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Isolation and characterization of ellagitannins as the major polyphenolic components of Longan (*Dimocarpus longan* Lour) seeds

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ABSTRACT

Longan (*Dimocarpus longan* Lour, syn. *Euphoria longan* Lam.) represents an important fruit in Northern Thailand and has significant economic impact. The fruit is either consumed fresh or as commercially prepared dried and canned products. The canning industry in Thailand produces considerable quantities of waste products, in particular Longan seeds. Because these seeds may be an exploitable source of natural phenolic antioxidants, it was of interest to identify, purify and quantitate the major potential antioxidant phenolics contained therein. The polyphenolic fraction from ground Longan seeds was obtained by extraction with methanol after delipidation with hexane. The hexane extract contained predominantly long-chain fatty acids with major contributions from palmitic (35%) and oleic (28%) acids. The polyphenolic fraction (80.90 g/kg dry weight) was dominated by ellagic acid (25.84 g/kg) and the known ellagitannins corilagin (13.31 g/kg), chebulagic acid (13.06 g/kg), ellagic acid 4-*O*-α-L-arabinofuranoside (9.93 g/kg), isomallotinic acid (8.56 g/kg) and geraniin (5.79 g/kg). Structure elucidation was performed with mass spectrometry and complete assignment of ^{1}H and ^{13}C NMR signals. The methanol extracts exhibited strong antioxidant capacities with an IC_{50} of 154 μg/ml for reactive oxygen species attack on salicylic acid and 78 μg/ml for inhibition of xanthine oxidase in the hypoxanthine/xanthine oxidase assay. The extracts were less effective in the 2-deoxyguanosine assay (IC_{50} = 2.46 mg/ml), indicating that gallates along with ellagic acid and its congeners exert their potential antioxidant effects predominantly by precipitation of proteins such as xanthine oxidase. This was confirmed for the pure compounds gallic acid, methyl gallate, ellagic acid and corilagin.

1. Introduction

Increased dietary intake of phenolic antioxidants from plants, especially fruits and vegetables, may reduce the incidence of some degenerative diseases such as cancer (Fekanich et al., 2000; Michels et al., 2000; Gonzalez and Riboli, 2010), and coronary heart disease (Oude Griep et al., 2010; Dauchet et al., 2010).

Longan (*Dimocarpus longan* Lour, syn. *Euphoria longan* Lam., Family Sapindaceae) fruits make a significant contribution to the Thai diet, especially in northern Thailand, where the cultivation of Longan is favoured over other fruits. In traditional medicine the flesh of the fruit is administered as a stomachic, febrifuge (antipyretic) or vermifuge (anthelmintic) and is regarded as an antidote for poison. A decoction of the dried flesh is taken as a tonic or as treatment for insomnia or neutrasthenic neurosis. In both North and South Vietnam, the "eye" of the Longan seed is pressed against a snakebite in the belief that it will absorb the venom.

Longan fruits are either consumed fresh or as commercially prepared dried and canned products. The canning industry produces considerable quantities of waste products, in particular Longan seeds, which may be an exploitable source of natural phenolic antioxidants. In comparison to pericarp Longan seeds represent a considerably higher proportion of the by-products arising from fruit processing in Thailand.

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; COLOC, correlation via long-range coupling (2D-NMR); COSY, correlated spectroscopy (2D-NMR); cROESY, compensated rotating-frame Overhauser spectroscopy (2D-NMR); DHBA, dihydroxybenzoic acid; DHHDP, dehydrohexahydroxydiphenoyl moiety; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; ESI, electrospray ionization; FRAP, ferric reducing ability of plasma; GC, gas chromatography; HHDP, hexahydroxydiphenoyl moiety (6,6′-dicarbonyl-2,2′,3,3′,4,4′-hexahydroxybiphenyl); HMBC, heteronuclear multiple-bond correlation (2D-NMR); HPLC, high-performance liquid chromatography; HSQC, heteronuclear single-quantum correlation (2D-NMR); IC_{50}, concentration for 50% inhibition; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; ORAC, oxygen radical absorbance capacity; SD, standard deviation; TMS, trimethyl silyl (GC–MS) or tetramethyl silane (NMR); TPTZ, 2,4,6,-tripyridyl-*s*-triazine complex; XHCORRD, heteronuclear correlation with remote proton decoupling (2D-NMR).

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The common antioxidants used in the food, cosmetic and pharmaceutical industries, e.g., butylated hydroxytoluene and butylated hydroxyanisole, are synthetic and may be carcinogenic (Tsuda et al., 1994). Although α-tocopherol, β-carotene and ascorbic acid are non-carcinogenic natural antioxidants, they are less effective than synthetic compounds and their isolation and purification costs are high. Therefore, the processing residues generated by the agricultural and food industries may represent alternative inexpensive raw materials from which other natural phenolic antioxidants can be isolated and purified.

Previous data on the content and profile of phenolic compounds in Longan seeds is rather fragmentary. Compounds which have been definitively identified, include gallic acid, ellagic acid and corilagin (Soong and Barlow, 2005; Rangkadilok et al., 2005) along with ethyl gallate, 1-β-*O*-galloyl-D-glucopyranose, brevifolin, methyl brevifolincarboxylate and 4-*O*-α-L-rhamnopyranosyl-ellagic acid (Zheng et al., 2009). In this paper we describe in detail the isolation and structure elucidation (with comprehensive NMR data) of the polyphenolic secondary plant substances from methanol extracts of Longan seeds.

We also report on the antioxidant capacity of these extracts and purified phenolic compounds therefrom, as determined in the hypoxanthine/xanthine oxidase assay, the 2-deoxyguanosine HPLC-based assay, as well as the DPPH, FRAP and ORAC assays.

2. Results

2.1. Lipid content

The long-chain fatty acid profile for the hexane extract of Longan seeds can be summarized as follows [% of total (SD)]: palmitic acid, 34.6 (0.14); linoleic acid, 10.05 (0.21); oleic acid, 27.95 (0.07); stearic acid, 10.85 (0.07); C19.1, 9.65 (0.07); C20.1, 5.05 (0.07) and C22.1, 1.80 (0.0).

2.1.1. Identification of phenolic compounds in Longan seeds

The analytical reverse-phase HPLC profiles for the methanolic extract of Longan seeds are shown for two detector wavelength settings in Fig. 1. A total of eleven phenolic components (peak and compound numbers **1**–**11**) were identified by MS and NMR analysis as the following known compounds: gallic acid (**1**), methyl gallate (**2**), corilagin (**3**), (−)-epicatechin (**4**), an A-type proanthocyanidin trimer (**5**), geraniin (**6**), isomallotinic acid (**7**), methyl brevifolin carboxylate (**8**), chebulagic acid (**9**), ellagic acid 4-*O*-α-L-arabinofuranoside (**10**), and ellagic acid (**11**). The structures of these compounds are depicted in Fig. 2.

HPLC–ESI-MS and nano-ESI-MS data for all isolated native compounds (**1**–**11**) are presented in Tables 1 and 2, respectively. For compounds **1**–**3** TMS–ether/ester derivatives were prepared, and their GC–MS data are summarized in Table 3. Compound **5** was not available in sufficient quantity for NMR and was identified on the basis of the molecular mass of 864 as a proanthocyanidin trimer containing three monomers of the catechin or epi-catechin type (monomer mass 290) with one A-type and one B-type linkage. Without NMR the stereochemical details could not be determined. Therefore, Fig. 2 shows only one of several possible isomers as an example. The dashed lines labeled a, b, c represent fragmentation schemes for negative ESI-MS–MS which lead to the following observed neutral-loss fragments (*m*/*z*): (a) $[M-H-C_{15}H_{14}O_6]^-$ = 573.1, $[M-H-C_{30}H_{22}O_{12}]^-$ = 289.1; (b) $[M-H-C_{24}H_{20}O_9]$ = 411.1, $[M-H-C_{21}H_{16}O_9]^-$ = 451.1; (c) $[M-H-C_8H_8O_3]$ *Euphoria longan* = 711.1.

^{1}H and ^{13}C NMR data were obtained for all compounds except **5**. The completely assigned NMR data for compounds **1**, **2**, **3**, **6**, **7**, **8**, and **10** are summarized in Tables 4–8 since data of this quality for CD_3OD solutions have not been available in the literature (see Section 3.1). Compounds **1** and **2** were confirmed as gallic acid and methyl gallate (Tables 4 and 5) by comparison of their NMR data with our reference data (Barreto et al., 2008). Compounds **4** and **11** (data not shown) were confirmed as (−)-epicatechin and ellagic acid, respectively, also by comparison with our detailed reference data (Khallouki et al., 2007). Compound **9** (data not shown) was confirmed as chebulagic acid with the stereochemistry as shown in Fig. 2 by comparison of the data from our Longan sample with our detailed reference data for samples obtained from extracts of *Terminalia* fruit (Pfundstein et al., 2010).

Compound **3** was confirmed as corilagin (Tables 3–5) by virtue of the following structural features confirmed by NMR. The β-glucose core is in the inverted $^1C_4$ conformation with all ring protons in equatorial positions (series of small vicinal ^3J couplings around the ring, relatively large ^4J couplings via equatorial W pathways). The chemical shifts indicate acylation at positions 1, 3, and 6. The galloyl substituent at C1 was confirmed by $^3J_{CH}$ = 3.2 Hz between galloyl C7 and glucose H1 and by the NOEs observed at galloyl H2,6 when irradiating glucose H2 (0.3%) and H3 (0.8%) and at glucose H6b when irradiating galloyl H2,6. The 3,6-(*R*)-HHDP substituent was confirmed by $^3J_{CH}$ = 2.9 Hz between carbon A-7 and glucose H3, $^3J_{CH}$ = 3.8 Hz for carbon B-7′ and glucose H6b (dihedral angle 5° in molecular model) and $^3J_{CH}$ = 0.8 Hz for B-7′ with glucose H6a (dihedral −112°). Furthermore, proton A-3 exhibits NOEs with glucose H2 (0.5%) and galloyl H2,6 (0.5%) while B-3′ shows no NOE effects. With the assignments for protons A-3 and B-3′ in hand, the assignments for all carbons in the HHDP skeleton were made on the basis of the measured long-range J_{CH} couplings (^{1}H-coupled ^{13}C spectrum and appropriate selective ^{1}H decouplings).

The NMR of compound **7** (Tables 3–5) indicated that it was very similar to corilagin (inverted β-glucose with same substitution pattern) but contained, instead of the 3,6-(*R*)-HHDP unit, a three-ring valoneoyl unit attached at positions 3 and 6 of glucose and featuring three aromatic proton singlets. In the literature (Lee et al., 1990) one finds two appropriate isomeric compounds: mallotinic acid, with the A and B rings of valoneoyl attached to positions 6 and 3 of glucose, respectively and isomallotinic acid, with the A and B rings attached in reverse order to positions 3 and 6, as shown in Fig. 2. The available NMR data for acetone-d_6 solutions of mallotinic acid (Saijo et al., 1989) and isomallotinic acid (Lee et al., 1990) provide complete ^{1}H and ^{13}C assignments for the glucose and galloyl moieties but only partial ^{13}C assignments for the valoneoyl group. Therefore, for a CD_3OD solution we performed complete *ab initio* signal assignments via 2D NMR and molecular modeling to confirm compound **7** as isomallotinic acid. The following ^3J coupling pathways were detected: ValA-H3 to ValA-C7 to Glc-H3 and ValB-H3′ to ValB-C7′ to Glc-H6b ≫ H6a. The latter result is consistent with the energy-minimized molecular model where the dihedral angle ValB-C7′ to Glc-H6b = 2° (large $^3J_{CH}$) while the dihedral to Glc-H6a = −114° (small $^3J_{CH}$). The ValB-C4′

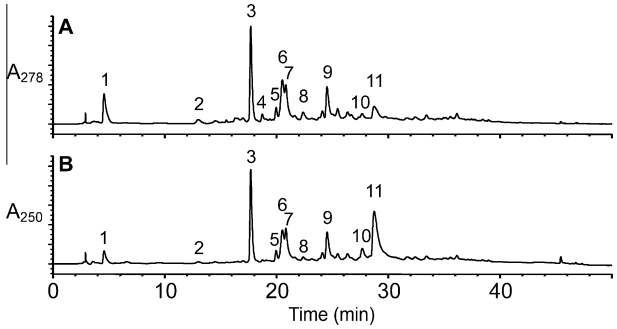

Fig. 1. Analytical HPLC chromatograms of the methanol extract of Longan seeds display absorption at 278 nm (A) or 250 nm (B) vs. retention time. The peaks labeled **1** to **11** correspond to the compounds listed in Table 1.