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Comparative HILIC/ESI-QTOF-MS and HPTLC studies of pyrrolizidine alkaloids in flowers of *Tussilago farfara* and roots of *Arnebia euchroma*

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ABSTRACT

The utility of HPTLC and HILIC/ESI-QTOF-MS for the determination of pyrrolizidine alkaloids (PAs) and their *N*-oxides (PANOs) was compared in the selected plant species: *Tussilago farfara* L. (TF, flower) and *Arnebia euchroma* (Royle) I.M. Johnst. (AE, root). HPTLC confirmed the postulated presence of PAs (saturated and unsaturated) or PANOs in the tested extracts. In accordance with previous studies, HILIC/ESI-Q-TOF-MS confirmed the presence of the toxic PA senkirkine and the saturated otonecine-type PAs, tussilagine and isotussilagine in the TF extract and 7-angeloylretronecine and 9-angeloylretronecine in AE extract. Moreover, the following alkaloids were identified in AE root: intermedine, intermedine-*N*-oxide, leptanthine-*N*-oxide, echimidine-*N*-oxide (or their corresponding stereoisomers) and traces of 7-angeloylretronecine and 9-angeloylretronecine-*N*-oxide. The study demonstrates the HILIC/ESI-Q-TOF-MS method to be a very useful tool for monitoring PAs and PANOs in the test samples, even when not all of the necessary standards are available. Quantitative analysis of senkirkine in TF flower by HILIC/ESI-QTOF-MS featured high resolution, high precision, high mass accuracy, and very high sensitivity with limit-of-detection (LOD) of 27.50 fg/μL and limit-of-quantitation (LOQ) of 91.60 fg/μL. The results from both methods may be used for the development or rejection of European Pharmacopoeia (X) monographs of both investigated species.

1. Introduction

T. farfara and *A. euchroma* species found wide use in traditional medicine. TF have been used in respiratory diseases among others in cough, asthma and bronchitis (Adamczak et al., 2013; Lebada et al., 2000). According to elaborated studies TF extracts exhibit antimicrobial (Hleba et al., 2014), anti-imflammatory and antioxidant activity (Ravipati et al., 2012). Flower buds of TF were used in Traditional Chinese Medicine (TCM) under the name *Kuandonghua*, while in Europe leaves extract of TF were used (Adamczak et al., 2013; Wu et al., 2008). Similar medical use like TF have AE extracts known in TCM as *Zicao* (Adamczak et al., 2013; Liao et al., 2015). This herbal medicine can be prepared from pulverized root of AE (Royle) I.M. Johnst. *Ruan Zicao*), as well as related species from Boraginaceae family: *Lithospermum erythrorhizon* Siebold & Zucc., *Onosma paniculata* Bureau & Franch. (Bauer and Franz, 2010) or *A. guttata* Bunge (Liao et al., 2015). The *Zicao* and AE extracts are characterized by important pharmacological activities confirmed in reasearch anti-inflammatory (roots) (Ashkani-Esfahani et al., 2012; Roeder and Rengel-Mayer, 1993), antimicrobial and antitumor (callus and cell suspension cultures) (Damianakos et al., 2012). *T. farfara* L. (Asteraceae) and *A. euchroma* (Royle) I.M. Johnst. (Boraginaceae) are known to contain PAs according to previous reports found in the other parts of plant (Adamczak et al., 2013; Lebada et al., 2000; Roeder and Rengel-Mayer, 1993). A study of the PAs and PANOs content in extract AE (root) and TF (flower) is certainly needed due to the wide use of AE and TF in Traditional Chinese Medicine.

Pyrrolizidine alkaloids are toxic plant secondary metabolites which drive away their natural enemies (insects, animals and other plants). Their presence was established in plants from over 14 families, including many species in the Asteraceae and Boraginaceae families (Dreger et al., 2009). Toxic and non-toxic derivatives

Abbreviations: PAs, pyrrolizidine alkaloids; PANOs, pyrrolizidine alkaloid-*N*-oxides; TF, *Tussilago farfara*; AE, *Arnebia euchroma*; HILIC, hydrophilic interaction liquid chromatography; ESI-QTOF-MS, electrospray ionization quadruple time-of-flight mass spectrometry; TCM, traditional Chinese medicine; CID, collision induced dissociation; ESI, electrospray ionization; TIC, total ion current Chromatogram; EIC, extracted ion current chromatogram; W, standard.

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can be distinguished depending on the structure; those with a 1,2-unsaturated necine ring: are regarded as toxic (Dreger et al., 2009).

The relationships between toxicity and structure of the PAs have been well studied. The most toxic of the PAs are the macrocyclic esters, less toxic are the non-macrocyclic diesters, while the monoesters are the least toxic. PAs can also be classified depending on structure: platynecine-type, retronecine-type, otonecine-type and its enantiomers (at C-7 position) – heliotridine (Dreger et al., 2009; Roeder, 1995). PAs which are 1,2-unsaturated exhibit carcinogenic and hepatoxic activities, pulmonary and kidney toxicity, and genotoxicity. They are transformed metabolically during of hydrolysis and oxidation to toxic *N*-oxides and by dehydrogenation to pyrroles (Dreger et al., 2009; El-Shazly and Wink, 2014). It is mandatory to define and control their presence in plant extracts and pharmaceutical formulations. Senkirkine and senecionine were identified in TF (Lebada et al., 2000), while 7-angeloylretronecine and 9-angeloylretronecine were found in AE (Roeder and Rengel-Mayer, 1993).

From a related species, *Lithospermum erythrorhizon* Siebold & Zucc. (Boraginaceae) Roeder and Rengel (1990) identified the presence of intermedine, myoscorpine and hydroxymyoscorpine. The presence of echimidine and monocrotaline was determined in *A. hispidissima* (Lehm.) A.DC. (Boraginaceae) and 7- and 9-tigloylretronecine, heliotrine, supinine, lycopsamine and europine in different species from *A. decumbens* (Vent.) Coss.& Kralik (Boraginaceae) (Sharma et al., 2009).

In TF extracts, Roeder (1995) indicates the presence of other non-toxic alkaloids, including isotussilagine, isotussilaginine, tussilagine and tussilaginine. Nedelcheva et al. (2015) examined the PAs present in TF leaf extract from Bulgaria by applying GC–MS analysis to the crude extract and found senkirkine, senecionine, integerrimine, and seneciphylline in trace amounts (at a level of 55 μ/g of dried sample). Adamczak et al. (2013) examined extracts from the leaves of 20 samples of TF growing in the Poland by high performance liquid chromatography with a photodiode array detector (HPLC-DAD) and confirmed low levels of senkirkine (mean 0.19 μg/g) and senecionine (mean 0.23 μg/g).

Differences in chemical composition were observed depending on plant origin. The variable content of PAs and PANOs in different parts of plants is also observed (Dreger et al., 2009). These differences were confirmed by examining TF parts: flower buds, leaves and flowers using GC–MS with an orbitrap mass analyzer (Xue et al., 2012). For the analysis of PAs, those based on TLC separation, followed by visualisation by colour reactions or by spectrophotometry, were used (Bartkowski et al., 1997; Dann, 1960; Mattocks, 1967; Mroczek et al., 2006). Different variants of gas chromatography (GC–MS) (Carvalho et al., 2013; Kempf et al., 2010; Nedelcheva et al., 2015; Roeder and Rengel-Mayer, 1993; Xue et al., 2012) and capillary electrophoresis (Lebada et al., 2000; Yu and Li, 2005) methods were also empolyed. Currently, the most commonly used technique involve HPLC method coupled with MS (Bartkowski et al., 1997) analyzers or more recently MS/MS (by quadrupole MS/MS or ion trap MS) (Avula et al., 2015; Chen et al., 2015; Cheng et al., 2011; Griffin et al., 2015; Jiang et al., 2009; These et al., 2013; Valese et al., 2016; Zhu et al., 2016).

According to the guidelines of the European Medicines Agency, the most frequently used method for the analysis of PAs is LC–MS combined with sample preparation by the solid-phase extraction (SPE) technique (Griffin et al., 2015; Mroczek et al., 2002, 2004b; Weston et al., 2013). In order to prepare the sample, microwave-assisted extraction and pressurized hot water extraction were also used (Jiang et al., 2009). Adamczak et al. (2013) draws attention to the conditions under which extraction of alkaloids occurs as well as other subsequent steps.

In some methods, like GC–MS in high operating temperatures decomposition of unstable PANOs may occur, which may have an impact on obtaining a false analytical results (Mroczek et al., 2002). To prevent these process Stelljes et al. (1991) performed reduction of PANOs with Zn dust in acidic solution to obtain PAs free bases. However, strong acidic conditions may also be responsible for creating some of decomposition products.

While others (Bartkowski et al., 1997; Mroczek et al., 2002) indicate the decomposition of the *N*-oxides of otonecine-type alkaloids is affected by temperature and by the potential interaction of the reagents used at the increased temperature (acetic anhydride, acetyl chloride, 4,4-dimethylaminopyridine) in the case of usage of colorimetric methods. It was suggested to use 4,4-dimethylaminobenzaldehyde, which does not give this effect, for the detection by TLC (Bartkowski et al., 1997; Mroczek et al., 2002). The based on the above conclusions, the HPLC method seems to be more advantageous due to reduced risk of decomposition of labile compounds (*N*-oxides, diesterified alkaloids) compared to the GC method (Zhu et al., 2016).

An increasing number of studies demonstrate the usefulness and advantages of the quantitative and qualitative analysis of PAs by LC–MS, especially using a quadrupole analyzer and an ion trap, those detection are characterized by high measurement sensitivity and better results for interpretation compared to previously used methods. In addition there is the possibility of fragmentation and further analysis of generated ions, which increases the accuracy of data interpretation.

A number of alkaloid analytical procedures using different variants of HPLC–MS were elaborated. Also combination of 2D-HPLC-UV and LC-MMI-TOF–MS methods were tried (Aydın and Letzel, 2013). For screening PAs in 17 selected species of the families Asteraceae, Boraginaceae, Fabaceae, tandem mass spectrometry was used, combined with MRM (multiple reaction mode). As a result, 121 previously unknown PAs were identified (These et al., 2013). Zhu and colleagues have developed UPLC-QTOF–MS method to estimate the amount of retronecine-type PAs in plant material (Zhu et al., 2016). This method does not require the use of standards, as it is based on the analysis of the obtained mass fragmentation patterns (retrorsine was used for method calibration).

Regarding *A. euchroma,* Liao et al. (2015) performed a QTOF-MS analysis of *Zicao* (three Arnebia species including AE) in negative ion mode for the presence of shikonins and shikonofurans. Fifty two compounds including thirthy two new were identified. However, the analysis of PAs in extracts of AE and TF by Hydrophilic Interaction Liquid Chromatography (HILIC), combined with an ESI-QTOF-MS detection system, has not been reported. The examples demonstrate the utility and appropriateness of the methods used in this study to identify PAs in the studied species. The benefit of the HILIC/ESI-QTOF-MS method is emphasized as a very precise and very sensitive tool for the qualitative and quantitative analysis of PAs.

2. Materials and methods

2.1. Reagent and chemicals

The mixture of standards (at a concentration of 0.2 mg/mL each) was prepared from: retrorsine-*N*-oxide (Sigma Chemical Corp. St. Louis, MO, USA), senkirkine and senecionine (Carl Roth, Karlsruhe, Germany). The following reagents were used for extraction and isolation: tartaric acid, 25% aqueous ammonia solution, and methanol (MeOH, analytycal grade). They were obtained from The Polish Reagents (POCh, Gliwice, Poland). For the CE-SPE method, Lichrolut™ (E. Merck, Darmstadt, Germany) SCX (500 mg, 3 mL) solid phase extraction columns were used and the following chemicals were utilized (analytical grade): 30% HCl water solution from The Polish Reagents (POCh, Gliwice, Poland) and MeOH from

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