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Short Communication

Determination of Four Major Saponins in Skin and Endosperm of Seeds of Horse Chestnut (*Aesculus Hippocastanum* L.) Using High Performance Liquid Chromatography with Positive Confirmation by Thin Layer Chromatography

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Abstract

Purpose: To separate and quantify four major saponins in the extracts of the skin and the endosperm of seeds of horse chestnut (*Aesculus hippocastanum* L.) using ultrasonic solvent extraction followed by a high performance liquid chromatography-diode array detector (HPLC-DAD) with positive confirmation by thin layer chromatography (TLC).

Methods: The saponins: escin Ia, escin Ib, isoescin Ia and isoescin Ib were extracted using ultrasonic extraction method. The optimized extraction conditions were: 70% methanol as extraction solvent, 80 °C as extraction temperature, and the extraction time was achieved in 4 hours. The HPLC conditions used: Zorbax SB-ODS-(150 mm × 2.1 mm, 3 µm) column, acetonitrile and 0.10% phosphoric acid solution (39:61 v/v) as mobile phase, flow rate was 0.5 mL min^{-1} at 210 nm and 230 nm detection. The injection volume was 10 µL, and the separation was carried out isothermally at 30 °C in a heated chamber.

Results: The results indicated that the developed HPLC method is simple, sensitive and reliable. Moreover, the content of escins in seeds decreased by more than 30% in endosperm and by more than 40% in skin upon storage for two years.

Conclusion: This assay can be readily utilized as a quality control method for horse chestnut and other related medicinal plants.

Introduction

The use of medicinal plants to heal diseases has been a common practice worldwide.[1] Its popularity is still maintained due to the cultural and historical reasons, and to the primary health care needs added by modern medicine.[1,2] For instance, beneficial effects including antimutagenicity,[3] chemoprotection, and antioxidant activity have been reported.[4]

Aesculus hippocastanum L., commonly known as "horse chestnut", is a native, stable and important elements of urban and rural landscapes, which is widely distributed all over the world.[1,5] It is used for a variety medical conditions, including malary, fever, bladder and gastrointestinal disorders. Moreover, the seeds extract have been prescribed traditionally for the treatment of several chronic venous insufficiency, to reduce its associated symptoms, including leg swelling and heaviness as well as vascular problems.[1,6] This extract consists mainly of escins and a mixture of triterpenoid saponins (α- and β-escin).

Saponins consist of four substances make up 60% of the total mixture, namely: escin Ia 24%, escin Ib 17%, escin IIa 13% and escin IIb 6%.[7] Thus, due to the different pharmacological properties and their wide use in the pharmaceutical, β-escin (which all are dependent on its concentration) has significant role making its analysis in herbal medicines important and deemed necessary.

Literature survey reveals that saponins in *Aesculus hippocastanum* L., plant have been widely studied, and about 30 individual compounds of saponins have been isolated and identified. Only escin Ia among the four escins is assigned as the marker species for Semen Aesculi as prescribed by the official Chinese

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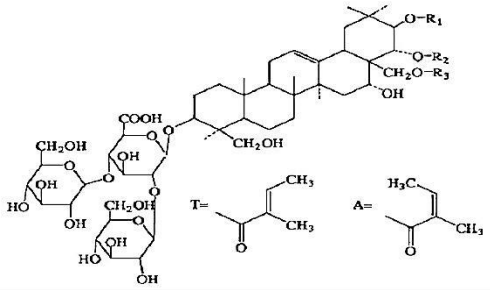
Pharmacopoeia of 2005 edition.[8] The structures of the four compounds are illustrated in Figure 1.



Saponins	R1	R2	R3
Escin Ia	T	-$COCH_3$	-H
Escin Ib	A	-$COCH_3$	-H
Isoescin Ia	T	-H	-$COCH_3$
Isoescin Ia	A	-H	-$COCH_3$

Figure 1. Chemical structures of four escins: escin Ia, escin Ib, isoescin Ia and isoescin Ib.

Several analytical methods for the analysis of *Aesculus hippocastanum* L., have been reported whether for the determination of the phenolic compounds, organic acids, and sugars in the dry extracts from the leaves using high performance liquid chromatography (HPLC) coupled with UV detector at 190 nm,[9] or at 340 nm for the analysis of esculin and fraxin in bark,[10] or at 210 nm for the analysis of β-escin extract.[11] Ultra performance liquid chromatography (UPLC) coupled with UV detector at 365 nm for coumarins analysis in *Aesculus hippocastanum* L. flowers has also been reported.[12] Ultraviolet (UV) method at 265 nm for the estimation of *Aesculus hippocastanum* L. extract has also been developed.[13] Capillary electrophoresis (CE) coupled with UV detector at 226 nm in dry, hydroalcoholic and hydroglycolic extracts of *Aesculus hippocastanum* L. have been reported.[14] Additionally, liquid chromatography coupled with mass spectrometry (LC-MSQT) for the determination of the content of phenolic compounds in leaf tissues of white and red *Aesculus hippocastanum* L. with leaf miner larvae before and after cameraria ohridella attack has been conducted.[15]

Recently, the application of ultrasound sonication has showed up promising results in plant extraction area. Moreover, ultrasound-assisted extraction, as an innovative technology, could enhance mass and heat transfer by disrupting the matrix cell walls mechanically, through creation shear forces, to promote the release of bioactive components from natural products.[16,17] It has been reported that a variety of nutritional materials such as saponins, phenolics, polysaccharides, essential oils, and carotenoids have been extracted successively with the aid of ultrasound methods.[18,19] Once compared to conventional solvent extraction, ultrasound sonication is considered a useful, inexpensive method in addition it can increase extraction yield and extraction efficiency, thus a noticeable reduction in solvent consumption resulting in saving extraction time and energy input with high reproducibility.[17] Additionally, the advantages of ultrasound sonication, compared with supercritical fluid (CO_2) extraction, are that ultrasound sonication can accelerate mass transfer by mechanical effects and simplify manipulation with cheaper equipment.[20]

In the present paper, HPLC method has been developed for the quantitative determination of four major saponins: escin Ia, escin Ib, isoescin Ia and isoescin Ib in the skin and the endosperm of seeds of *Aesculus hippocastanum* L. extract. TLC has also been conducted herein for the identification of individual escins.

Materials and Methods

Chemicals and reagents

Standards of escin Ia, escin Ib, isoescin Ia and isoescin Ib were obtained from National Institute for the Control of Pharmaceutical and Biological Products of China. HPLC-grade methanol (≥99.96%), acetonitrile (≥99.96%), ethyl acetate, and isopropanol were purchased from Merck (Merck, Darmstadt, Germany). Ortho-phosphoric acid (85%) was purchased from Sigma-Aldrich (St Louis, MO, USA). Phosphotungstic acid ($H_3PW_{12}O_{40}$, HPW) was purchased from Sinopham Chemical Reagent Co., Ltd. Ultrapure water (resistivity, 18.2 $M\Omega cm^{-1}$) was produced by a Milli-Q system (Millipore, USA) and was used throughout for the preparation of solutions. Centrifuge (model 2100) was purchased from Kubota (Tokyo, Japan).

Sample preparation

Seeds of *Aesculus hippocastanum* L. growing in Ukraine were collected from healthy trees that were harvested in September, 2012 and 2014. The dried seeds were divided into two parts; skin and endosperm. The two parts were air-dried, ground, sifted through 0.30 mm mesh screen before extraction to obtain a uniform particle size. After collected, the samples were immediately submitted to the ultrasonic extraction step.

Ultrasonic extraction

Ultrasonic extraction was conducted by mixing 2 g of the powdered sample and 150 mL of 70% methanol in a flask. It was then placed in an ultrasonic bath for 4 hours at 80 °C. The extraction was repeated three additional times in order to enhance the extraction yield and the extracts were then combined. The combined extracts was centrifuged (2580 rcf) for 10 min. The obtained supernatant was evaporated to dryness using a rotary evaporator at 50 °C. Finally, the residue was dissolved in 50 mL of 70% methanol and then filtered through a 0.2 µm nylon membrane filter prior HPLC analysis.

Standard solutions preparation

Standard stock solutions containing 1 mg mL^{-1} were prepared by dissolving approximately 10 mg of each pure compound in 10 mL methanol. The calculations were carried out using the following equation:

$$x = \frac{Cx \times Vs \times 1000}{k \times M} \left[{}^{g}/_{kg} \right]$$

C_x – concentration of escin in the test sample
V_s – volume of extract
M – mass of prepared sample
k - coefficient of concentration of the extract
x – content of escin (g) per 1 kg of plant material

HPLC conditions

HPLC analysis was carried out using Agilent instrument (Agilent1200, USA) equipped with a quaternary solvent delivery system, autosampler, column oven and diode array UV/Vis detector. Zorbax SB-ODS-150 mm×2.1 mm, 3 µm column, (Agilent Technologies, USA) was used isocratically with a binary mixture of acetonitrile and 0.10% orthophosphoric acid solution (39:61 v/v) at a flow rate of 0.5 mL min^{-1}. Detection was achieved at 210 and 230 nm. The injection volume was 10 µL, and separation was carried out isothermally at 30 °C in a heated chamber. Identification was conducted by comparing the retention times of the components of the sample and standards of escins. The quantitative determination was conducted by external calibration method.

Identification by TLC

Standards of escins (100 µg mL^{-1} each) and the extract obtained were spotted on RP-18 Silica coated TLC plate (Merck, 20 × 20 cm) with a 10 mm space between each spot. The spots were then air dried before solvent development. The plate was developed in a mixture of ethyl acetate, isopropanol, and water at a ratio of (40:40:30 v/v) for approximately 60 min in a developing chamber. After the plate was air dried, a 25% alcoholic solution of phosphotungstic acid reagent was used to develop escin spots. The identification of escins was confirmed based on comparison with the R_f values with the escin standards and the quantitative concentrations were obtained from the HPLC analysis.

Results and Discussion

The HPLC chromatographic conditions were developed and optimized using saponin standards and real *Aesculus hippocastanum* L. sample to get the best separation in a reasonable separation time. Reversed phase HPLC was applied according to the previous work reported by Chen *et al*., 2007 by varying the ratios of water and acetonitrile in the mobile phase which provided a better improvement in separation and significant enhancement in peak shape.[13]

Figure 2 (A and B) shows the chromatograms of the *Aesculus hippocastanum* L. samples (endosperm & skin) harvested in 2014 under the same HPLC conditions. All the saponin peaks are well resolved from each other.

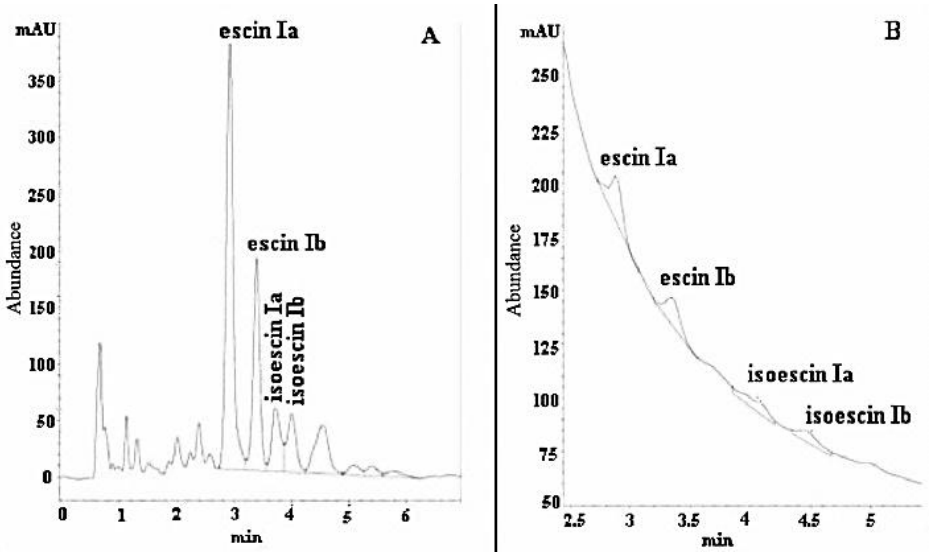


Figure 2. HPLC chromatograms of extracts of *Aesculus hippocastanum* L. endosperm (A); extracts of *Aesculus hippocastanum* L. skin, both harvested in 2014 (B). Please refer to text for HPLC conditions.

The presence of escins in the endosperm and the skin of *Aesculus hippocastanum* L. have been confirmed by TLC method. Spots were evaluated in comparison with the escin standards (the spot of purple color with R_f = 0.71). However, the color and the sizes of escins of the endosperm spots had much more intensity than spots of escins from the skin. This may indicate a greater accumulation of saponins in the seeds endosperm of *Aesculus hippocastanum* L.

The qualitative determination using TLC method showed the presence of escin Ia, escin Ib, isoescin Ia and isoescin Ib. The contents of escins in the skin and in the endosperm of *Aesculus hippocastanum* L. samples for the years 2012 and 2014 were compared. We can say that

Aesculus hippocastanum L. seed's skin has considerably less escins content (0.32 ± 0.012 g kg^{-1} and 0.19 ± 0.009 g kg^{-1}) than the seed's endosperm (52.05 ± 0.67 g kg^{-1} and 34.9 ± 0.51 g kg^{-1}), for the years of 2012 and 2014, respectively.

Also, it has been noticed that the content of escins in the skin and in the endosperm of seeds that were gathered in 2012 exceeds the content of escins in the skin and the endosperm of those stored till 2014.

Quantification of saponins content

The newly developed HPLC method has been applied for the determination the four saponins in different *Aesculus hippocastanum* L. extract samples (each sample was extracted twice and analysed thrice, n = 3). It is clear that among the four saponins, escin Ia is the most abundant saponin in *Aesculus hippocastanum* L. samples. Additionally, it is found that the contents of the four major saponins in the *Aesculus hippocastanum* L. samples are different. It is may be attributed to various factors such as geographical source, cultivation, harvest, storage and processing of the herb.

Conclusion

The presence of escins in the endosperm and skin of all samples of *Aesculus hippocastanum* L. have been confirmed by TLC method. The quantitative determination of four major saponins (escin Ia, escin Ib, isoescin Ia and isoescin Ib) has been conducted using HPLC method in the skin and endosperm of *Aesculus hippocastanum* L. samples for the years 2012 and 2014. The main site of localization of escins in *Aesculus hippocastanum* L. seeds is in its endosperm. Its content is decreased by more than 30% in endosperm and more than 40% in skin during storage for two years. Finally, the applicability of TLC means that the procedure is effectively portable and could be undertaken on site (e.g. in a motor vehicle situated near a plantation) for particularly rapid results.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

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