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Isolation and structure elucidation of phenolic compounds from longan (*Dimocarpus longan* Lour.) seed by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

High-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC–ESI-MS) analysis revealed the phenolics profile of longan (*Dimocarpus longan* Lour.) seed. Gallic acid, ellagic acid, monogalloyl-glucose, monogalloyl-diglucose, digalloyl-diglucose, penta- to heptagalloyl-glucose, ellagic acid-pentose conjugate, galloyl-HHDP (Hexahydroxydiphenoyl)-glucopyranose, pentagalloy-HHDP-glucopyranose, procyanidin A-type dimer, procyanidin B2 and quercetin-3-*O*-rhamnoside were found to be present in longan seed along with a number of, as yet, unknown compounds. The results illustrate the rich array of phenolic compounds in longan seeds which could be utilized as health-beneficial bioactive compounds rather than just discarded as waste.

Keywords: Ellagitannins; Gallotannins; HPLC–ESI-MS; Longan seed; Phenolics; Proanthocyanidins

1. Introduction

Oxidative stress is believed to be an important contributing factor to the pathology of atherosclerosis, cancer and tissue damage in rheumatoid arthritis, as well as neurodegenerative diseases and the aging processes [1,2]. Phenolics have been reported to scavenge oxygen-derived free radicals as well as to inhibit lipid hydroperoxide formation [3,4]. Some epidemiologic studies have shown a correlation between an increased consumption of phenolic antioxidants and a reduced risk of cardiovascular disease, neurodegenerative disease and certain types of cancer [5,6]. In addition, tannins are reported to possess biologically multifunctional properties including antioxidant, antibacterial, antiviral, anti-inflammatory, anti-HIV, antitumor activities, and inhibitory effects on various enzymes [7–13].

Fruit seeds are known to contain many phenolic compounds capable of protecting them from oxidative damage and defending them against yeast, fungi, virus and bacteria that might inhibit their germination. Considering the developing nutraceutical industry and escalating demand for natural functional food additives, fruit seeds could be further assessed and utilized in view of their phenolics content which have been claimed to impart health benefit effects. In a previously reported study [14], longan seed was determined to possess potent antioxidant activity which could be ascribed to its phenolic content of 6300 mg/100 g seeds (dry matter). Previous work [15] has also indicated a level of 23 mg/100 g of gallic acid and 156 mg/100 g of ellagic acid in longan seeds.

The traditional usages of longan seeds as folklore medicine, that the seeds are administered to counteract heavy sweating and the pulverized kernel serves as a styptic [16], and the suggested therapeutic effects of the phytonutrients, advocate further research on the possibility of processing longan seeds into biopreservatives or therapeutic functional food ingredients. Phenolics with different structures are likely to have different chemical and biological properties and there may have positive implication for human health. Therefore, it is important to clearly identify the individual phenolic

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compounds in fruit seeds before use as functional food ingredients so that their single or synergistic effect can be studied.

Reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection is most frequently used for the separation and detection of phenolic compounds from plants [17–21]. MS is used for unambiguous characterization of phenolic compounds, eliminating artifacts arising from co-eluting compounds with similar UV spectra. An ESI interface may be connected to a HPLC system. ESI is a gentle ionization method generating mainly deprotonated molecules $[M-H]^-$ of the compounds analyzed in the negative ion mode for rapid determination of the molecular mass [22,23]. The mild ionization can be complemented by invoking fragmentation-induced collisions in the interface itself or by resource to LC-tandem MS as realized with the use of a triple quadrupole system. A collision-induced dissociation (CID) mass spectrum confirms the identity of the compound as it is characteristic of the structure of the parent compound. Longan seeds have been reported to contain gallic acid, ellagic acid, corilagin and acetonyl-geraniin [24–26]. However, in this study, some 26 phenolic compounds in longan seed, including those phenolic acids but not the ellagitannins reported above have been characterized by the use of mass spectrometry for their structure elucidation.

2. Experimental

2.1. Chemicals and materials

Longans (*Dimocarpus longan* Lour.) imported from Thailand, were purchased on separate occasions at local markets in Singapore. Ellagic acid (approx. 95%) was purchased from Sigma (St. Louis, MO, USA). Gallic acid (98%) was purchased from Acros Organics (Fairlawn, NJ, USA). Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden). Absolute ethanol, acetic acid and HPLC grade methanol were purchased from Merck (Darmstadt, Germany).

2.2. Sample pretreatment

The seeds from longan fruits at the maturity stage best for consumption were freeze-dried (−50 °C, 24 h) and ground using a stainless-steel grinder. All the samples were stored in vacuum-packaged polyethylene pouches at −20 °C until required for analysis.

2.3. Seeds extraction

An amount of 20 g (∼22 longan seeds) of freeze-dried ground longan seed was accurately weighed and refluxed three times with 50 ml of ethanol:water (50:50, v/v) in a water bath at 70 °C for one hour. The pooled extracts were passed through a Whatman filter paper no. 4 and the filtrate was concentrated to circa 60 ml on a rotary evaporator below 40 °C under reduced pressure. The aqueous residue was defatted three times with 60 ml of chloroform, and then freeze-dried.

2.4. Column chromatography

Fractionation was carried out according to the method described by Wettasinghe and Shahidi [27]. A 200 mg portion of the above freeze-dried aqueous residue was dissolved in 4 ml of HPLC grade methanol and chromatographed on a Sephadex LH-20 column (45 cm × 2.5 cm I.D.) eluted with methanol. Methanolic fractions (10 ml each) were collected in test tubes and their absorbance was measured at 280 nm by the use of UV spectrophotometer (UV mini 1240 Shidmazu). Based on the absorbance data, eluates were pooled into major fractions, solvent evaporated and re-dissolved in 1 ml of HPLC grade methanol. Samples were prepared as above in duplicate. The samples were filtered through a 0.45 μm filter prior to injection (20 μl) to the HPLC system.

2.5. HPLC-DAD and ESI-MS analysis

The TSP 4000 HPLC system was coupled with an on-line degasser, P4000 quaternary pump, AS3000 autosampler, and UV6000LP PDA detector, as well as connected in series with a Finningan MAT LCQ quadrupole ion trap mass analyzer (San Jose, CA, USA) fitted with an ESI source. The separation was performed on a Shim-Pack VP-ODS column (250 mm × 4.6 mm I.D.; Shimadzu, Kyoto, Japan) with a guard column (GCP-ODS, 10 mm × 4.6 mm I.D.). Solvent gradients were formed by varying the proportion of solvent A [water–acetic acid (97:3, v/v)] to solvent B (methanol). Solvent B was increased to 10% in 10 min and subsequently increased to 70% in 40 min at a flow rate of 0.9 ml/ min. For DAD, the phenolic compounds were detected at both 280 nm and 360 nm. For MS, the heated capillary and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was operated at 80 psi as sheath gas and 20 psi for auxiliary gas flow. MS (full scan) from *m*/*z* 50–2000 with a scan speed of 1 s/scan was used to measure the $[M-H]^-$ ions, revealing the molecular masses of the components. MS–MS was used to break down the most abundant $[M-H]^-$ ion from MS with dependent CID and MS^3 was used to break down the most abundant fragment ion from MS–MS with CID. Tandem mass spectrometry was performed using helium as the collision gas, operated at 0.8 mTorr, and the collision energy was set as 50%. All mass spectrometry data were acquired in the negative ionization mode.

3. Results and discussion

3.1. Sephadex LH-20 column chromatography of crude longan seed extracts

Sephadex LH-20 column chromatography has been widely used for the fractionation of natural plant extracts [28–30]. The separation of phenolics on the Sephadex gel column is fast and efficient [28]. Phenolic compounds are separated by filtration (molecule sieve effect of the gel) and

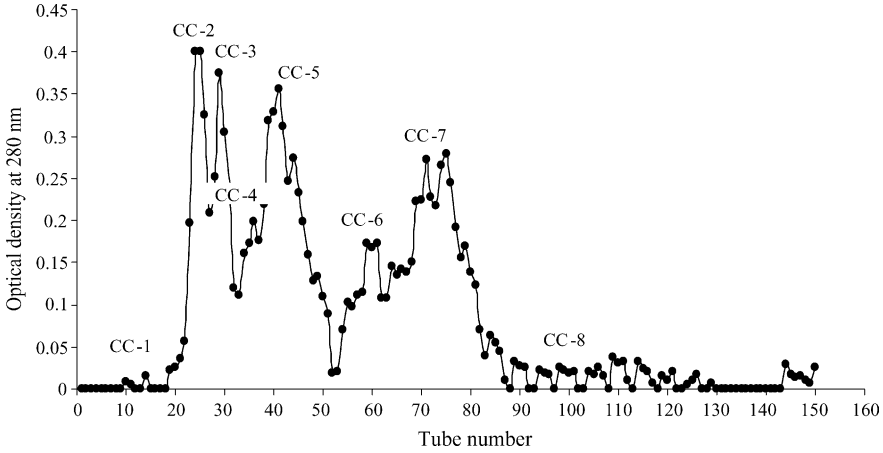

Fig. 1. Column chromatography fractions profile for longan seed crude extracts.

adsorption (hydrogen bonding). Fractionation of crude longan seed extract by column chromatography yielded a total of eight fractions (labeled CC-1 to CC-8), according to their absorbance readings at 280 nm (Fig. 1). HPLC-DAD analysis has demonstrated a good separation of the various compounds in each fraction.

3.2. Characterization of phenolics from longan seed extracts by HPLC–MS

The total ion chromatograms (TICs) of longan seed extracts were initially acquired by the HPLC–ESI-MS in both positive and negative modes. Negative ionization was used since complex adduct formation occurred in the positive ion mode. The TICs of longan seed fractions detected with negative ion mode was shown in Fig. 2. Fragmentation of vegetable tanning agents at negative mode was proposed by Zywicki et al. [31]. Hydrolyzable tannins from leaves of *Betula pubescens* was also characterized by the HPLC–MS in the negative ion mode [32].

Longan seed was found to contain 14 positively identified phenolic compounds along with 12, as yet, unknown compounds as shown in Tables 1 and 2 with the structures in Fig. 3. Following comparison of mass spectra with authentic standards, gallic acid anion (*m*/*z* 169) and ellagic acid anion (*m*/*z* 301) were characterized by the ESI-MS. Gallic acid anion decarboxylates to form a fragment of *m*/*z* 125; ellagic acid anion generates intensive product ions at *m*/*z* 257 and 229. Mass spectrometric analysis revealed a molecular anion of ellagic acid–pentose conjugate at *m*/*z* 433; this ion eliminated a pentosyl unit giving rise to a product ion of *m*/*z* 301 which followed the fragmentation pattern of ellagic acid.

Monogalloyl- (*m*/*z* 331), pentagalloyl- (*m*/*z* 939), hexagalloyl- (*m*/*z* 1091) and heptagalloyl-glucose esters (*m*/*z* 1243) were found to be present in longan seed extracts. The heptagalloyl-glucose anion *m*/*z* 1243 eliminated a galloyl ester group (*m*/*z* 1091) from where a second galloyl moiety (*m*/*z* 939) was eliminated. Elimination of a galloyl ester group (*m*/*z* 787) and water (*m*/*z* 769) were observed for the pentagalloyl-glucose anion *m*/*z* 939. The glucose was

Table 1
HPLC–ESI-MS data for phenolic compounds in longan seed extracts

Peak no.	Phenolic compounds	$[M-H]^-$ (*m*/*z*)	MS^2 ions (*m*/*z*)	MS^3 ions (*m*/*z*)	Isomers
I	Gallic acid	169	125		n.d.[a]
II	Monogalloyl-glucose	331	271, 211, **169**[b]	125	n.d.
III	Monogalloyl-diglucose	493	**331**, 313, 271, 211	313, 241, 169	n.d.
IV	Digalloyl-diglucose	645	**493**, 483, 313, 271	331, 271, 211	n.d.
V	Pentagalloyl-glucose	939	**769**	617	2
VI	Hexagalloyl-glucose	1091	**939**	769	2
VII	Heptagalloyl-glucose	1243	**1091**, 939		2
VIII	Ellagic acid	301	284, 257, 229		n.d.
IX	Ellagic acid-pentose conjugate	433	**301**	284, 257, 229	n.d.
X	Galloyl-HHDP-glucopyranose	633	463, **301**	284, 257, 229	≥2
XI	Pentagalloyl-HHDP-glucopyranose	1241	1179, 633		n.d.
XII	Procyanidin A-type Dimer	575	**449**, 423, 289	287	n.d.
XIII	Procyanidin B2	577	451, 425, 407, 289		≥3
XIV	Quercetin-3-*O*-rhamnoside (quercitrin)	447	**301**	271, 255, 179, 151	n.d.

[a] n.d.: Not detected.
[b] *m*/*z* in bold was subjected to MS^3 analysis.

eliminated from the monogalloyl-glucose anion *m*/*z* 331 giving a gallic acid anion *m*/*z* 169. The daughter ions *m*/*z* 271 and 211 formed during the fragmentation of mono- to pentagalloyl-glucose have been previously described [31]. Monogalloyl-diglucose (*m*/*z* 493) and digalloyl-diglucose (*m*/*z* 645) have been identified by analyzing their fragmentation patterns. The addition of a glucosyl unit to anion *m*/*z* 331 gave rise to monogalloyl-diglucose (*m*/*z* 493) from where a second galloyl ester group was added to form digalloyl-diglucose (*m*/*z* 645).

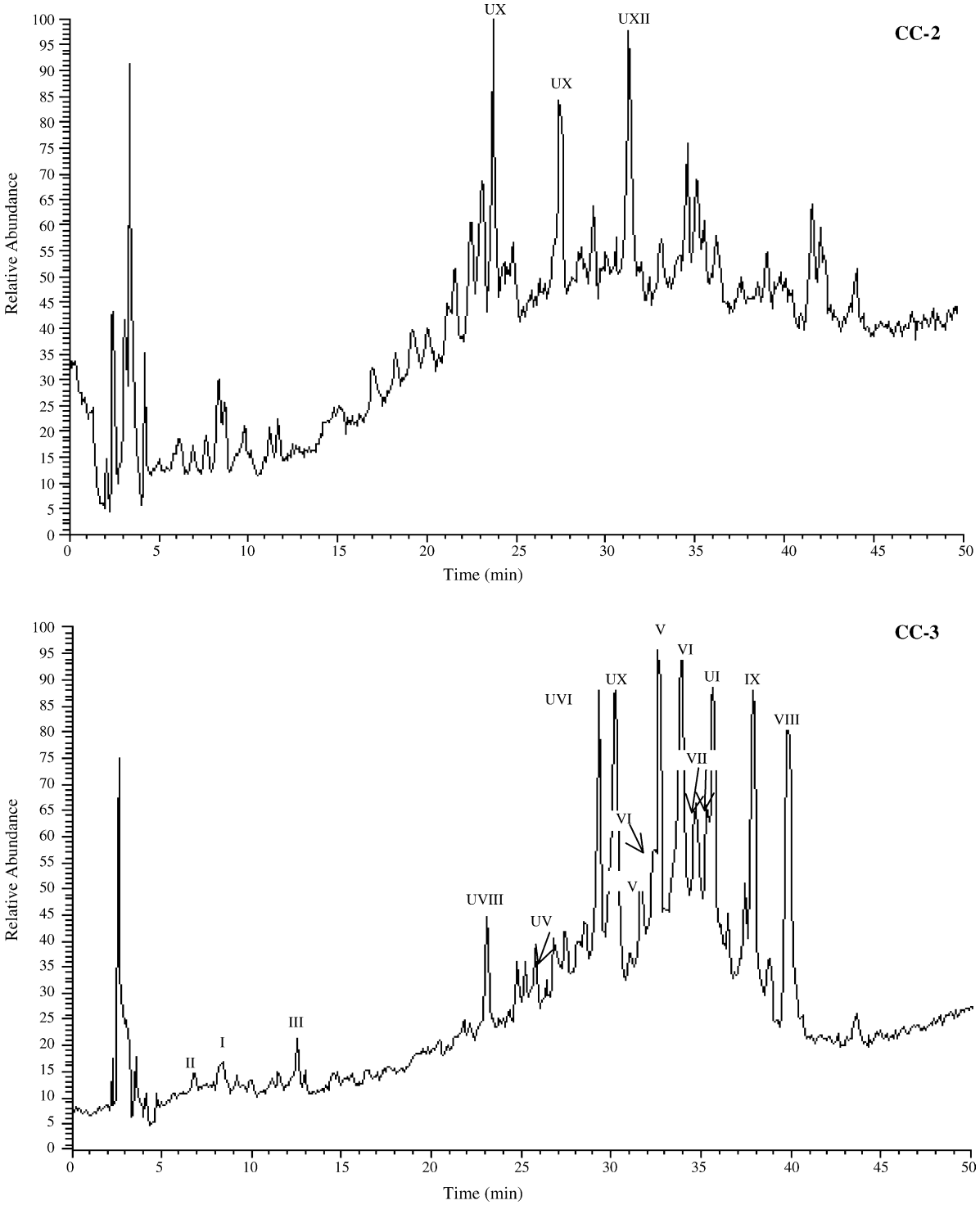

Fig. 2. TICs of longan seed fractions detected with negative ESI-MS in full scan mode. Roman numerals refer to individual compounds in longan seed extract listed in Tables 1 and 2.

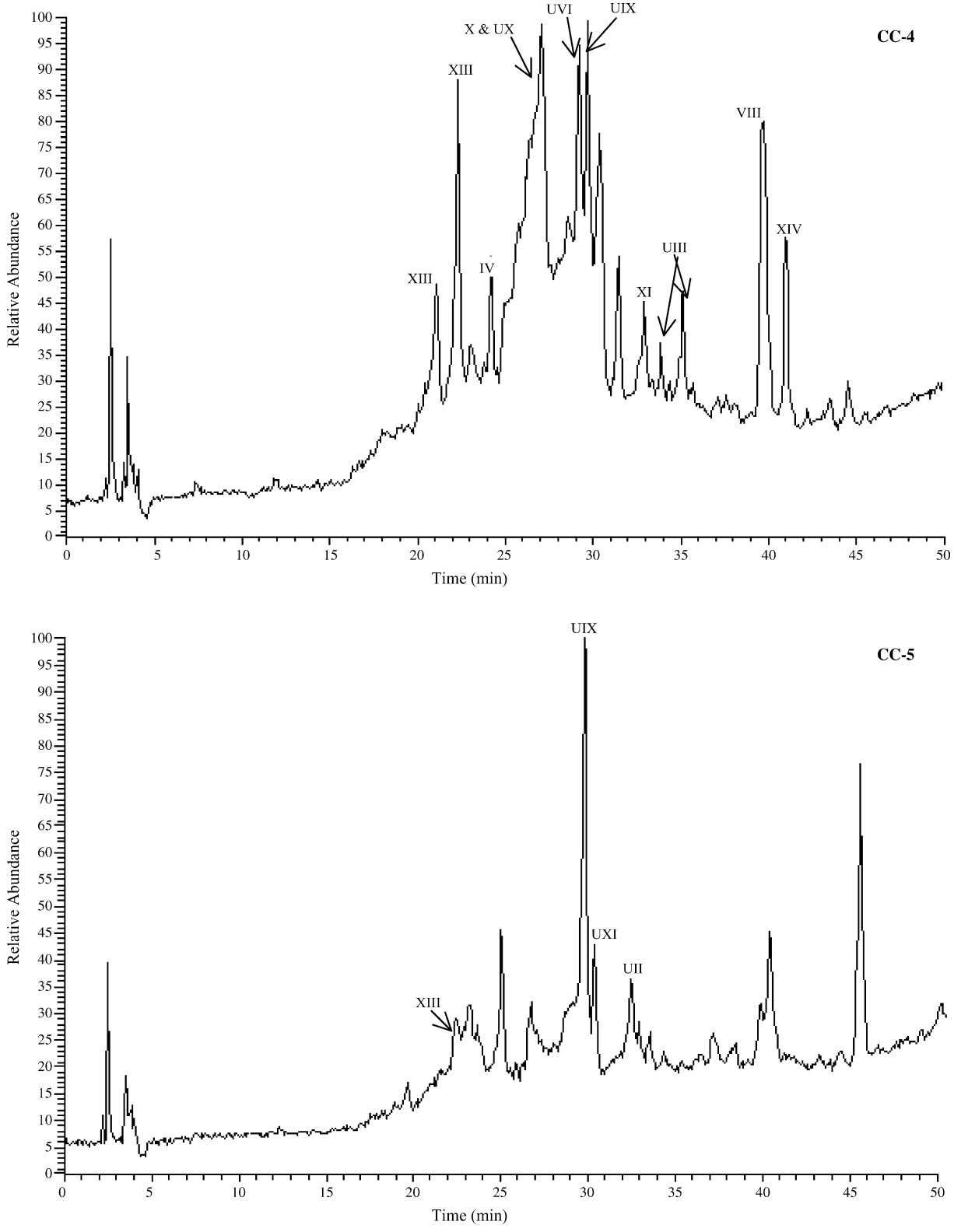

Fig. 2. (*Continued*)

Comparison of the mass spectra with those reported by Salminen et al. [32] reveals the presence of galloyl-HHDP-glucopyranose (*m*/*z* 633) and pentagalloyl-HHDP-glucopyranose (*m*/*z* 1241) in longan seed extracts. The latter (*m*/*z* 1241) contained fragment of *m*/*z* 633 which gave product ions at *m*/*z* 463 and 301 after losing a gallic acid and glucosyl unit respectively. The ion *m*/*z* 301 has a similar fragmentation pattern as ellagic acid confirming the presence of ellagitannin.

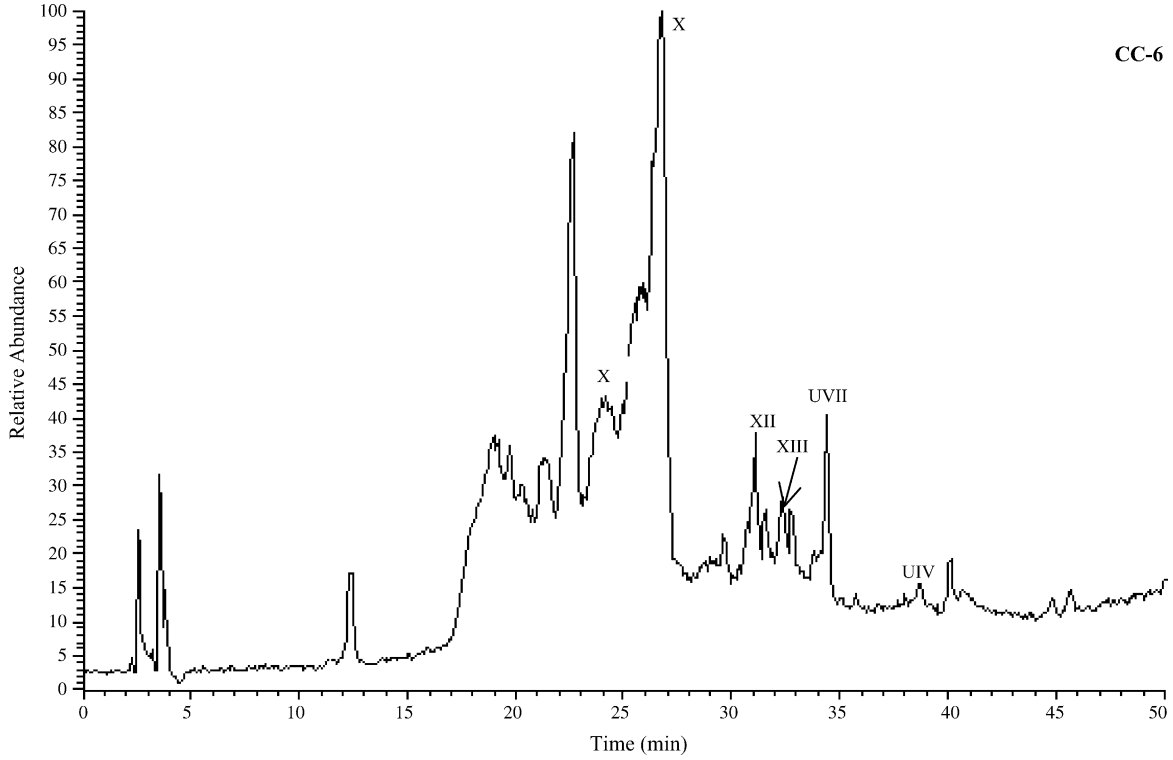

Fig. 2. (*Continued*).

The intensive peaks at *m*/*z* 985, 977, 969, 953, 951, 907, 801, 651 and 625 remained unidentified, but they may belong to ellagitannins illustrated by bearing the fragments of *m*/*z* 633 and *m*/*z* 301 (Table 2). The fragmentation pattern of ellagitannins was less clear than the gallotannins as ellagitannins display an enormous structural variability that results from the manifold possible sites for the linkage of HHDP residues with the glucose moiety and particularly by their strong tendency to form a multitude of dimeric and oligomeric derivatives being inter-connected by C–C and C–O–C linkages [33]. Characterization of these unknown compounds could be important for studying their structure-biological property relationship.

The mass spectra showed a molecular anion at *m*/*z* 447 which was subsequently fragmented giving an aglycone fragment of *m*/*z* 301. This could be identified as quercetin attached to a desoxy hexose. Comparison of mass spectrometric data with the study of Schieber et al. [34] revealed the presence of quercetin-3-*O*-rhamnoside in longan seeds. In addition, molecular anions at 477, 407 and 319 were observed but their identities have not yet been resolved.

The dimers procyanidin have been identified by observing the prominent molecular anion at *m*/*z* 577 and 575. Fragmentation of these anions yielded a monomer catechin (*m*/*z* 289 and 287) due to the cleavage of the interflavanoid C–C linkages with losses of 288 amu. Alternatively, a series of

Table 2
HPLC–ESI-MS data for unknown compounds in longan seed extracts

Peak no.	Phenolic compounds	$[M-H]^-$ (*m*/*z*)	MS^2 ions (*m*/*z*)	MS^3 ions (*m*/*z*)
UI	Unknown	319	273	
UII	Unknown	407	275	
UIII	Unknown	477	417, **315**[a], 265, 169	169, 163
UIV	Unknown	625	**301**	257, 229
UV	Unknown	651	633, 481, 463	
UVI	Unknown	801	**757**, 631	633, 301
UVII	Unknown	907	**633**, 435	301
UVIII	Unknown	951	933, 301	
UIX	Unknown	953	935, 633, 481, 301	
UX	Unknown	969	925, 881, 633	
UXI	Unknown	985	**953**, 935, 633, 301	801, 757
UXII	Unknown	997	907, 633, 463, 301	

[a] *m*/*z* in bold was subjected to MS^3 analysis.

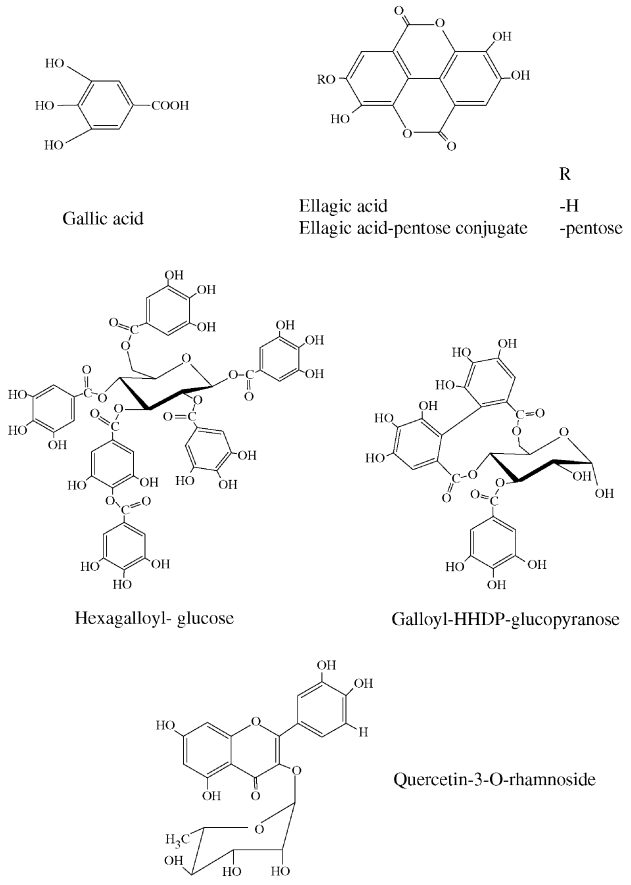

Fig. 3. Chemical structures of phenolic compounds.

fragments may evolve from a RDA (Retro-Diels-Alder) fission of the flavonoid nucleus [35,36] giving rise to a fragment of *m*/*z* 425 from anion *m*/*z* 577. This product (*m*/*z* 425) eliminates water, probably from ring C in position C3/C4, resulting in a fragment ion of *m*/*z* 407 (Fig. 4). Consistent with the fragmentation pattern reported by Tomás-Barberán et al. [37], the molecular anions *m*/*z* 577 and 575 were identified as procyanidin B2 and procyanidin A type dimer respectively. The latter showed a characteristic mass spectra fragment at 575 $[M-H]^-$, *m*/*z* 423 (RDA fragment), *m*/*z* 287 and 285. This A-type dimer showed similar mass spectra to those of the B-type dimers but 2 amu less as it possesses two inter-catechin bond.

Based on the mass spectra, fractions 2–6 were determined to contain phenolic compounds as listed in Tables 1 and 2. Some compounds were found to be spread over several fractions.

4. Conclusions

In the present study, HPLC–ESI-MS analysis has revealed the wide array of phenolic compounds present in longan seed comprising gallic acid, ellagic acid, monogalloyl-glucose, monogalloyl-diglucose, digalloyl-diglucose, penta- to hepta-galloyl-glucose, ellagic acid-pentose conjugate, galloyl-HHDP-glucopyranose, pentagalloy-HHDP-glucopyranose, procyanidin A-type dimer, procyanidin B2 and quercetin-3-*O*-rhamnoside along with 12, as yet, unknown compounds but most likely ellagitannins. The molecular ions of individual phenolic compounds and their fragmentation spectra are valuable confirmatory data when compared with the authentic standards or reference data. The analytical method allows the positive identification of phenolic acids, flavonoids, gallotannins, ellagitannins and proanthocyanidins under the same chromatographic conditions. Quantification of oligomeric tannin components is hampered by the lack of pure standards however longan seeds have been previously reported

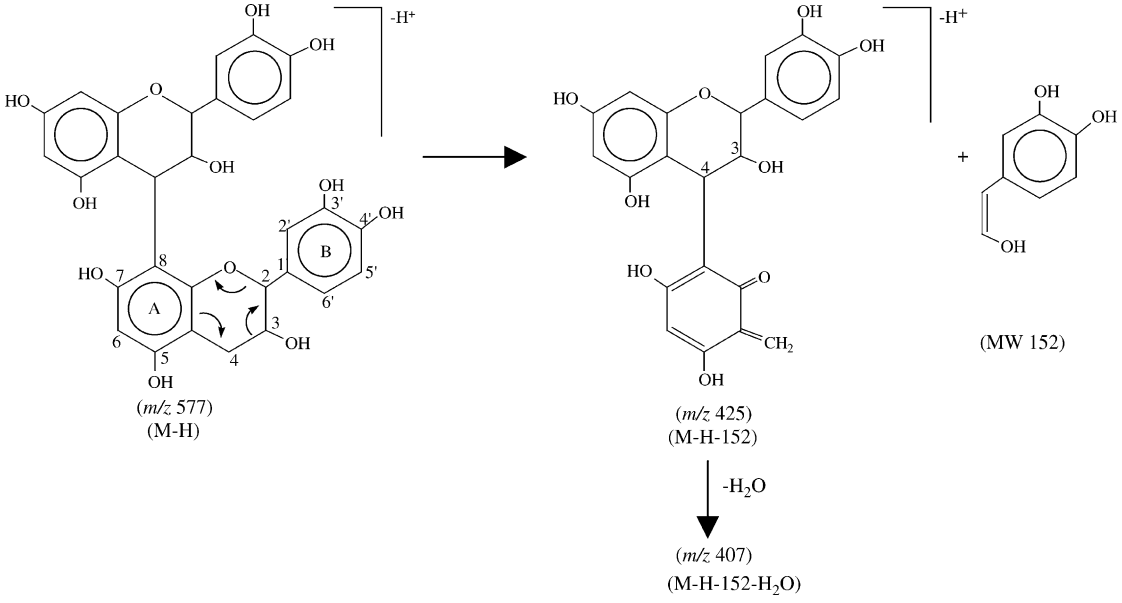

Fig. 4. Postulated RDA fragmentation of Procyanidin B2.

by the authors to contain considerable amounts of total phenolics content that contribute to antioxidant activity. Phenolic compounds of several different types in longan seed extract might form a synergistic multilevel defence system as each phenolic is unique in terms of its structure and chemical and biological properties. The study breaks new ground and may mark the beginning of longan seeds exploitation for their contents of functional components. However, further investigation of individual phenolic compounds, their in vivo antioxidant activity and the different antioxidant mechanism, as well as the biological properties is necessary before applications of longan seeds in pharmaceutical and food products can be positively advocated.

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