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# Phenylpropanoid profiling reveals a class of hydroxycinnamoyl glucaric acid conjugates in *Isatis tinctoria* leaves

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ABSTRACT

The brassicaceous herb, *Isatis tinctoria,* is an ancient medicinal plant whose rosette leaf extracts have anti-inflammatory and anti-allergic activity. Brassicaceae are known to accumulate a variety of phenylpropanoids in their rosette leaves acting as antioxidants and a UV-B shield, and these compounds often have pharmacological potential. Nevertheless, knowledge about the phenylpropanoid content of *I. tinctoria* leaves remains limited to the characterization of a number of flavonoids. In this research, we profiled the methanol extracts of *I. tinctoria* fresh leaf extracts by liquid chromatography – mass spectrometry (LC-MS) and focused on the phenylpropanoid derivatives. We report the structural characterization of 99 compounds including 18 flavonoids, 21 mono- or oligolignols, 2 benzenoids, and a wide spectrum of 58 hydroxycinnamic acid esters. Besides the sinapate esters of malate, glucose and gentiobiose, which are typical of brassicaceous plants, these conjugates comprised a large variety of glucaric acid esters that have not previously been reported in plants. Feeding with $^{13}C_6$-glucaric acid showed that glucaric acid is an acyl acceptor of an as yet unknown acyltransferase activity in *I. tinctoria* rosette leaves. The large amount of hydroxycinnamic acid derivatives changes radically our view of the woad metabolite profile and potentially contributes to the pharmacological activity of *I. tinctoria* leaf extracts.


## 1. Introduction

*Isatis tinctoria* L. (Brassicaceae), commonly known as woad, is a biennial herbaceous plant that was imported into Europe in antiquity. Woad was cultivated for centuries to produce the blue dye indigo and as a medicinal plant for the treatment of inflammatory diseases. The import of cheaper indigo from *Indigofera suffruticosa* in the late 17th century, and the production of synthetic indigo since the late 1890s, finally led to the disappearance of woad cultures in Europe (Hamburger, 2002). However, in traditional Chinese medicine, Banlangen (*Isatis* root extract) and Daqingye (*Isatis* leaf extract) from the taxonomically closely related *Isatis indigotica* Fort. have remained popular herbal drugs for the treatment of inflammatory diseases (Mohn et al., 2009). In recent decades, there has been a renewed interest in the potential of *I. tinctoria* extracts as new active ingredients for anti-inflammatory phytopharmaceuticals. As a result, many pharmacological studies have been carried out on woad and its bio-active constituents. A broad pharmacological profiling of the lipophilic and polar extracts from fresh and dried *I. tinctoria* roots and leaves in various animal models showed that the lipophilic extracts from dried leaves displayed the strongest anti-inflammatory potential (Hamburger, 2002). These

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extracts have been the subject of comprehensive metabolite profiling and a large number of alkaloids, fatty acids and carotenoids has been identified (Mohn et al., 2009). The only phenylpropanoids described in these lipophilic extracts from dried leaves were ferulic and sinapic acid, syringic aldehyde, and minor amounts of pinoresinol. This study was extended with a profiling of the polar extracts of the same dried leaves, in which eight flavone C-glucosides were identified and several other potential, but not further identified flavonoids were suggested (Mohn et al., 2009). To date, a metabolite profiling that targets the phenylpropanoids in *I. tinctoria* leaves has not been reported. Nevertheless, the rosette leaves of plants of the Brassicaceae family are known to accumulate, in addition to flavonoids, a wide range of hydroxycinnamic acid derivatives and mono- and oligolignols (Dima et al., 2015; Morreel et al., 2014). Recently, the pharmacological activity of Banlangen has been attributed to phenylpropanoids, and, more specifically, to a group of lignan metabolites (Chen et al., 2015; Yang et al., 2013). Similarly, flavonoids and other polyphenols may contribute significantly to the pharmacological activity of *I. tinctoria* leaf extracts. In this paper, an HPLC-ESI-MSn-based analysis of the phenylpropanoids and benzenoids in polar extracts of fresh *I. tinctoria* leaves is described. We report the characterization of 99 phenylpropanoid derivatives including flavonoids, hydroxycinnamic and hydroxybenzoic acid derivatives, and mono- and oligolignols.

## 2. Results

### 2.1. Structural elucidation of 99 phenylpropanoid derivatives from *I. tinctoria* rosette leaves

A non-targeted metabolite profiling of the polar extracts of *I. tinctoria* first-year rosette leaves was carried out. The analyses of the leaf extracts were performed on 30 biological replicates by HPLC-Hybrid Ion Trap-Orbitrap Mass Spectrometry. A representative chromatogram is shown in Fig. 1. Accurate masses were obtained in the Orbitrap while, in parallel, MS2 fragmentation spectra were recorded in the LTQ linear ion trap. In order to obtain additional information for the elucidation of the structures, a few samples were subjected to further fragmentation (see Experimental section). Ninety-seven phenylpropanoids and 2 benzenoids that were detected in at least half of the 30 replicates were structurally elucidated. Two levels of structural elucidation, i.e. structural annotation and structural characterization, were distinguished (Sumner et al., 2007). For 95 compounds, a structural annotation (SA) was obtained based on the combination of the exact mass of the LC-MS features and the interpretation of the corresponding MSn spectra. For four other compounds, a structural characterization (SC) was performed, based solely on the exact mass and the correlation in intensity of the compound with a structurally annotated compound. For these latter compounds, MSn data were not available or limited, only indicating that the compound was a hexoside or that it was ionized as a formic acid adduct. Overall, three main categories of phenylpropanoids were distinguished: flavonoids (18 compounds), phenylpropanoic acid conjugates (58 compounds), and mono- and oligolignol hexosides (21 compounds). Together with two benzenoids, these 99 compounds are listed in Table 1. Although it was not the focus of this study, we also annotated the glucosinolates and the remaining major peaks in the chromatograms (added to Table 1). These peaks can be useful as relative retention time references for the phenylpropanoids described above. For all the compounds listed in Table 1, the detailed structural elucidation and the relative abundance in the 30 samples are given in the Supplemental Data and Supplemental Table 1.

### 2.2. Flavonoids

With the exception of chrysoeriol-7-*O*-glucoside (**15**), all detected flavonoids were derivatives of flavone-6-*C*-glucosides in which the aglycone corresponded to luteolin, apigenin, or chrysoeriol. For most of these compounds, the carbohydrate moiety on the *C*-6 position was a diglucoside with a rare 1 → 3 glycosidic linkage. The simple 6-*C*-glucose(3←1)glucose derivatives of luteolin, apigenin and chrysoeriol (**7**, **9**, **11**) have previously been described in dried *I. tinctoria* rosette leaves and confirmed by co-chromatography with reference standards. They were named isoorientin-, isovitexin-, and isoscoparin-3″-*O*-glucoside, respectively (Mohn et al., 2009). The mass spectral fragmentation of these compounds is characterized by a strong cross-ring cleavage of the C-linked hexoside resulting in a neutral loss of 90 Da (base peak), the cleavage of the 1 → 3 glycosidic linkage resulting in the loss of 180 Da, and the combination of both. Characteristically, a hexose cross-ring cleavage resulting in a loss of 120 Da, typically seen in 6-*C*-monoglucosides or 6-*C*-diglucosides with the more common 1 → 2 glucosidic linkage, is prevented here by the linkage of a hexose to the *O*-3″ position (Deng et al., 2008). We also detected derivatives of these 6-*C*-dihexosides with an additional hexosylation of the *O*-7 position (**3**, **5**), an esterification with a hydroxycinnamic acid derivative such as *p*-coumaric, ferulic, or sinapic acid (**16**, **17**, **18**), or both (**12**, **13**, **14**). The O-linked glucoses in compounds **3** and **5** were revealed by the addition to the characteristic mass spectrum of the C-6 bound 1 → 3 diglucosides described above, of a dehydrohexose loss (neutral loss of 162 Da), typically seen in O-linked flavonoid hexosides, and the combination of this dehydrohexose loss with losses of 90 and 180 Da (Fig. 2). Because the 7-hydroxyl group is the favored glucosylation position for flavones, we annotated these compounds as 7-*O*-glucosides of isovitexin-3″-*O*-glucoside (**3**) and of isoscoparin 3″-*O*-glucoside (**5**).

Three isoscoparin-3″-*O*-glucoside derivatives were revealed in which a *p*-coumaric, ferulic, or sinapic acid was esterified to the 3″-O-linked glucose (**16**, **17**, **18**). The MS2 spectra of these compounds were characterized by the cleavage of the hydroxycinnamoyl ester bond and additional peaks that were highly similar to the isoscoparin-3″-O-glucoside spectrum (Fig. 2). The absence of neutral losses involving a free 3″-O-linked glucoside (neutral loss of 180 and 270 Da) indicated the position of the hydroxycinnamoyl group on this 3″-O-linked glucose but the exact site could not be deduced from the spectra. Compounds **12**, **13** and **14** showed identical molecular formulas, which corresponded to feruloylated derivatives of isoscoparin 3″-*O*-glucoside-7-*O*-glucoside or sinapoylated derivatives of isovitexin-3″-*O*-glucoside-7-*O*-glucoside. In compound **14**, the neutral losses involving the O-linked hexose (loss of 162 Da and combined loss of 162 and 90 Da) were substituted by losses of 162 + 176 Da (m/z 623) and 162 + 176+90 Da (m/z 533), indicating that the 7-O-linked hexose was feruloylated. This compound was annotated as isoscoparin-3″-*O*-glucoside-7-*O*-feruloyl glucoside. In compound **13**, the presence of a ferulic acid ester was revealed by a large loss of 176 Da but in compound **12**, no direct indication of the identity of the hydroxycinnamic acid group (sinapic or ferulic acid) was available. In compounds **12** and **13**, neutral losses of 162 Da and 180 Da suggested that the O-linked glucose and the (3←1) linked glucose were free. The main difference in the fragmentation of compounds **12** and **13** resided in the intensity of the 90 Da neutral loss fragment. In compound **12**, this fragment was the base peak, similar to what was observed in the other flavone 3″-*O*-glucoside derivatives, whereas in compound **13**, this fragment was relatively weak (9% of the base peak), suggesting an effect of the hydroxycinnamoylation on the fragmentation of the C-linked hexose. For a loss of 90 Da to occur, the esterification cannot be on the 4″ or 6″ position of the C-linked hexose, and only the hydroxyl group at the