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Qualitative and quantitative determination of ligustilide as bioactive marker in apiaceous botanicals

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Abstract: Variety of bioactivities has been associated with ligustilide present in root of *Angelica sinensis*, and predominantly used for the treatment of irregular menstrual cycles and premenstrual syndrome. Recent pharmacological studies showed that medicinal plants containing ligustilide, have anti-inflammatory effects and contribute to the improvement of cognitive functions, alleviate brain damage, inhibit tumor necrosis factor of some cell lines, and have nephron-protective effects and neuroprotective activity.

In this work, quantification of ligustilide using quantitative 1H NMR (qHNMR) in sealed tubes was performed. Four plant species were investigated: *A. sinensis, Ligusticum porteri, Ligusticum striatum,* and *Ligusticum sinense*. Modified supercritical CO_2 extraction of essential oil from root of four investigated species, was performed. qHNMR spectroscopy showed following percentage of ligustilide: *L. porteri* essential oil 3.74 (%); *L. sinense* essential oil 1.16 (%); *L. striatum* essential oil 6.61 (%) and *A. sinensis* essential oil 14.56 (%). The highest percentage of oil was obtained from the root of *L. porteri* but the highest percentage of ligustilide was obtained from *A. sinensis* essential oil.

INTRODUCTION

Phthalides such as the prototypical dihydro-phthalide (Z)-ligustilide (3-butylidene-4,5-dihydro-2-benzofuran-1-one) (Figure 1) are designated frequently as marker compounds of medicinal plants from the most prominent genera of the Apiaceae family, including Ligusticum and Angelica (Deng, 2005). Typically, depending on the phytochemical depth of the studies, ligustilide and its congeners are broadly associated with observed and ethno-botanically documented bioactivities of the plants and their preparations.

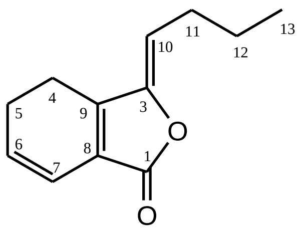

Figure 1. Z-ligustilide

Angelica sinensis is one of the 15 most commonly used Traditional Chinese Medicines (TCMs), predominantly in formulations for the treatment of irregular menstrual cycles and premenstrual syndrome (Wei, Zenq, Gu, *et al.*, 2016). Monograph of the root of *A. sinensis* was included in European pharmacopoeia as well. Recent pharmacological studies showed that medicinal plants containing ligustilide, have anti-inflammatory effects (Chung, Choi, and Seo, *et al.*, 2012; Ma and Bai, 2013) improves the cognitive functions, alleviate brain damage (Feng, Lu, Wu, *et al.*, 2012), inhibit tumor necrosis factor of some cell lines (Shi, Xiao, Yin, *et al.*, 2015), and have nephron-protective effects and neuroprotective activity (Bunel, Antoine, Nortier, *et al.*, 2015; Wenxia, Yuzhi, Xiao, *et al.*, 2016**)**. The extraction techniques have focused on hexanes, petroleum ether and other similar solvents as the initial strategy due to the relatively non polar characteristic of the majority of the phtalides. Furthermore, many phtalides are part of the essential oil composition of this botanicals and steam distillation was frequently used in order to obtaine this compounds (Cui, Fenq, and Hu, 2006). One of the problem that occure during the extraction and isolation of this compounds, especially ligustilide, is due to its unstable nature.(Beck and Chou, 2007). This chemical and physical property makes preparations and conservation of the herbal products containing ligustilide very difficult. Many studies reported a considerable number of degradation products of ligustilide have been identified and their structures confirmed (Friesen, McAlpine, Chen, *et al.*, 2015; Schinkovitz, Pro, Main, *et al.*, 2008). One recent study on ligustilide stability, confirmed stability of this compound in essential oil and drug itself, but high instability of isolated compound was detected.(Quiroz-Garcia, Figueroa, Cogordan, *et al.*, 2005) The light strongly influenced the ligustilde transformation into its dimer – diligustilide, compond without any activity. The use of botanical dietary supplements with ligustilide as main component is increasingly popular, due to the number research studies that confirmed very important activities of this bio-active compounds. In paralel, with discovery of the herbal drugs containing ligustilide, the challenge is also to discover novel – non destructive analytical methods, that can easily enable quantitative determination of ligustilide, respecting its unstable nature (Zou, Chen, Zhao, *et al.*, 2018).

In this work, quantification of ligustilide in several botanicals, potentialy containing ligustilide, using quantitative ^{1}H NMR (qHNMR) was performed.

EXPERIMENTAL

Plant Material

Four ligustilide-rich species were investigated: *A. sinensis, L. porteri, L. striatum* and *L. sinense*. Plant materials, were purchased from Chinatown, Chicago, IL. The plants material were identified through a series of comparative macroscopic, organoleptic, and TLC analyses against an authentic *A. sinensis* voucher sample (BC440), *L. porter* voucher sample (BC576), *L. striatum* voucher sample (BC572) and *L. sinense* voucher sample (BC575), deposited at the UIC/NIH Center for Botanical Dietary Supplements Research, Chicago, IL. During experimental period the plant material was stored in a dry place, in the absence of light and in a cold location.

Supercritical Fluid Extraction

Extraction of essential oil from root of four investigated species, with modified supercritical CO_2 extraction method was performed. Supercritical fluid extraction (SFE) was performed on Speed SFE instrument, model 7070 (Applied Separation Inc. Allentown, PA), consisting of a column oven, air pressure regulator and 195 mm × 75 mm i.d. stainless steel column connected to a NESLAB RTE 7 refrigerated bath (Thermo Electron Corporation, Waltham, MA). Compressed air and CO_2 gases were purchased from Airgas Inc., Radnor, PA. The extraction column was filled with powdered plant material (69 g). Glass wool was added at each end of the column. As modifier, methanol was added at a concentration of 5% to the part of column where the CO_2 entered into the column. The extraction temperature was set to 50°C. Extraction was performed at 250 psi with static extract time of 30 min at a flow rate of 0.5 mL/min, 4 times for each sample. The SFE extract was then collected in glass vials and stored in a -20°C freezer.

Quantitative Nuclear Magnetic Resonance (qNMR) spectroscopy

Samples were dissolved in 600 μL of $CDCl_3$ using an analytical syringe (Valco Instruments, Baton Rouge, LA, USA). NMR experiments were performed on: Bruker Avance-360 MHz. The ^{1}H NMR experiments for stability evaluation and qHNMR quantification of ligustilide from the SFE extracts was performed using standard proton acquisition program (“zg 30”). Spectral width (SW) was 12 ppm, the shift of the center of the spectrum (O1P) was 7.9 ppm, acquisition time (AQ) was set to 2.77 s, receiver gain (RG) value was set to 512, and relaxation delay (D1) was 1 s. The spectra were processed and analyzed using MestReNova v9.0.1 (Mestrelab Research, Santiago de Compostela, Spain) software. A calibration curve was generated using dimethyl sulfone ($DMSO_2$, lot# BCBH9813V, Fluca analytical) as the external standard at concentrations ranging from 0.28 mM to 15.13 mM. The qHNMR data were processed as follows: baseline/Polynominal fit, zero-filling to 512 k prior to Fourier transformation of the FID and Zhu_Bax method, Lorenzian/Gaussian factor 0.25, manual peak by peak picking and integration.

RESULTS AND DISCUSSION

Most of phthalides, ligustilide included, are components of the essential oils, as they are mostly non-polar molecules. Extraction methods, conventionally used, includes steam distillation. One of the disadvantages of this method using water or water vapor is that essential oils undergo chemical alternation. Relatively high temperature could easily destroy sensitive compound and change oil composition and consequently its quality (Duric, Liu, Chen, *et al.*, 2019).

Therefore, it is very important that natural proportion of the component in essential oil is maintained during the extraction from plant material by any procedure (Răileanu, Todan, Voicescu, *et al.*, 2013; Turek, and Stintzing, 2013).

In this work essential oils from four different plant species were obtained by supercritical CO_2 fluid extraction. Modification of supercritical CO_2 method includes addition of modifier MeOH (5%), into column with plant material, in order to enhance the solubility of lipophilic components. Static extraction time from 20 minutes was prolonged to 30 minutes per every extraction. Flow rate from 2.0 mL/min was decreased to 0.5 mL/min., which aims to extend the contact of the solvent and plant material (Table 1).

Table 1. Comparison of extraction conditions Between Standard and Modified Method

Extraction conditions	Standard	Modified
Extract temperature	50°C	50°C
Vessel temperature	120°C	120°C
Pressure	250 psi	250 psi
Static extract time	20 min	30 min
Flow rate	2 mL/min	0.5 mL/min
Modifier	no	5% MeOH

After extraction with CO_2 was performed, four essential oils of different shades of pale yellow color were obtained. The yield of essential oils, ranged from 0.35 g/100g to 7.3 g/100g of dry plant material (Figure 2). Prior to analyses of the Z-ligustilide content in the essential oils, obtained essential oils were stored at -20°C.

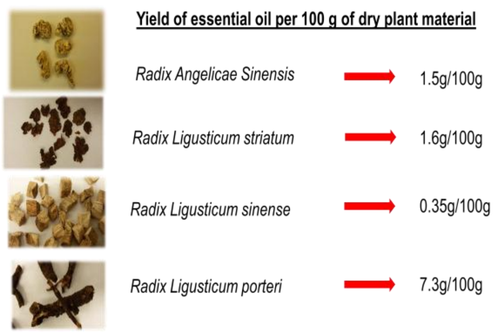

Figure 2. Yield of essential oil obtained by SFE extraction using modified method.

Extraction with supercritical fluid allows to obtain an essential oil at relatively low temperature, 50°C in our case, which could explain better yield of the oil itself. Since Z-ligustilide was a compound of interest in this study, after essential oils were obtained, percentage of Z-ligustilide were quantified using qHNMR (Gödecke, Napolitano, Rodriguez Brasco, *et al.*, 2013). Quantification of Z-ligustilide was done thanks to the characteristic signal of this compound doublet of triplet at δ 6.286 (1H, H-7) and calibration with dimethyl sulfone ($DMSO_2$) as external standard (EC qHNMR) (Pauli, Chen, Simmler, *et al.*, 2014; Pauli, Gödecke, Jaki, *et al.*, 2012).

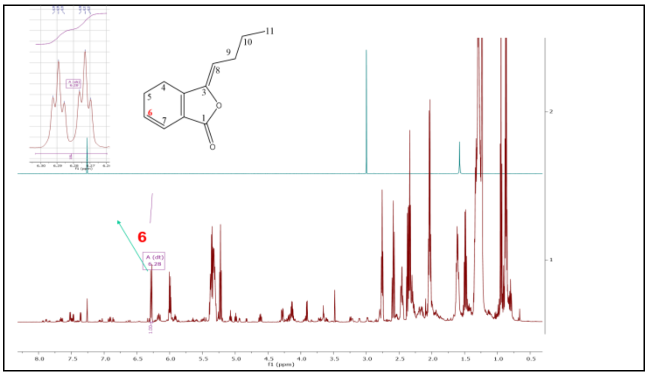

Figure 3. HNMR spectra of essential oil of *Angelica sinensis* with characteristic peak at δ 6.286 (1H, H-7).

Although the essential oil has a complex structure and consequently overlapped NMR spectra, the characteristic signal was possible to identified (Simmler, Napolitano, McAlpine, *et al.*, 2014). Figure 3 shows the HNMR spectra of essential oil of *A. sinensis*.

Quantitative analysis was performed using absolute quantification (100% method). This method allows the determination of the mass of a compound with known structure (Z-ligustilide in our case) in an accurately weighed sample. It involves the use of a calibrant of known exact weight and purity. When using an external calibrant (EC), the general calculation of purity (P) was done according to following formula:

$$P_{analyte} = \frac{I_{analyte} \times N_{EC} \times M_{analyte} \times W_{EC} \times P_{EC}}{I_{EC} \times N_{analyte} \times M_{EC} \times W_{sample}}$$

where, P is the purity of the analyte (in %), I is the absolute integral value, N is the number of protons in the integrated signal, M is the molar mass, W is the gravimetric weight (in mg), EC is the external calibrant. The calculated percentage of ligustilide in four investigated essential oils were presented in Figure 4.

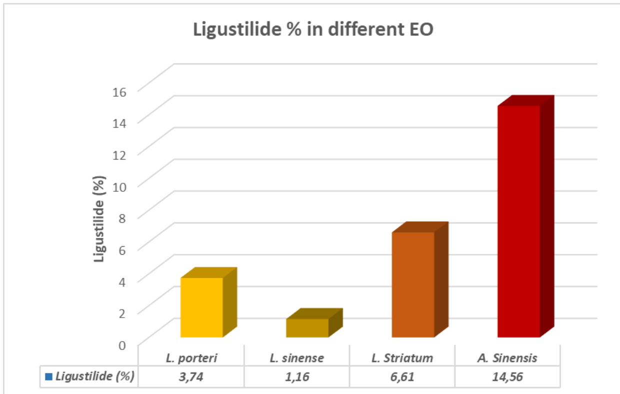

Figure 4. Ligustilide content (%) in different essential oils obtained from plant species belonging to Apiaceae family

Quantification for each oil was made in triplicate. Previous work with non modified method of supercritical CO_2 extraction indicate 10% of ligustilide in essential oil of *A. sinensis* (Yi, Liang, Wu, *et al.*, 2009; Zhou, and Li, 2001). This shows that modified method is much more efficient because it gives a higher yield of ligustilide. The highest amount of essential oil was found in plant species *L. porteri* (7.3 g of essential oil/100 g of dry plant material) but the essential oil with the highest percentage of ligustilide was oil obtained from *A. Sinensis* (14.56%). Calculating the quantity of ligustilide per 100 g of plant material it comes out that *L. porteri* is the species with the highest amount of ligustilide with 0,27g of ligustilide/100g of plant material, followed by *A. sinensis* 0,21g/100g, *L. striatum* 0,11g/100g and L. sinense 0,004 g/100 g. Although *L. porteri* has been used for treatment of wild range of illness in ethno medicine, there is scarce scientific data about its activity and pharmaceutical usage (Beck and Chou, 2007). The results about ligustilide content in *L. porteri*, obtained in this research, open new possibilities for use of this plant species as a source of ligustilide.

CONCLUSIONS

Although plenty of pharmaceutical effects of *Z*-ligustilide have been reported, study on the activities of *Z*-ligustilide is still inadequate. The main reason is the instability of *Z*-ligustilide, and its easily transformation into other degradation products by oxidation, isomerization, and dimerization at an elevated temperature. Different extraction techniques involve laborious operations and consume large amounts of organic solvents. More over these techniques used a high temperature which is unfavorable to ligustilide. The extraction of essential oils using supercritical fluids present an alternative to conventional methods, and it is much faster, more simple and efficient. This article summarized research outcomes involving optimization of parameters of supercritical extraction for maximum recovery of analytes. The SFE method and conditions applied in this work prove to be more efficient in obtaining essential oil with major percent of Z-ligustilide. The environmental friendliness of this technique with non toxic carbon dioxide as main solvent, represent u huge advantage towards organic solvents. qHNMR technique and method of quantification using external standard, allowed to quantified this compound in the essential oil and herbal preparations. Nondestructive nature of NMR, prove to be optimal analytical method for determination of ligustilde in the essential oil sample. Also, this method could be used for the tracking of the Z-ligustilide degradation in different herbal formulations. This is very important from the point of view of the instability of this molecule, especially with regard to the fact that it is a bioactive marker for many apiaceous botanicals.

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Summary/Sažetak

Ligustilid je označen kao glavna aktivna komponenta biljne vrstie *Angelica sinensis*, koja se prevashodno koristi kod neredovnog menstrualnog ciklusa i predmenstrualnog sindroma. Najnovija farmakološka ispitivanja potvrđuju da ljekovite biljke koje sadrže ligustilid, imaju protuupalno djelovanje, poboljšavaju kognitivne funkcije, ublažavaju oštećenje mozga nakon hipoksije, inhibiraju faktor nekroze tumora određenih ćelijskih linija, imaju nefron-zaštitne učinke i neuroprotektivnu aktivnost. U ovom radu provedeno je kvantitativno određivanje ligustilida, pomoću kvanti 1HNMR metode. Ispitane su četiri vrste biljaka: *Angelica sinensis, Ligusticum porteri, Ligusticum striatum* i *Ligusticum sinense*. Ekstrakcija eteričnog ulja iz korijena četiri ispitivane vrste, provedena je modificiranom metodom sa superkritičnim CO_2. qHNMR analiza pokazala je sljedeći postotak ligustilida: eterično ulje *L. porteri* 3,74 (%); eterično ulje *L. sinense* 1,16 (%); eterično ulje *L. striatum* 6,61 (%) i eterično ulje *A. sinensis* 14,56 (%). Najveći postotak ulja dobiven je iz korijena *L. porteri*, ali najveći postotak ligustilida sadržavalo je eterično ulje *A. sinensis*.