NPC Natural Product Communications

2017
Vol. 12
No. 2
213 - 216

Rapid and Efficient Extraction and HPLC Analysis of Sesquiterpene Lactones from *Aucklandia lappa* Root

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Received: May 25th, 2016; Accepted: December 13th, 2016

The root of *Aucklandia lappa* Decne, family Asteraceae, is widely used in Asian traditional medicine due to its sesquiterpene lactones. The aim of this study was the development and optimization of the extraction and analysis of these sesquiterpene lactones. The current Chinese Pharmacopoeia reports a monograph for "Aucklandiae Radix", but the extraction method is very long and tedious including maceration overnight and ultrasonication. Different extraction protocols were evaluated with the aim of optimizing the maceration period, solvent, and shaking and sonication times. The optimized method consists of only one hour of shaking plus 30 minutes of sonication using 100% MeOH as solvent. ^{1}H NMR spectroscopy was used as a complementary analytical tool to monitor the residual presence of sesquitepene lactones in the herbal material. A suitable LC-DAD method was set up to quantify the sesquiterpene lactones. Recovery was ca. 97%, but a very high instability of constituents was found after powdering the herbal drug. A loss of about 20% of total sesquiterpenes was found after 15-20 days; as a consequence, it is strongly endorsed to use fresh powdered herbal material to avoid errors in the quantification.

Keywords: *Aucklandia lappa*, Sesquiterpene lactones, Recovery, HPLC-DAD, NMR, Quality control.

Aucklandia lappa Decne. (Family *Asteraceae*) is a perennial plant, native to East Asia, growing in the Himalayas, Burma, China and India. The root (木香, Mu Xiang, also called Costus Root) is widely used in Traditional Chinese Medicine and a monograph is reported in the current Chinese Pharmacopoeia [1]. It is mainly used for treatments of digestive ailments, including gastric and abdominal pain, loss of appetite, indigestion, diarrhoea, anorexia, nausea and vomiting [2]. The plant is also utilized to treat asthma and cough, coronary heart disease, acute pancreatitis, acute cholecystitis and hepatitis [3]. The sesquiterpenes costunolide and dehydrocostus lactone are considered the major active compounds [4, 5] of "Aucklandia root", and many pharmacological activities have been attributed to their presence, such as anti-ulcer [6], anti-cancer [7], hepatoprotective [8] and cytotoxic properties [5]. Furthermore, they have also been found to exhibit antiangiogenic [9], anti-inflammatory [10], antimicrobial, fungicidal [10, 11] and immunomodulatory activities [12].

At present, this plant is widely used in the European market, which justifies the need for a simple and rapid HPLC method for quality control of the herbal drug. The HPLC assay described in the Chinese Pharmacopoeia monograph reports the quantification of the two major sesquiterpene lactones, but the sample extraction method is very long and tedious including a maceration overnight and diverse steps of ultrasonication. The main purpose of our work was the optimization of the extraction method for the sesquiterpene lactones in the roots and the consequent quantitative analysis of commercial samples of "Aucklandia root".

Different extraction methods of the powdered herbal material were evaluated. They were all identified with a number (1, 2, 3, 4, 5) and a letter indicating the type of extraction, namely "S" for sonication bath and "U" for ultrasonication probe. In parentheses, the time of extraction expressed in minutes and/or hours is reported. In all the experiments 0.30 g of powdered roots was tested after addition of 50 mL of MeOH.

The following methods were investigated:
-Method 1S (30): 24 hours of maceration with shaking, plus 30 minutes in the sonication bath
-Method 1U (30): 24 hours of maceration with shaking, plus 30 minutes of ultrasonication with a probe
-Method 2S (30): 30 minutes in the sonication bath
-Method 2U (30): 30 minutes of ultrasonication with a probe
-Method 3S (15+15): 15 minutes of sonication plus 1 hour of maceration with shaking, plus 15 minutes of sonication
-Method 3S (30): 1 hour of maceration with shaking, plus 30 minutes of sonication
-Method 3U (15+15): 15 minutes of ultrasonication with a probe plus 1 hour of maceration with shaking, plus 15 minutes of ultrasonication with a probe
-Method 3U (30): 1 hour of maceration with shaking, plus 30 minutes of ultrasonication with a probe
-Method 4S (15+15): 15 minutes of sonication plus 16 hours of maceration with shaking, plus 15 minutes of sonication
-Method 4U (30): 16 hours of maceration with shaking, plus 30 minutes of ultrasonication with a probe
-Method 5E-S: 48 hours of maceration with shaking, plus 30 minutes in a sonication bath
-Method 5E-U: 48 hours of maceration with shaking, plus 30 minutes of ultrasonication with a probe

The first set of experiments was started with *Method 1U (30),* which is that described in the Chinese Pharmacopoeia monograph for "Aucklandiae Radix". This method was used as a reference for a preliminary analysis of the data. Recovery of costunolide and dehydrocostus lactone with this method was considered to be 100%. Five different methods of maceration were tested using increasing times (0, 1, 16, 24, 48 hours) of mechanical stirring and followed by 30 minutes of ultrasonication, as reported in the official monograph of the Chinese Pharmacopoeia. A considerable loss of MeOH due to evaporation was observed in all samples and, consequently, ultrasonication was replaced by sonication.

Preliminary experiments gave contradictory results, probably because the sesquiterpene lactones degraded very quickly after powdering the herbal material due to atmospheric oxidation. Consequently, HPLC analyses, in triplicate, of the same sample during different days after powdering were carried out. A gradual loss in the content of the main constituents was observed. After 12 days the residual percentage of active ingredients was ca. 90%, and after 18 days, ca. 80%. The herbal material, therefore, when powdered undergoes rapid degradation.

Accordingly, sample Auck1 was tested immediately after the pulverizing process, using: Method 1S (30), Method 1U (30), Method 2S (30), Method 2U (30), Method 3S (15 + 15), Method 3U (15 + 15), Method 4S (15 + 15), and Method 4U (15 +15). Table 1 reports the results of the quantitative analyses performed by HPLC-DAD expressed as percentages of the active constituents.

Table 1: Quantitative results obtained from sample Auck1 after extraction assays based on different times of maceration combined with centrifugation or ultracentrifugation.

Sample Auck1	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Method 1S (30)	1.00± 0.05	1.38± 0.09	2.37± 0.07
Method 2S (30)	0.98± 0.03	1.34± 0.05	2.32± 0.04
Method 2U (30)	0.85± 0.07	1.18± 0.08	2.02± 0.08
Method 3S (15+15)	0.96± 0.09	1.35± 0.07	2.31± 0.08
Method 3U	0.87± 0.02	1.25± 0.04	2.12± 0.03
Method 3S (30)	1.01± 0.03	1.44± 0.02	2.45± 0.02
Method 3U (30)	0.85± 0.08	1.22± 0.05	2.07± 0.06
Method 4S (15+15)	0.96± 0.05	1.38± 0.07	2.33± 0.06
Method 4U	0.85± 0.03	1.20± 0.03	2.05± 0.03
Method 4S (30)	0.86± 0.03	1.27± 0.09	2.13± 0.06
Method 4U (30)	0.96± 0.04	1.41± 0.06	2.37± 0.05

According to the above results:

1. The sonication bath seems to be able to extract both costunolide and dehydrocostus lactone to either the same extent or even better than the ultrasonication probe.
2. Sonication or ultrasonication gave the same quantitative results
3. The best extraction method was 3S (30), with only one hour of shaking, followed by 30 minutes in the sonication bath.

To assess further the influence of maceration time, the extraction methods 3S (30), 2 S (30), and 1S (30) were repeated with samples Auck2 and Auck3. Data are reported in Tables 2 and 3.

Table 2: Repeated assays with sample Auck2.

Sample Auck2	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Method 2S (30)	0.91± 0.08	1.36± 0.06	2.27± 0.07
Method 3S (30)	1.01± 0.06	1.47± 0.04	2.47± 0.05
Method 1S (30)	0.83± 0.02	1.18± 0.01	2.01± 0.01

Table 3: Repeated assays with sample Auck3.

Sample Auck3	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Method 2S (30)	1.85± 0.07	1.76± 0.08	3.61± 0.05
Method 3S (30)	1.78± 0.09	1.68± 0.07	3.46± 0.06
Method 1S (30)	2.17± 0.03	2.03± 0.02	4.21± 0.02

The best extraction method for Auck3 was 1S (30), corresponding to 24 hours of maceration with shaking, plus 30 minutes in the sonication bath. For Auck2 it seems that method 3S (30) was the best. Similar results were obtained with Auck4 (Table 4).

Table 4: Extraction assays with sample Auck4.

Sample Auck4	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Method 2S (30)	0.82± 0.09	1.31± 0.08	2.13± 0.07
Method 2U (30)	0.82± 0.03	1.28± 0.06	2.09± 0.04
Method 1S (30)	0.89± 0.05	1.37± 0.04	2.26± 0.05
Method 1U (30)	0.86± 0.08	1.32± 0.07	2.18± 0.04

^{1}H NMR experiments were performed directly on the pulverized herbal drug to confirm the exhaustive extraction of active constituents. The exhausted powdered Auck samples were treated with DMSO-d6. In the investigated samples no characteristic signals of costunolide or dehydrocostus lactone were found in the range between δ 8 and 5.5, after extraction of the sample using the method 3S (30).

In conclusion, sonication is less invasive then ultrasonication and the extraction method 3S (30) is the best one for the extraction of the main active constituents of Aucklandia root. The optimized method is able to extract more than 97% of the total of active principles.

A simple HPLC-DAD method was used for the evaluation of the sesquiterpene lactones. Both costunolide (Rt 7.55) and dehydrocostus lactone (Rt 8.83 minutes) were easily identified by comparison of their retention times with those of reference standards.

Six different commercial samples of *A. lappa* were evaluated. The Chinese monograph for "Aucklandiae Radix" reports a minimum content of 0.6% of costunolide and a minimum of 1.8% for the sum of costunolide and dehydrocostus lactone with respect to the dried herbal drug. Quantitative analyses were performed by HPLC–DAD and the optimized method 3S (30) was used for the extraction of the roots. All results are reported in Table 5.

Table 5: Extraction assays with further samples.

Sample	% costunolide (g/100g)	% dehydrocostus lactone (g/100g)	% costunolide plus % dehydrocostus lactone (g/100g)
Auck5	0.65± 0.02	0.95± 0.03	1.61± 0.02
Auck6	1.06± 0.05	1.30± 0.6	2.36± 0.05
Auck7	0.81± 0.03	0.68± 0.04	1.49± 0.02
Auck8	0.02± 0.04	2.15± 0.06	2.17± 0.05
Auck9	1.31± 0.06	1.35± 0.03	2.66± 0.05
Auck10	1.53± 0.08	1.46± 0.07	2.99± 0.04

Of the tested samples, only Auck5 and Auck7 contained less than 1.8% of costunolide plus dehydrocostus lactone.

HPLC-DAD and NMR spectroscopy were used as integrative analytical tools to develop the best extraction method of sesquiterpene lactones from *A. lappa* root. A very high instability of both constituents was found after powdering the herbal drug with 80% residual percentage of active constituents after 15-20 days. Accordingly, it is strongly recommended to use fresh powdered herbal drug material to avoid errors in the quantification of constituents. The optimised, rapid and efficient extraction method is 3S (30), namely the powdered material is macerated for 1 h with shaking, followed by 30 minutes of sonication (total time of extraction is 1 h and 30 min).

The developed extraction and HPLC analytical methods were adequate for the quality control of Aucklandia root in order to guarantee the integrity and stability of the products and assess efficacy and safety.

Experimental

Apparatus: Extractions were performed using an electronic Sonorex RH 100 SH ultrasonic bath (Bandelin, Berlin, Germany) and a Bandelin electronic Sonoplus, using the mechanical shaker HS 250 BASIC (Ika Labortechnik, Staufen, Germany). A mortar was used to powder the roots before extraction. An Agilent 1100 HPLC system coupled with DAD detector was used for chemical profile and quantitative analysis. NMR spectra were recorded using a Bruker DRX (Koln, Germany) spectrometer operating at 400.13 MHz and a Bruker Avance-600 spectrometer operating at 600.13 MHz (14.1T), both using a 5 mm inverse probe equipped with a z-shielded gradient. Data processing was achieved using TOPSPIN software package 1.3.

Chemical and reagents: Dimethylsulfoxide-d_6 (99.9% purity) and MeOH were HPLC grade from Sigma Aldrich (Seelze, Germany). Water was purified by a Milli-Qplus system from Millipore (Milford, MA, USA). The HPLC column used was a Zorbax® Eclipse XDB C18, 150 × 4.6 mm, 5 µm (Agilent, Palo Alto, CA, USA). The following standards were used: Costunolide, code Y0001307 and dehydrocostus lactone, code 38384 both CRS and given by EDQM (Strasbourg, France).

Herbal drug samples: Two samples (46393, named Auck1 and 31401, named Auck2) were commercial herbal drugs from Shenyang, China, and sent by EDQM. A commercial Chinese sample, Auck3, was donated by Phytax (Schlieren, Switzerland) while 5 samples from China (Auck5=410079, Auck6=750079, Auck7=110079, Auck8=671375, Auck9=130079), one from Austria (Auck10=112014) and one from India (Auck11= 21657100) were provided by Plantasia (Oberndorf, Austria).

Preparation of extracts: All the investigated samples were whole roots, which were firstly cut into transverse slices and subsequently powdered in a porcelain mortar. Extracts were obtained by macerating 0.3 g of herbal drug with 50 mL of MeOH, with mechanical shaking for 1 h. After maceration overnight, the samples were than sonicated for 30 min using an ultrasonic bath. The total weight of preparations was monitored at the beginning and end of the extraction process (after cooling of the sample) and MeOH was replenished in the case of loss of weight. The obtained liquid extracts were mixed well and filtered through a membrane filter (nominal pore size 0.45 µm) before analysis.

Preparation of samples for HPLC-DAD analysis: The extracts of *A. lappa* were sonicated for 10 min in an ultrasonic bath and then centrifuged for 4 min at 14,000 rpm, prior to injection. Subsequently, the standard solutions were sonicated for 2 min before injection. Reference solutions were prepared as follows: (a): 5.0 mg of costunolide CRS was dissolved in 5 mL of MeOH, shaken well, diluted to 50 mL with the same solvent and finally mixed well. b): 2.5 mg of dehydrocostus lactone CRS was dissolved in 5 mL of MeOH, shaken well and diluted to 25 mL with the same solvent and finally mixed well.

Preparation of samples for ^{1}H NMR analysis: The herbal material, both before and after extraction, was freeze-dried for 12 h in order to remove the residual water. Fifty mg of the dried powdered herbal material was put in glass tubes and treated with 0.6 mL of DMSO-d_6. Tubes were manually shaken and finally filtered before analysis.

Sonication and ultrasonication process: Sonication was performed using a bath and the temperature never exceeded 25°C. Ultrasonication was performed by immersion of the probe directly into the sample with a maximum amplitude of 50% and a frequency of 20 KHz, at room temperature. At the end of the process, the samples became very hot; as the extracts were in direct contact with the air there was a possible increase in the oxidative processes and evaporation of the solvent.

Qualitative and quantitative HPLC–DAD analysis: The analysis was performed using a Zorbax® Eclipse XDB C18, 4.6 x 150 mm, 5 µm column, at 24°C. The mobile phase was composed of methanol (solvent A) and water at pH 3.2 (solvent B), 13:7 v/v. The flow rate was 1.0 mL/min and the detection wavelength was set at 225 nm. The injection volume was 10 µL. Identification of the 2 main constituents was performed by comparison with the retention times and UV spectra of the reference standards and of the data reported in the literature. Quantitative analysis of the constituents was performed using external standards. Costunolide and dehydrocostus lactone were used to obtain the calibration curve in a range of 0.220-2.200 µg/mL and 0.407-4.070 µg/mL, respectively. The standards were weighed accurately and dissolved in MeOH to obtain stock solutions, which were then diluted. The linearity of the calibration curves were expressed by the values of R^2 (0.99992) for both standards.

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