Identification of Phenolic Compounds in *Aquilaria crassna* Leaves Via Liquid Chromatography-Electrospray Ionization Mass Spectroscopy

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In this study, we extracted *Aquilaria crassna* with aqueous ethanol and water and analyzed the extracts via liquid chromatography diode array detection and electrospray ionization mass spectrometry (LC–ESI–MS) methods. Phenolics were separated using semi-micro HPLC and were identified as iriflophenone 3,5-Cβ-diglucoside (1), iriflophenone 3-Cβ-glucoside (2), mangiferin (3), iriflophenone 2-O-α-rhamnoside (4), genkwanin 5-O-β-primeveroside (5), genkwanin 5-O-β-glucoside (6), genkwanin 4′-methyl ether 5-O-β-primeveroside (7), and genkwanin (8) via a comparison with authentic samples. The collision-induced dissociation (CID)-MS/MS spectra of these polyphenols and the unknown chromatographic peaks were detected using hybrid ion trap time-of-flight (IT-TOF) mass spectrometry. The results of the present study demonstrated that LC–ESI–MS can be useful for the specific quality control of extracts of extracted *A. crassna*.

Keywords: *Aquilaria crassna*, agarwood leaves, LC–ESI–MS, quality control, polyphenols, laxative effect

Introduction

*Aquilaria* is a woody plant in the Thymelaeaceae family native to Southeast Asia, and some members of the genus, represented by *A. crassna* and *A. sinensis*, are known as incense trees (Ng, Chang, and Kadir, 1997). The depletion of wild trees due to indiscriminate cutting of agarwood has resulted in these trees being listed and protected as an endangered species. In Thailand, *A. crassna* has been systematically cultivated to ensure a constant production of resin and processed for perfume. In addition, the leaves have been used as tea. The polyphenols present in *A. crassna* leaves could be an important source of bioactive components. The major polyphenols in this material are glycosides of flavonoids, benzophenones, and xanthones. Researchers have recently clarified the laxative properties of agarwood leaves (AL) and identified mangiferin (3) and 5-O-β-primeveroside (5) as the active components (Hara et al., 2008; Kakino et al., 2010). The functional food applications of AL require an exact understanding of not only the chemical components, but also the AL properties such as their chemical natures, sizes, solubilities, and the degrees and positions of glycosylation, which influence their pharmacokinetics and pharmacodynamics in humans. In addition, it is necessary to identify the AL phenolics present in these suppliable materials (*A. crassna* and *A. sinensis*) and control the standard qualities of the extracts prepared via standardized conditions. Due to the
lack of standard methods for sample preparation, extraction, and analysis, there is no general consensus on a standard protocol for the quantitation of phenolic compounds in AL. Our present study focused only on the identification and not the quantitation of these phenolic compounds in AL in 60% ethanol extracts (ALEE) and hot water extracts (ALWE). The objective of our study was to identify the phenolic compounds and generate characteristic chromatographic fingerprints of ALEE and ALWE via liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS) with multi-stage analysis (MS²). Our study provides useful information necessary for the generation of standardized AL materials for in vitro and in vivo studies and for the authentication of AL-based food products.

**Experimental Methods**

*General experimental procedures* The instruments used in this study were DGU-20A3, LC-20AP, CBM-20A, SPD-M20A, and SIL-20A LC instruments for semi-micro HPLC (Shimadzu, Kyoto, Japan) and a Shimadzu hybrid IT-TOF mass spectrometer (Shimadzu).

*Plant material* *A. crassna* was collected in Pechaboon, Thailand, in October 2009 and was identified by M. Inuma, one of the authors. A voucher specimen has been deposited at the herbarium of API Co. (Gifu, Japan).

*Extraction procedures* The dried and chopped leaves of *A. crassna* (50 g) were separately extracted with either 60% (v/v) ethanol (1.0 L, 24 h × 1, room temperature) or water (1.0 L, 1 h × 1, 95°C), and then were filtered. The extracts were concentrated in vacuo at 50°C to yield alcohol and water AL extracts [ALEE (11.3 g) and ALWE (11.1 g)], respectively.

*Reagents for HPLC* All solvents were HPLC grade and purchased from Sigma Aldrich Co. (St. Louis, MO). EA, catechin, epicatechin, and quercetin standards were also purchased from Sigma Aldrich Co. Reagent grade formic acid (98%) was purchased from Nacalai Tesque Inc. (Tokyo, Japan). Ultra-pure water was prepared using a Millipore Milli-Q purification system (Bedford, MA).

*Sample preparation for LC–MS* ALEE and ALWE (each 40.0 mg) were dissolved in 50% ethanol and water (20 mL each), respectively, and injected directly for HPLC–MS analysis.

*HPLC analysis* We performed HPLC analysis using a Shimadzu HPLC system. Chromatographic separation was performed on a Capcell Pak UG120 (5 μm, 2.0 i.d. × 250 mm; Shiseido, Tokyo, Japan). Mobile phase A was water containing 0.1% acetic acid, and mobile phase B was CH₃CN containing 0.1% acetic acid. The column temperature was 40°C. The HPLC flow rate was 0.2 mL/min. A sample solution of 1 μL was injected into the HPLC system. A mobile phase gradient was used with the percentage of B in A varying as follows: initial concentration, 10% B; 30 min, 50% B; and 40 min, 50% B.

**IT-TOF MS analysis** The diode array analysis was performed on a SPD-M20A (Shimadzu; Kyoto, Japan) scanning in the range 200 − 400 nm. CID-MS experiments were performed on a hybrid IT-TOF mass spectrometer with an ESI interface (Shimadzu). The negative ESI conditions were as follows: high voltage probe, −3.5 kV; nebulizing gas flow, 1.5 L/min; CDL temperature, 200°C; heat block temperature, 200°C; and drying gas pressure, 200 KPa. CID parameters were chosen as 70% for the CID energy and 50% for the collision gas parameter. We used N₂ gas for CID. The detector voltage of TOF was 1.6 kV. A solution of trifluoroacetic acid and sodium hydrate was used as the standard sample to adjust the sensitivity and resolution and to perform the mass number calibration (ion trap and TOF analyzer).

**Results and Discussion**

*General* The common solvents used for the extraction of phenolic compounds from foods include water, metha-
nol, aqueous acetone, ethanol, and ethyl acetate (Naczk and Shahidi, 2004). Because our primary aim is to use AL as a food material in Japan, we performed the preliminary extractions with water and various concentrations of aqueous ethanol at various temperatures. The results of the comprehensive HPLC analysis (data not shown) indicated that the 60% aqueous ethanol at room temperature and water at 95°C extracts (ALEE and ALWE) were similar and yielded the most peaks in the HPLC–diode array detection (DAD) chromatogram when recorded at 330 nm (Fig. 1, [A] a and [B] a). The peaks showed absorbance wavelengths (200 – 400 nm) typical of phenolics, including benzophenones (1, 2, and 4), xanthone (3), and flavones (5 – 8) (Fig. 2, a – c). However, 80% ethanol is also the accepted solvent for the most efficient extraction to investigate one of the laxative principles (5) (data not shown). The extraction scheme, facilitated by high density ethanol, requires custom equipment and is not a practical method that can be used in API Co. (Gifu, Japan). We discuss the comparative study of ALEE and ALWE in this paper.

To obtain good resolution of the peaks in a reasonably short analysis time, we screened different mobile phases and semi-micro column compositions. We found that one of the suitable eluting solvent systems was acetonitrile and 0.1% aqueous acetic acid, and the suitable column was a Capcell Pak UG120 (5 μm, 2.0 i.d. × 250 mm; Shiseido; Tokyo, Japan). The optimized LC conditions permitted good separation of eight target polyphenols as well as other phenolic compounds within 35 min.

The wavelength for the comparison of the chromatograms of ALEE and ALWE was 330 nm which is the λmax of 5, and we performed a DAD analysis, scanning in the range 190 – 400 nm. For the MS analysis, we utilized both positive and negative ion modes of ESI, since dual mode results would give promising information for the determination of the molecular composition, and the negative mode would provide extensive information via CID fragmentations. The product ion spectra of the pseudomolecular ions [M+H]+ and [M−H]− as well as the selective ion chromatograms for the aglycons, were obtained by conducting CID-MS/MS experiments. The chromatograms of the MS total ion current (TIC) and the selective ion monitoring (SIM) mode in positive mode are shown in Fig. 1. The MS+ data of 1 – 5 are summarized in Table 1.

Identification of 1 – 8 by HPLC in ALEE and ALWE
HPLC profiles of ALEE and ALWE monitored at 330 nm showed that peaks numbered 1 – 8 were completely separated (Fig. 1). These peaks were identified as iriflophenone 3,5-C-β-diglucoside (1), iriflophenone 3-C-β-glucoside (2),

<table>
<thead>
<tr>
<th>Peak</th>
<th>Rt (min)</th>
<th>Isolated m/z</th>
<th>MS+ type</th>
<th>Major product ions (m/z)</th>
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<tr>
<td>1</td>
<td>6.9</td>
<td>571 [M−H]+</td>
<td>MS+</td>
<td>533 (100), 433 (57)</td>
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<tr>
<td></td>
<td></td>
<td>569 [M−H]−</td>
<td>MS+</td>
<td>449 (21), 431 (13), 359 (57), 329 (100)</td>
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<tr>
<td></td>
<td></td>
<td>449 [M−H]−</td>
<td>MS+</td>
<td>329 (100)</td>
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<tr>
<td></td>
<td></td>
<td>359 [M−H]−</td>
<td>MS+</td>
<td>239 (100)</td>
</tr>
<tr>
<td>2</td>
<td>7.4</td>
<td>409 [M+H]+</td>
<td>MS+</td>
<td>391 (100), 325 (64), 313 (64)</td>
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<td></td>
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<td>407 [M−H]−</td>
<td>MS+</td>
<td>287 (100)</td>
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<tr>
<td>3</td>
<td>9.3</td>
<td>423 [M+H]+</td>
<td>MS+</td>
<td>369 (17), 357 (17), 351 (26), 327 (41), 303 (21), 299 (17), 273 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>421 [M−H]−</td>
<td>MS+</td>
<td>331 (49), 301 (100), 271 (23), 259 (17)</td>
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<td></td>
<td></td>
<td>310 [M−H]−</td>
<td>MS+</td>
<td>273 (25), 272 (25), 258 (100)</td>
</tr>
<tr>
<td>4</td>
<td>11.8</td>
<td>393 [M+H]+</td>
<td>MS+</td>
<td>339 (100), 247 (62)</td>
</tr>
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<td>247 (100)</td>
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<td></td>
<td></td>
<td>391 [M−H]−</td>
<td>MS+</td>
<td>245 (100), 151 (15)</td>
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<td>MS+</td>
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<td>MS+</td>
<td>283 (100), 268 (24)</td>
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<td></td>
<td></td>
<td>283 [M−H]−</td>
<td>MS+</td>
<td>268 (100)</td>
</tr>
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</table>

* Peak numbers are the same as in Fig. 1.

Relative abundances (%) are given in parentheses.

![Fig. 2. Three-dimensional HPLC analysis of major chemical compounds in agarwood leaves.](image)

a time, 5.0 – 40.0 min; wavelength, 190 – 400 nm; intensity max, 180 mAU, b time, 10.0 – 40.0 min; wavelength, 240 – 400 nm; intensity max, 5 mAU, c UV spectra of key peaks [1 (benzophenone), 3 (xanthone), and 5 (flavone)].
mangiferin (3), iriflophenone 2-O-α-rhamnoside (4), genkwanin 5-O-β-primeveroside (5), genkwanin 5-O-β-glucoside (6), genkwanin 4′-methyl ether 5-O-β-primeveroside (7), and genkwanin (8) by comparison with standard samples. The composition of ALEE (Fig. 1, [A]) and ALWE (Fig. 1, [B]) were similar except for the ratios of representative polyphenols (1 – 8). Because our study focused on identification and not quantitation, the differences in the ratio of each polyphenol with the peak of 3 were discussed. For quality control of the AL extracts, it is important to identify exact information on the bioactive polyphenols bearing laxative properties (3 and 5). We found evidence for the existence of both compounds in both extracts as well as the effective extraction of 5 with hot water, strongly indicating that both extracts are substantial functional food materials with significant laxative effects. The other evident differences were the ratios of the other polyphenols such as 1 (ALEE < ALWE), 2 (ALEE < ALWE), 7 (ALEE < ALWE), and 8 (ALEE > ALWE). These differences were acceptable when we considered the structures of 1 – 8 and the solvents used for the extraction. Phenolic glycosides, represented as 1 – 7 in AL, are well known to dissolve in hot water. In contrast, genin (8) was more soluble in organic solvents, thus reflecting the analytical results.

**DAD analysis of major polyphenols in the ALEE**

Three-dimensional HPLC analysis showed absorbance wavelengths typical of phenolic compounds. Figure 2 presents the UV spectra of three majorities (Fig. 2a, maximum mAU: 180) and the others (Fig. 2b, maximum mAU: 5), as well as UV spectra of 1, 3, and 5 (Fig. 2c). The results indicated that the LC−DAD fingerprint of the AL extract existed as three major peaks of two benzophenones (1 and 2) and xanthone (3) and the other minor peaks of benzophenones (4) and flavones (5 and 8).

**ESI-MS/IT-TOF analysis of ALEE and ALWE**

Figure 1 shows the chromatograms of TIC and SIM in the positive mode (A, ALEE and B, ALWE). Not all compounds in AL were detected in the DA detector, e.g., due to lack of conjugated systems in some compounds in the extract. However, the TIC chromatograms of ALEE (Fig. 1, [A] b) and ALWE (Fig. 1, [B] b) showed superimposable features with those of the respective chromatograms at 330 nm (a) except for the initial 5 min. The TIC chromatograms also supported the compositional similarity of ALEE and ALWE.

The major phenolic compounds in AL existed as C-glucosides (1 – 3) and O-glucosides (4 – 7), and the aglycons of 4 – 7 were iriflophenone (1,3,4,4′-tetrahydroxy benzo[phenone, MW 246) for 4, genkwanin (8: 5,4′-dihydroxy-7-

Conclusions

In conclusion, we have identified the major phenolic compounds present in agarwood leaves (AL) and established their characteristic chromatographic profiles and compositional similarities for 60% ALEE and ALWE. Among the identified compounds, genkwanin 5-O-β-primeveroside (5) and mangiferin (3) showed laxative activities via different mechanisms.

Because it is meaningful to evaluate chemical compositional similarities and biological properties of various extract forms, these methods should aid in the standardization of AL materials for *in vitro* and *in vivo* studies. The chromatographic fingerprinting of AL should also be useful for the authentication of AL-based food products. The information provided by our study will aid in the evaluation of the importance of AL consumption on human health.

**References**


