthocyanins (**Figure 5**). Thus, it is worthwhile to consider utilization of this agricultural waste for the production of functional food ingredients or phytochemicals for medicinal exploration. We also compared the ORAC values of the flesh and the pericarps and found that the pericarps contain about 20 times higher antioxidant activity and 10 times more total phenolics than that in the flesh on dry weight basis. The higher antioxidant capacity in the pericarps is also observed in other tropical fruits such as dragon fruits (red and white flesh) and cempedak (*21, 22*). Liu and co-workers reported a much higher antioxidant content in the apple skins than that in the flesh and suggested that it may be beneficial to consume unpeeled apples for better health benefits (*23*). For mangosteen and many other fruits, the pericarps are not edible. Utilization of these micronutrients will then rely on chemical extractions and separations. Fruit pericarps or skin contain high amounts of bioactive compounds, and many of them are phytoalexins in response to microbial or other forms of environmental stress (*24*). The interest on the nutritional benefits of these phytoalexins was rekindled lately upon the discovery that resveratrol, a phytoalexin found in grape skin, can be used as a calorie restriction mimetic for chronic disease prevention (*25*). The secondary

metabolites found in mangosteen pericarps warrant further investigation regarding their therapeutic and chronic disease prevention potential and mechanisms.

ABBREVIATIONS USED

OPC, oligomeric proanthocyanidins; ORAC, oxygen radical absorbance capacity; DP, degree of polymerization; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; ESI, electrospray ionization.

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Received for review April 21, 2007. Revised manuscript received July 11, 2007. Accepted July 11, 2007. We thank the National University of Singapore for a Start-up Grant (R-143-000-244-101) of financial support and scholarship (to C.F.).

JF071166N