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Identification and Quantification of Polyphenolic Compounds in Longan (*Euphoria longana* Lam.) Fruit

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Regular consumption of fruit and vegetables is associated with a lower risk of some chronic diseases including various forms of cancer and cardiovascular diseases. The health-promoting potential of these foods may be due, in part, to the phytochemical bioactive compounds present in the plants. Fruit of *Euphoria longana* Lam. (longan) are consumed throughout Asia and are a major crop in Thailand. In the present study phytochemicals were extracted with 70% methanol from peel, pulp, and seed tissues of longan fruit, and the major components were identified as gallic acid, corilagin (an ellagitannin), and ellagic acid. A high-through-put reversed phase HPLC method was developed to determine the content of these three compounds in different parts of the longan fruit and among different cultivars. The analyses showed that there was a large variation in the contents of gallic acid, corilagin, and ellagic acid in different plant tissues and cultivars. Seed contained the highest levels of the three phenolics, and pulp contained the lowest. Among commercial cultivars, Biewkiew and Edor contained the highest levels of gallic and ellagic acid while Srichompoo contained the highest content of corilagin. These three cultivars may be used in directed breeding and cultivation programs and to develop concentrated longan seed extracts to promote good health. Utilization of this byproduct material will support the use of thousands of tons of waste longan seeds after the production of canned longan pulp.

KEYWORDS: ***Euphoria longana*; ellagic acid; gallic acid; corilagin; HPLC; LC-MS**

INTRODUCTION

Euphoria longana Lam. (longan) is a member of the Sapindaceae family. This family also includes litchi (*Litchi chinensis* L.), rambutan (*Nephelium lappaceum* L.), and horse chestnut (*Aesculus hippocastanum* L.). Longan is a subtropical fruit, which is widely grown in China and South East Asia including Thailand, Vietnam, and the Philippines. Longan, known as 'Lumyai' in Thailand, is a major Thai fruit export. Thailand, China, and Taiwan are the main centers of commercial longan production (*1*). In Thailand, the matured fruit can be harvested from July to September. The fruit peel is brown or light-brown with white translucent flesh. The seed is round and black with a circular white spot at the base. The flesh is sweet and juicy; therefore, it can be consumed in both fresh and processed products such as canned longan in syrup or as dried fruit.

Longan fruit are extensively consumed in Thailand; however, there is still limited information on the pharmacological activities. In Chinese medicine the flesh of the longan is used as a stomachic, febrifuge, vermifuge, and also as an antidote for poison (*2*). A decoction of the dried flesh is also used as a tonic and for the treatment of insomnia and neurasthenic neurosis (*2*). There is also limited information on the analysis of the bioactive compounds in longan fruit. A methanol extract of longan arillus exhibited anxiolytic-like effects; it was shown to contain adenosine, which may also account for the reported sedative and analgesic effects (*3*). The seeds have previously been shown to contain the hydrolyzable tannins (ellagitannins) corilagin and acetonyl-geraniin (*4*, *5*). Corilagin (**Figure 1**) has been widely studied for its pharmacological activities in extracts of plants such as *Phyllanthus amarus* (*6*) and *Acer nikoense* (*7*) and also as a pure isolated compound. It has been shown to lower the blood pressure of spontaneously hypertensive rats (SHR) through blocking noradrenaline release and (or) by direct vasorelaxation (*4*). In addition, corilagin has been shown to have antifungal activity against *Candida glabrata* strains (*8*), to potently inhibit HIV-1 replication in HeLa $CD4^+$ cells (*6*), and inhibit tumor necrosis factor-α (TNF-α) release (*7*). Corilagin also remarkably reduced the β-lactam antibiotic resistance in methacillin-resistant *Staphylococcus aureus* (MRSA) (*9*). Ac-

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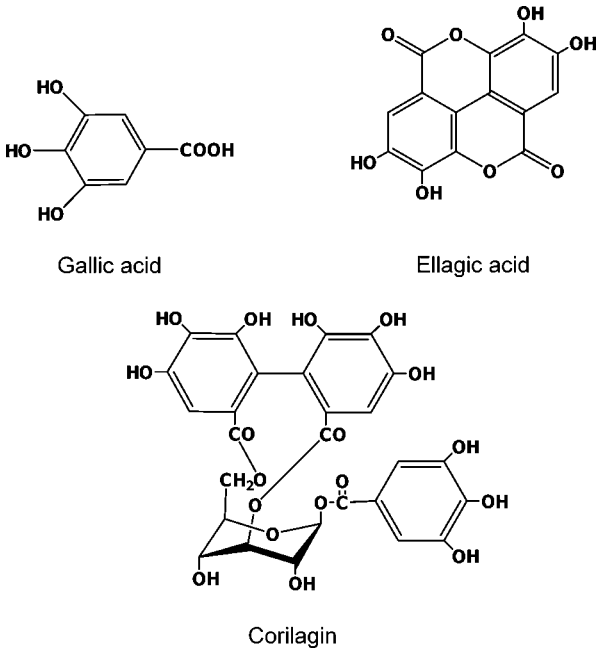

Figure 1. The structures of gallic acid, ellagic acid, and corilagin.

etonyl-geraniin has been shown to reverse the orthostatic hypotension in SHRs that were awake through an effect on the noradrenergic nerve and subsequent release of noradrenaline (*5*).

For all these reasons, it is important to study the active compounds in longan fruit, especially the potentially beneficial polyphenolic compounds, since this plant may be used as a new natural source of these bioactive compounds. Therefore, the objectives of the present study were to develop suitable methods for extraction, purification, and identification of the major active compounds from the fruit of *E. longana* and also to determine the concentration of these active compounds in different plant tissues and commercial cultivars for the selection of the best cultivars containing high levels of these bioactive compounds for further studies.

MATERIALS AND METHODS

Plant Materials. *Euphoria longana* fruit (nine cultivars) were freshly harvested from the field in August (Faculty of Agricultural Production, Maejo University, Chiang Mai) and transported to the Laboratory of Pharmacology (Chulabhorn Research Institute), Bangkok. All fruit were washed and separated into two groups for analyses, i.e., fresh and dried fruit. For the dried fruit, all fresh fruit (whole) were placed in the oven at 80 °C for 48 h (following the conventional method to produce dried longan for export). Fresh and dried fruit were then separated into three different parts (peel, pulp, and seed) and crushed into small pieces using a pestle and mortar or a blender. Ten fruit of each cultivar (same size) were combined for chemical analysis.

Chemicals. Gallic acid, ellagic acid, tetrahydrofuran, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Standard corilagin was a gift from Prof. Tomofusa Tsuchiya and Dr. Sumiko Shiota, Shujitsu University, Japan. HPLC grade methanol and acetonitrile, and formic acid, were obtained from Merck (Darmstadt, FR Germany). Milli-Q deionized water (Branstead, Newton, MA) was used throughout this experiment.

Sample Extraction. Each part of the fruit (fresh and dried tissues) was accurately weighed, 400 mg, into 15 mL plastic tubes (two replicates per sample). Methanol (70%) (5.0 mL) was added and vigorously shaken for 20 s. The samples were then left at room-temperature overnight, with occasional shaking to ensure effective extraction. The next day, samples were centrifuged (2000*g*, 3 min, 25 °C), and the supernatants were transferred into 10 mL volumetric flasks. The residues were then re-extracted with 4.0 mL 70% methanol. All extracts were combined and filtered through a 13 mm, 0.45 μm PVDF membrane (Orange Scientific, Belgium) prior to HPLC analysis.

HPLC−UV/Vis Detection Analyses. The HPLC analyses were performed using an HP1100 HPLC system with a thermostatically controlled column oven, a binary pump, and a diode-array detector (Hewlett-Packard, Palo Alto, CA). A 125 × 4 mm i.d., 5 μm reversed phase column, LiChrospher RP-18 was connected to a 4 × 4 mm LiChrospher RP-18 guard column (Macherey-Nagel, Germany). The compounds were eluted with a gradient system of 0.4% formic acid (solvent A): methanol (solvent B) at a flow rate of 1.0 mL/min. The temperature of the column was 25 °C with the UV detection at 270 nm. The injection volume was 10 μL. The gradient system started from 0 min (100%A) to 2 min (95%A), 5 min (70%A), 8 min (66%A), 11 min (45%A), 14 min (45%A), 17 min (100%A) and maintained at this ratio until 20 min.

Calibration Curves. All standards (gallic acid, ellagic acid, and corilagin) were dissolved in methanol to produce five concentrations of gallic acid (0.008, 0.03, 0.06, 0.1, 0.14 mg/mL), five concentrations of ellagic acid (0.005, 0.01, 0.03, 0.06, 0.1 mg/mL), and five concentrations of corilagin (0.006, 0.012, 0.063, 0.125, 0.63 mg/mL). The detection limit of each compound was determined at the concentration where S/N ratio was >3.

Recovery. Crude samples were spiked with known amounts of reference standard solutions (each concentration $n = 2$), gallic acid (at the concentrations 0.05 and 0.08 mg/mL), ellagic acid (at the concentrations 0.02 and 0.04 mg/mL), and corilagin (at the concentrations 0.03 and 0.06 mg/mL) and then extracted following the method previously described (each concentration $n = 2$).

HPLC−UV/Vis Detection with Electrospray Mass Spectrometry. Selected samples were separated on a 250 × 4.6 mm, 5 μm Phenomenex Luna C_{18} reversed phase column with a Phenomenex Luna C_{18} 'Security-Guard' column. The mobile phase consisted of a water/tetrahydrofuran/TFA (98:2:0.1) (solvent A) and acetonitrile (solvent B). A linear gradient was started from 0 min (83%A), to 7 min (75%A), 15 min (65%A), 20 min (50%A), 25 min (100% B), 30 min (100%B), 35 min (83%A) and maintained at this ratio until 45 min. All mass spectra were obtained using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) coupled to a Jasco PU-1585 triple pump HPLC equipped with an AS-1559 cooled autoinjector, CO-1560 column oven and UV-1575 UV detector (Jasco[UK] Ltd., Great Dunmow, UK). The detector was set at 270 nm, and the HPLC column temperature was maintained at 25 °C. The 1.0 mL/min mobile phase flow exiting the HPLC column was split using an ASI 600 fixed ratio splitter valve (Presearch, Hitchin, UK) so that approximately 200 μL/min entered the mass spectrometer. Mass spectra were obtained in both positive ion and negative ion electrospray modes using a Micromass Z-spray ion source. The electrospray probe was operated at 3.5 kV with a cone voltage 24 V. The source and desolvation temperatures were 140 °C and 350 °C, respectively. The nitrogen nebulizing and drying gas flow rates were optimized at 15 L/h and 500 L/h, respectively. Spectra were recorded (in centroid mode) between m/z 50−1500 with a scan duration of 1.5 s/scan and an interscan time of 0.1 s. MS1 was set to unit mass resolution or better (LM and HM resolution parameters both set to 15.0). Spectra were processed using MassLynx 3.4 software (Micromass, Manchester, UK).

RESULTS AND DISCUSSION

The initial studies on comparisons of the extraction of the longan phytochemicals compared hot water (as in the preparation used for Chinese herbal medicines) and 70% methanol. It was found that 70% methanol was the most suitable solvent for reproducible and effective extraction of the active compounds under investigation(gallic acid, ellagic acid, and corilagin) (data not shown). Some phenolic compounds such as ellagic acid are very slightly soluble in water; therefore, using an alcohol mixture solvent would improve the solubility of these compounds. The structures of these three phenolics are shown in **Figure 1**. Formic acid (0.4% aqueous) in combination with a methanol gradient was found to be an effective mobile phase for HPLC

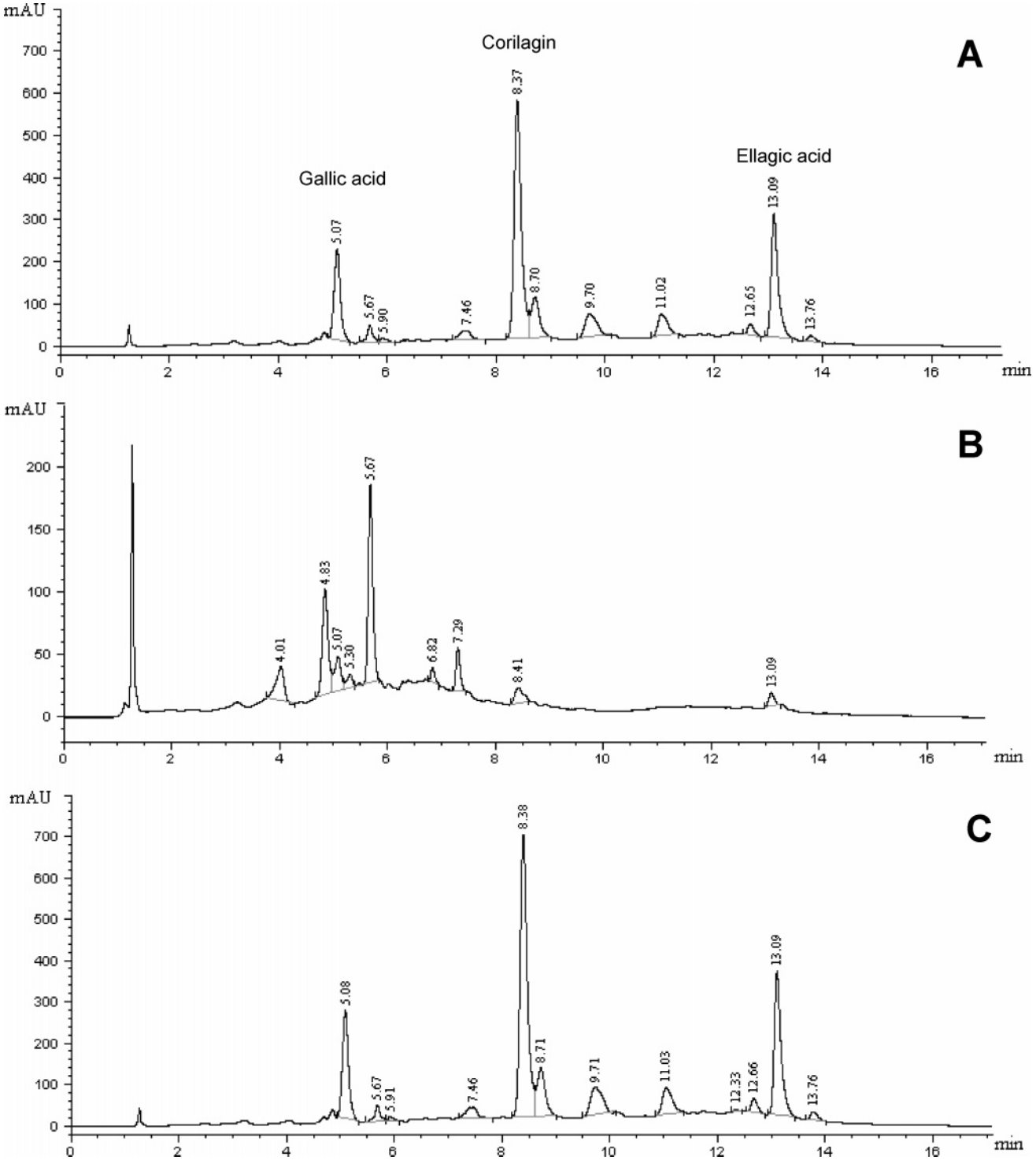

Figure 2. Example HPLC chromatograms of different parts of *E. longana* dried fruit: A, peel; B, pulp; C, seed. Retention time (t_R) gallic acid = 5.07 min, t_R corilagin = 8.37 min, and t_R ellagic acid = 13.09 min.

separation (good separation with short running time). The percentage recoveries for gallic acid, ellagic acid, and corilagin were 94−96%, 97%, and 98−101%, respectively. The combination of the extraction and HPLC methods was consistent for the separation of these three compounds from different tissues of *E. longana* fruit (**Figure 2**). Calibration curves were linear with $R^2 = 0.9996$ (gallic acid), 0.9993 (ellagic acid), and 0.9995 (corilagin). The detection limit of gallic acid, ellagic acid, and corilagin were 0.31, 0.10, and 0.34 μg/mL, respectively.

The HPLC fingerprint of *E. longana* peel (**Figure 2A**) was similar to that of seed (**Figure 2C**), where gallic acid, corilagin, and ellagic acid were major compounds, while pulp (**Figure 2B**) contained small amounts of these three phenolic compounds. However, pulp contained higher levels of unidentified compounds at RT 4.83, 5.67, and 7.29 min (**Figure 2B**). The data from LC-MS analyses, in negative ion mode, confirmed the identities of gallic acid, corilagin, and ellagic acid in the samples. In pulp, the peak at 5.67 min had a $[M - H]^-$ at *m*/*z* 254 indicative of a nitrogenous compound (λ_{max} = 285 nm), and that at 7.29 min produced a $[M - H]^-$ at *m*/*z* 477 indicative of a non-nitrogenous compound (λ_{max} = 297 nm). For peel and seed, the peak at 9.70 min had diode array spectra similar to

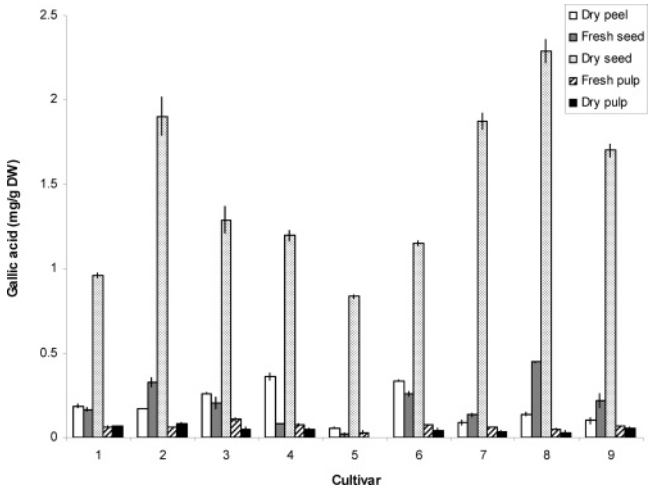

Figure 3. The contents of gallic acid in different fresh and dried tissues: peel, pulp, and seed in nine cultivars of *E. longana* fruit. Values represent mean of two replicates with standard error of means. All data were calculated on a DW basis. 1 = Baiyok, 2 = Edor, 3 = Biewkiew, 4 = Pungtong, 5 = Petsakorn, 6 = Srichompoo, 7 = Dangklom, 8 = Baidam, 9 = Haew.

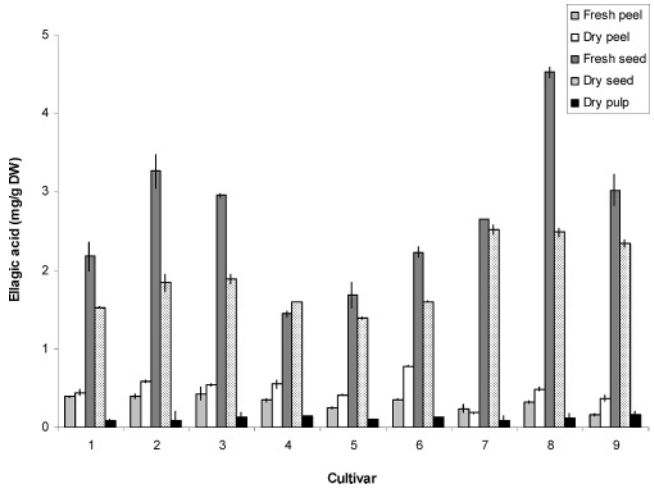

Figure 4. The contents of ellagic acid in different fresh and dried tissues: peel, pulp, and seed in nine cultivars of *E. longana* fruit. Values represent mean of two replicates with standard error of means. All data were calculated on a DW basis. 1 = Baiyok, 2 = Edor, 3 = Biewkiew, 4 = Pungtong, 5 = Petsakorn, 6 = Srichompoo, 7 = Dangklom, 8 = Baidam, 9 = Haew.

that of corilagin (λ_{max} = 226, 266 nm); this compound may be another ellagitannin present in this fruit. The peak at 11.02 min can be assigned to a flavone (λ_{max} = 276, 355 nm) and the peaks at 12.65 and 13.76 min may be some ellagic acid derivatives (λ_{max} = 254, 365 nm). In addition, longan fruit also contained small amounts of epicatechin (peak at 8.70 min). This is the first report identifying these three beneficial compounds in different tissues of longan fruit. Lin et al. (*10*) and Hsu et al. (*5*) found that longan seed contained the ellagitannins corilagin and acetonyl-geraniin as the active compounds. Neither acetonyl-geraniin (MW = 992) nor its precursor geraniin (MW = 934) were found in any of the longan samples analyzed in this study, either by LC−UV/vis detection or by LC-MS (both positive ion and negative ion data evaluated). However, under these conditions a 70% methanol extract of *Geranium robertianum* L. leaves was found to contain high levels of geraniin, showing that the extraction and LC-MS conditions were appropriate for detecting these other ellagitannins if present (data not shown).

There was a large variation in the contents of the three active compounds, gallic acid, ellagic acid, and corilagin among cultivars tested. Fresh and dried pulp and seed of *E. longana* contained gallic acid while peel contained gallic acid only in dried tissues (**Figure 3**). The contents of gallic acid were higher in dried tissues (peel and seed) than fresh tissues. However, fresh pulp contained higher levels of gallic acid than dried pulp (calculated on DW basis) except for cultivar Edor. Pulp of cultivar Petsakorn and fresh peel of all cultivars contained very small amounts of gallic acid (<0.3 μg/mL) or none. Gallic acid content was highest in dried seed (0.8−2.3 mg/g DW) (**Figure 3**). The contents in dried seed were 5−10 times higher than fresh seed.

In contrast to gallic acid content, fresh and dried peel and seed contained ellagic acid at high levels whereas dried pulp contained less ellagic acid (the amounts in fresh pulp were also lower than 0.1 μg/mL) (**Figure 4**). Dried peel contained higher ellagic acid than fresh peel (calculated on DW basis) as was the case for gallic acid contents. Ellagic acid in dried peel was 1−1.5 times higher than fresh peel except for cultivar Dangklom. However, fresh seed contained higher levels of ellagic acid (1.4−4.5 mg/g DW) than dried seed (1.4−2.5 mg/g DW) (**Figure 4**). A possible reason for an increase of gallic acid and ellagic acid in dried peel tissues may be due to degradation of other ellagitannins or gallotannins by nonenzymatic or enzymatic hydrolysis, releasing free forms of gallic acid and ellagic acid. Unpublished data obtained in our study also found that corilagin standard solution was hydrolyzed to release gallic acid and ellagic acid after storage at −20 °C for a period of time (more than 6 months). Free forms of ferulic acid and total phenolics in corn were shown to increase by 550% and 54%, respectively, with thermal processing at 115 °C for 25 min (*11*). However, a decrease in ellagic acid, in the same time, in dried seed may be due to an enzymatic browning (polyphenoloxidase) reaction when plant cell is broken during drying process (all parts of fruit turned brown). The greater losses of ellagic acid observed in raspberries during frozen storage were also found to be related to a severe cellular disruption in these fruit, which was produced by a release of the enzyme polyphenoloxidase linked to the cell walls (*12*). The dehydration process (80 °C, 48 h) before inactivation of this enzyme in the present study may have damaged plant cell walls, which led to a decrease of ellagic acid. Other reasons may be due to the distribution of ellagic acid from the inner part (seed) to other parts of the fruit (pulp and peel) or ellagic acid may bind with some polysaccharides (e.g. pentose etc.) or proteins in longan seed to form insoluble derivatives that are then lost by precipitation or cannot be detected at the wavelength (270 nm) used in this method. Moreover, total phenolic contents of oven-dried (40, 60, and 80 °C) blanched apple peel have been also shown to be significantly lower than that of the fresh peel (*13*). The results from the present study indicated that in dried seed, cultivar Baidam had the highest contents of both gallic acid and ellagic acid followed by Edor, Dangklom, and Haew (**Figures 3**, **4**).

Gallic acid and ellagic acid are widely studied for their pharmacological activities such as antiplasmodial, antimicrobial, antioxidant, and cancer-preventing compounds (*14*−*23*). Recent research studies have been more specific on the mechanism in cancer prevention of these two phenolic acids (*15*, *17*−*18*, *20*). Grape seeds, a byproduct of wine production, contain high concentrations of gallic acid, whereas the waste grape skins had a much lower gallic acid content (*23*). Good dietary sources of

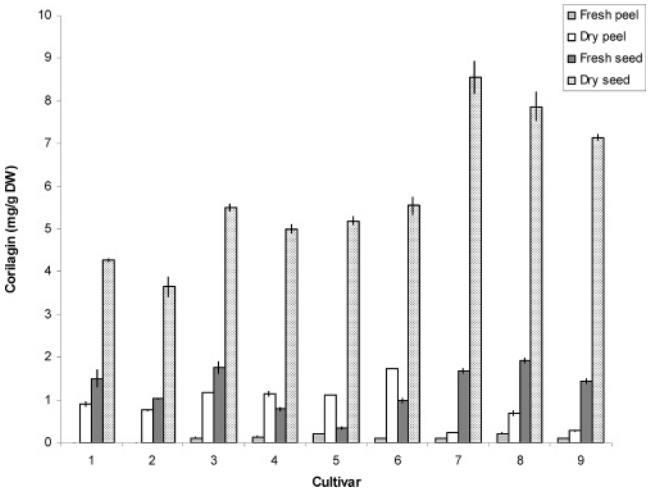

Figure 5. The contents of corilagin in different fresh and dried tissues: peel and seed in nine cultivars of *E. longana* fruit. Values represent mean of two replicates with standard error of means. All data were calculated on a DW basis. 1 = Baiyok, 2 = Edor, 3 = Biewkiew, 4 = Pungtong, 5 = Petsakorn, 6 = Srichompoo, 7 = Dangklom, 8 = Baidam, 9 = Haew.

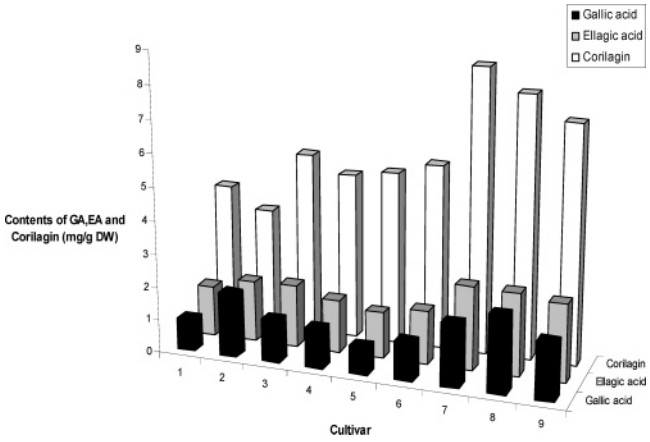

Figure 6. The contents of gallic acid (GA), ellagic acid (EA), and corilagin present in dried longan seed of the nine *E. longana* cultivars. 1 = Baiyok, 2 = Edor, 3 = Biewkiew, 4 = Pungtong, 5 = Petsakorn, 6 = Srichompoo, 7 = Dangklom, 8 = Baidam, 9 = Haew.

ellagic acid are strawberry and red raspberry, as both free ellagic acid and more complex ellagitannins (*24*−*25*). The concentration of gallic acid found in longan seed in the present study (0.8−2.3 mg/g DW) was higher than that reported in grape seed (10-99 mg per 100 g DW) by Yilmaz and Toledo (*23*). In addition, ellagic acid concentration in dried longan seed (1.4−2.5 mg/g DW) was also higher than ellagic acid concentration in berries, especially red raspberry (>160 mg per 100 g DW) and strawberry (>40 mg per 100 g DW) (*24*).

Peel and seed also contained corilagin at high levels while pulp contained low levels (0.08−0.15 mg/g DW in dried pulp; data not shown in figure) (**Figure 5**). Corilagin content in dried tissues was also 2−10 times higher than fresh tissues. The highest corilagin content was found in dried seed (3.7−8.6 mg/g DW). An increase of corilagin in dried seed may be due to the same reasons as in the case of the increases of ellagic acid and gallic acid in dried peel (hydrolysis of other ellagitannins, larger molecules). To understand the degradation or hydrolysis process, standards ellagic acid and corilagin should be treated under the same conditions for both drying process (80 °C for 48 h), extraction method, and then analyzed for the contents of gallic acid, ellagic acid, and corilagin. Seed of cultivars Dangklom and Baidam contained the highest content of corilagin (**Figure 5**). Among the commercial cultivars, Bewkaew and Srichompoo contained high levels of this compound. The results in the present study also indicated that corilagin was present as the major phenolic compound in longan seed (>50%) followed by ellagic acid and gallic acid (**Figure 6**). Corilagin has been shown to possess several important pharmacological activities especially in relation to lowering blood pressure. Therefore, these dried longan seed may be a useful alternative source for extraction of the three beneficial compounds, gallic acid, ellagic acid, and corilagin. However, the analyses of some other polyphenols or bioactive compounds present in longan pulp need to be studied further in detail by collecting and purifying these compounds from HPLC and then using NMR (nuclear magnetic resonance) to identify the structures. From the present data, the peak at 7.29 min ($[M - H]^-$ at *m*/*z* 477) may suppose to be a quercetin glucoronide (*m*/*z* 477), which was also found in raspberry fruit (*26*).

In conclusion, simple and reproducible extraction and high through-put HPLC methods were developed in this study to determine the three major phenolics, gallic acid, ellagic acid, and corilagin, in different tissues of longan (*E.longana* Lam.) fruit. The major compound in seed was corilagin followed by ellagic acid and gallic acid. In contrast to the other parts, pulp contained high levels of unidentified compounds with low levels of these three phenolic compounds. Longan seed and peel are byproducts of canned longan manufacture, and they clearly have the potential for development as extracts or pure compounds to promote good health, for example, as sources of dietary antioxidants, antihypertensive drugs, and cancer-preventing compounds. However their utilization and toxicity should be further studied. At present these investigations are also being carried out in our laboratory. Therefore, there is much scope for the utilization of longan seed and peel for creating new beneficial health products for nutraceutical markets.

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