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Research article

Preliminary results on the separation of the different parts of *Ligustrum lucidum* Ait fruit and the main bioactive compounds analysis

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ABSTRACT

Oleanolic acid (OA) and ursolic acid (UA) possess anti-inflammatory, antioxidative, antiprotozoal, antimutagenic and anticancer properties and are the main bioactive compounds in the fruit of *Ligustrum lucidum* Ait. The aim of this paper was to study the method of separating the whole fruit into the main ingredients containing OA and UA for the later effective extraction with reduced organic solvents and easy separation and purification. In the present study, the sarcocarps and exocarps were separated from the whole fruits (designated exo-sarcocarps, the mass percentages (w/w, dry weight basis), 33.1%), testae (13.5%) and cores (48.7%) by using methods separated. The contents of OA and UA in whole fruits, exo-sarcocarps, testae and cores were analyzed. The OA and UA extraction yields were highest in exo-sarcocarps vs. yields from whole fruits, testae or cores. The spective yields of OA and UA from exo-sarcocarps were 24.34 ± 2.09 and 7.82 ± 0.09 mg/g; these yields were about 4 times higher than OA yields and about 4 times higher than UA yields from fruit.

1. Introduction

The fruit of *Ligustrum lucidum* Ait is a well-known traditional Chinese medicinal plant (Chinese name "Nv-zhen-zi") (National Pharmacopoeia Committee, 2010) that exhibits immunomodulatory, anti-inflammatory, hepatoprotective, antitumor and antiaging activities (Pollier and Goossens, 2012; Zhang et al., 2013) and has been used for prevention and treatment of several diseases (Hao et al., 1992; Niikawa et al., 1993; Liu et al., 1994). A number of methods for isolating active components from the fruit have been described in literature (Gao, 2007; Lin et al., 2007; Gao et al., 2009; Tanahashi et al., 2009; Yang et al., 2010; Fu et al., 2010). Oleanolic acid (OA) and ursolic acid (UA) are the main bioactive components in the fruit (Zhang and Li, 1992; Shi et al., 1998) and have attracted a lot of attention because of their diverse biological effects (Xiang and Gu, 2002; Ramachandran and Prasad, 2008). The structures of the OA and UA are shown in Figure 1.

Extraction is one of the key processes to purify target compounds from herbal plants or fruits. Extraction influences the mass transport and subsequent extraction efficiency. However, the best starting material influences the extraction efficiency and cost more.

OA and UA have been extracted from fruits by several conventional methods, such as maceration, reflux, Soxhlet extraction and ultrasound-assisted extractions (Shi et al., 1998; Wang et al., 2003; Lin et al., 2007; Valachovic et al., 2001; Yang et al., 2013.). However, these extraction methods often use raw materials with low OA and UA contents, which make the extraction complicated, consume large volumes of solvent and entail lengthy extraction procedures, causing loss or low yields of OA and UA.

Although bioactive compounds are generally in low concentrations in whole fruits, the amounts of these active compounds vary between the different parts of a given fruit. Therefore, the efficient and high-yield extraction of bioactive compounds from parts of the fruit that contains the highest amounts of OA and UA can improve the effect and value in the use of those bioactive components.

A fruit is made up of three parts: exo-sarcocarp (the mixture of the exocarp and mesocarp), testae and cores. In this paper, methods were investigated to separate the fruit into these three parts, the extractions of OA and UA from the three parts and the contents of OA and UA and the yields from the three parts of the fruit were compared. The significance of this work is to reduce organic solvents for extraction and save the cost for the subsequent industrial extraction of active ingredients. Moreover, the

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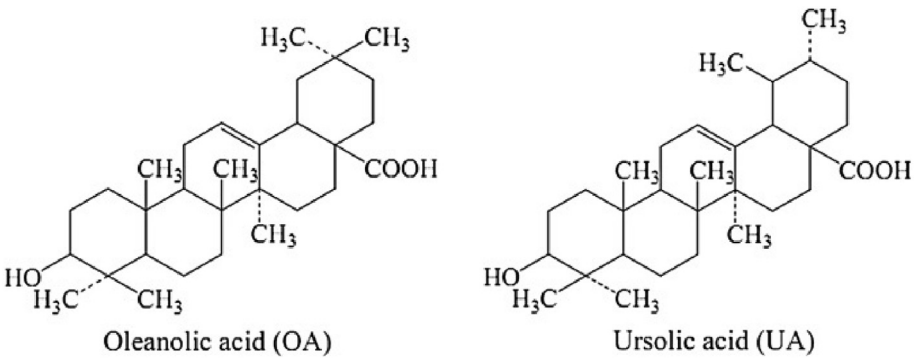

Figure 1. Chemical structures of OA and UA.

content of active components can be increased effectively, which is convenient for subsequent separation and purification after extraction.

2. Results and discussion

2.1. Preparation and separation of the exo-sarcocarp, testa and core parts of the fruit

The contents of OA and UA in the fruit of *Ligustrum lucidum* Ait from different parts were different, which are approximately 6.39–13.40 mg/g and 1.68–3.77 mg/g, respectively (Li et al., 2005; Li and Zhu, 2009). They are usually found in low concentrations in whole fruit from the same part. However, the contents of OA and UA differs in different parts of the fruit. Therefore, to efficiently obtain high-yield extracts of these two bioactive compounds, determining the part of the fruit that contains the highest OA and UA content is necessary. Another factor to consider is that the two acids in the fruit not only occur as free acids, but also as aglycone precursors of triterpenoid saponins, in which they are linked to one or more sugar chains (Liu 1995; Szakiel et al., 2003, 2005). OA and UA are not soluble in water or acid solution but are soluble in ethanol, while their saponin aglycone precursor forms are insoluble in ethanol. If saponin precursor forms could be extracted, this could increase yield in cases where the yields of OA and UA are not high. Therefore, after washing the fruit with water, the fruits were immersed in a pH 1–4 H_2SO_4 solution and heated, hydrolyzing the sugars attached to the two acids, freeing the OA and UA acids. This made the fruits fluffy and easier to separate into exo-sarcocarps and nutlets after agitation using a blender.

Although the density of exo-sarcocarps separated from the fruit was lower than the nutlet density, they both rose together with the rise in water level, exiting the reactor together with water outflow, and were filtered through a 0.125 mm filter screen to obtain the exo-sarcocarps of the fruits until the outflow ceased. After pretreatment and separation of exo-sarcocarps from nutlets as mentioned in Section 2.4 above, exo-sarcocarps and nutlets from the boiled fruit were separately freeze-dried. The nutlets were milled and passed through a 0.30 mm sieve to separate them into testae and cores. The dried materials, consisting of exo-sarcocarps, testae and cores, are shown in Figure 2. The percentages of mass (w/w, dry weight basis) of the isolated fractions were approximately: exo-sarcocarps, 33.1%; testae, 13.5%; cores, 48.7%.

2.2. Chromatographic analysis

Using the described HPLC conditions, the calibration, linear ranges for the two reference compounds were determined and are presented in Table 1.

2.3. Comparison of the content of UA and OA of four samples (fruits, exo-sarcocarps, testae and cores)

UA-SE (ultrasound-assisted solvent extraction) has been successfully used for the extraction of several important bioactive compounds from plant materials (Huang et al., 2011). UA-SE may enhance the extraction efficiency due to its disruption of cell walls, reduction of particle size and enhancement of the mass transfer of the cell contents as a result of cavitation bubble collapse (Wu et al., 2001; Melecchi et al., 2006). Therefore, UA–SE was selected for the extraction of OA and UA from the four samples. The contents of UA and OA in the extraction solution of four sample types were determined using the HPLC conditions described

Table 1. Regression equations, correlation coefficients of the linear calibration graphs (R^2) for the two analytes by HPLC.

Analytes	TR (min)[a]	Linear range (μg)	Regression equation (Y = *a* X + *b*)[b]	R^2
OA	13.430 ± 0.086	0.1–20	Y = 704.76773 X +3.941030.994	0.994
UA	14.164 ± 0.059	0.2–20	Y = 500.48021X + 2.70708	0.998

[a] Retention times (TR) are the mean values from twenty replicates ±SD.
[b] In the regression equation Y = *a* X + *b*, X denotes the quantity of the compounds (μg), Y is the peak area, *a* is the slope and *b* is the intercept of the regression line.

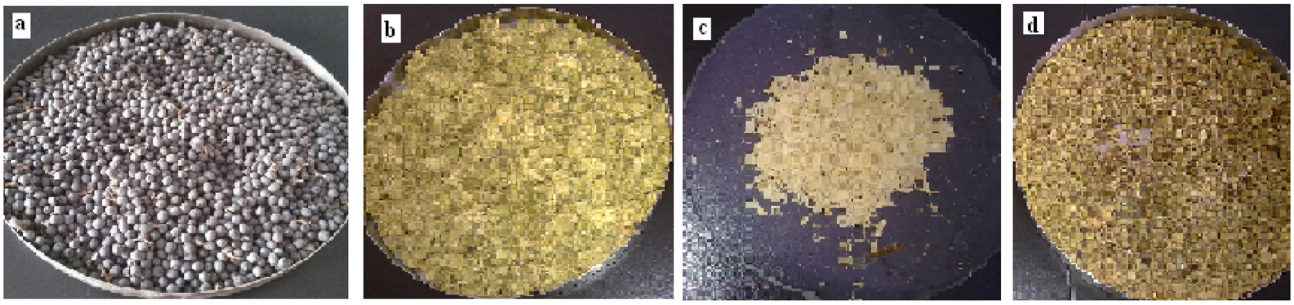

Figure 2. The exo-sarcocarps (b), testa (c) and core (d) are separated from the fruits of *Ligustrum lucidum* Ait (a).

Table 2. Contents of UA and OA in Ligustri Lucidi Fructus for the two analytes by HPLC.

Samples	Contents/mg/g (dried samples)[a]	
	OA	UA
dried whole fruits	7.18 ± 0.43	2.68 ± 0.04
dried cores	Not detected	Not detected
dried testae	Not detected	Not detected
dried exo-sarcocarps	24.34 ± 2.09	7.82 ± 0.09

[a] Contents are the mean values from three replicates ±SD (n = 3).

below according to the methods detailed in Sections 2.5 below. The contents of UA and OA of four sample types are presented in Table 2.

The extraction of organic ingredients from plant materials is directly dependent on their abundance within the starting plant materials. Our results for whole fruit OA and UA yields agree with findings in previous studies detailing OA and UA extractions from the *Ligustrum lucidum* Ait fruit (Shi et al., 1993; Cai et al., 2011; Xia et al., 2012; Ren et al., 2015). However, comparisons of the amounts of UA and OA (Table 2) between the four samples studied in this work (whole fruits, exo-sarcocarps, testaes and cores), it is observed that the OA and UA yields extracted from the exo-sarcocarps were the highest of all samples, with extraction yields of 24.34 ± 2.09 mg/g and 7.82 ± 0.09 mg/g for OA and UA, respectively, which were approximately 4 times as high as that of OA and about 3 times as high as that of UA from whole fruits. These OA and UA yields from testae and cores were not detected.

The results of the present study show that the OA and UA yields extracted from exo-sarcocarps were the highest of all samples and differ significantly from the test all samples ($P < 0.05$). This result also shows that the distribution of UA and OA is almost all in the exo-sarcocarps. Therefore, exo-sarcocarps were selected to be the starting material for the extraction of OA and UA in all subsequent experiments.

Under the UA-SE conditions, the OA and UA yields of the exo-sarcocarps were 24.34 ± 2.09 mg/g and 7.82 ± 0.09 mg/g. These yields were more than four times higher than those of OA and about four times higher than those of UA from whole fruits.

In addition, with the above conditions, the comparison of the total extraction yields of ex-osarcocarps and whole fruits (two extracts), and the contents of UA and OA in the two extracts were studied. Total extract yields, the contents of UA and OA in two extracts and the appearance of two extracts were presented in Table 3 and Figure 3.

Table 3 shows that the total extraction yields of the exo-sarcocarps (9.89%) were lower than those of the fruit (11.65%); however, comparing the contents of UA and OA between the two extracts, it was obviously observed that the content of OA and UA in the extracts of the exo-sarcocarps were higher, with 281.5 mg/g of dried extracts of OA and 96.82 mg/g of dried extracts of UA, respectively, which were approximately four times higher than that in the whole fruits (72.11 mg/g of dried extracts and 26.21 mg/g of dried extracts). Figure 3 shows that the exo-sarcocarps extract was a gray white powder and the whole fruits extract was a green oily substance. The appearance of the extracts of the whole fruits showed that the fruit extracts contain a lot of oil and fat (using HPLC were tested), which leads to the low content of OA and UA in fruit extracts. On the other hand, many oils and fats in fruit extracts will also lead to be difficulties in separating OA and UA from the whole fruit extracts. Thus, compared to whole fruits, the exo-sarcocarps are a better material for extracting OA and UA. Therefore, the exo-sarcocarps are a better material for exracting OA and UA extractions via the UA-SE extraction procedure, reducing the organic solvent, achieving high efficiency and low-cost extraction of OA and UA.

3. Experimental

3.1. Preparation fruit of Ligustrum lucidum Ait

The whole wet fruit of *Ligustrum lucidum* Ait was collected in November 2015 on the campus of Tianshui Normal University (Tianshui, Gansu Province, China) and dried for approximately 3 h at 105 °C to a constant mass.

3.2. Separated methods for the exosarcocarp, testa and core parts of the fruit of Ligustrum lucidum Ait

1.0 kg of fruit was washed with water and placed in a H_2SO_4 solution of pH 1–4 to immerse the fruit, then placed in an F20-H thermostatic reactor with a blade stirrer at 70 °C for 30 min and, after adding of an equal volume of water, the mixtures were quickly stirred using a rotational speed of 1000–1600 rpm for 20 min, then stirred at a reduced speed of 40 rpm. Subsequently quick pouring of the mixtures into water made the exosarcocarp part of the fruit rise together with the rise in water level and exit the reactor together with water outflow. The outflow was then filtered through a 0.125 mm filter screen to obtain the exo-sarcocarps of the fruit. After the water flow was stopped and stirred, the remaining mixture in the reactor was poured through the same filter screen. The whole fruits, separated exosarcocarps and nutlets were freeze-dried. The freeze-dried nutlets were milled and run through a 0.30 mm sieve and then separated into testae and cores. The freeze-dried whole fruits, exosarcocarps, testae and cores were separately powdered and sieved through a 0.25 mm (60 mesh) sieve and subsequently packed in plastic bags and stored at -4 °C in the refrigerator for later use. The separated methods is shown in Figure 4.

3.3. Methods used for determination and extraction of UA and OA from four samples

3.3.1. Methods used for determination of UA and OA

OA and UA HPLC analysis was carried out on a Agilent 1260 HPLC system with an Eclipse Plus C-18 analytical column. The mobile phase was composed of methanol: acetonitrile: 0.5% ammonium acetate in water (55:27:18, v/v) with a flow rate of 1.0 mL/min (Srivastava and Chaturvedi, 2010; Tian et al., 2010; Shi et al., 2005). Before use, all mobile phases, standard solutions and extracts samples were degassed by ultrasonication and filtered through a 0.45-μm nylon membrane. The column was kept equilibrium for 20 min before each analysis. Standard and sample solutions were injected into HPLC system respectively. The column temperature was kept at 20 °C, and the eluting compounds from the column was detected at 210 nm (Xia et al., 2012). The eluates were identified by comparing their retention time to those of the standards. The content of the eluates were calculated according to the standard curve. The sereies of standard solutions of OA and UA (10–2000 μg/mL

Table 3. The comparison the total extraction yields and contents of OA and UA in the two extracts from exo-sarcocarps and whole fruits.

Extracts	total extraction yields % (w/w)	contents/mg/g (dried extract)[a]	
		OA	UA
From dried whole fruits	11.65	72.11 ± 4.53	26.21 ± 2.12
From dried exo-sarcocarps	9.89	281.5 ± 5.63	96.82 ± 2.15

[a] Contents are the mean values from three replicates ±SD (n = 3).

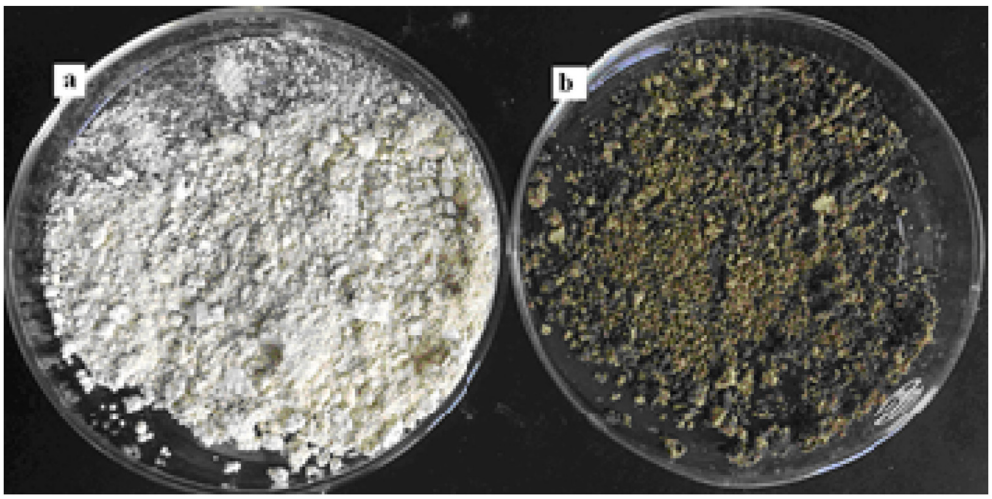

Figure 3. The appearance of two extracts.

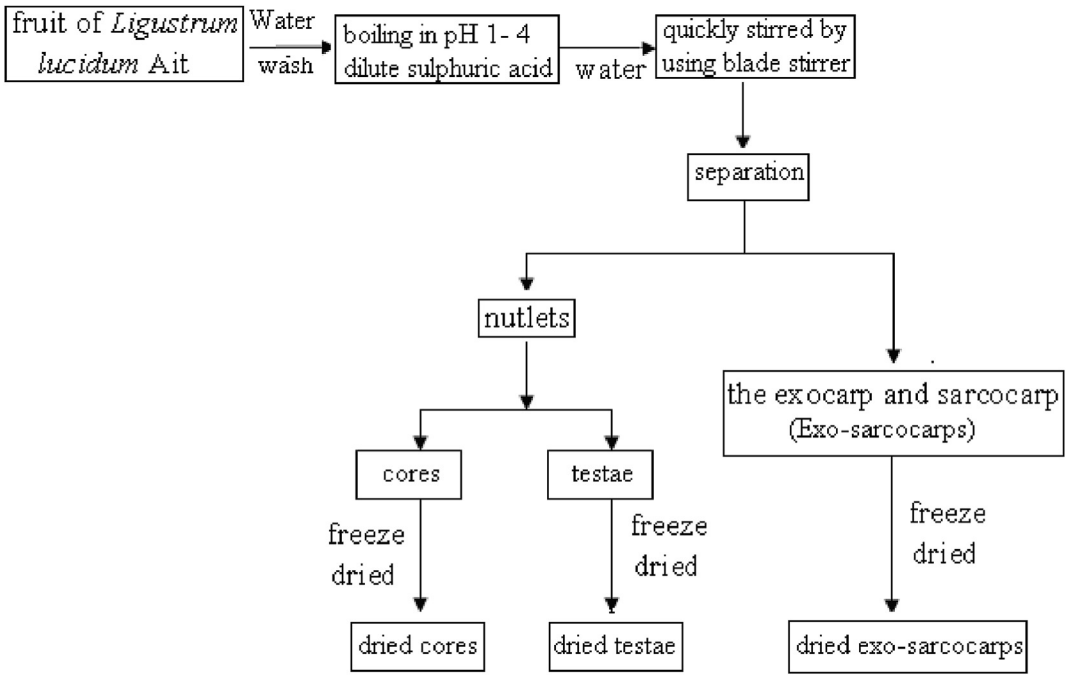

Figure 4. The separated methods for the exosarcocarp, testa and core parts of the fruit of *Ligustrum lucidum* Ait.

OA and 20–2000 μg/mL UA) were prepared respectively by gradient dilution of stock solution in methanol. Then taking the quantity of the compounds as the X-axis, the peak area as the Y-axis, the standard curves of OA and UA were drawn. Eevery 10 μL sample solutions were injected into HPLC and measure their corresponding peak area. To reliably measure the contents of OA and UA, the process repeated six times and the average of six peak area was took. The regression equations of the standard curves were represented by the linear equation $Y = a\ X + b$, where Y is the peak area, X is the quantity of the compounds, a is the slope, and b is the intercept of the regression line.

3.3.2. Ultrasound extraction methods for UA and OA from four samples

When the contents of UA and OA of four crude samples of fruit and fruit parts were extracted and determined by ultrasound extraction (Xia et al., 2012) and HPLC, each powered sample (1.0 g) was weighed and placed in an extraction glass cup, and then mixed with 30 mL 95% ethanol solvent. After soaking for 10 min to allow the solvent to wet the samples, a φ6 amplitude transformer was inserted into the extraction glass cup with the sample and extraction solvent, and the cup was placed in the ultrasonic device. The ultrasonic working conditions were as follows: the pre-set irradiation time was 3s, the interval time was 3s and the continuous working time was 20 min at room temperature. After extraction, the samples were centrifuged at 4000xg for 10 min, the supernatants were collected. The extraction was carried on 3 times. The collected supernatants were combined and concentrated into 50 mL. All sample solutions were ultrasonically degassed and filtered through a 0.45-μm nylon membrane. A 10 μL of sample solution were individually injected into the chromatographic system for the quantification analysis of the two compounds in the samples. All experiments were carried out in triplicate and the average values ±SD (standard deviation) were reported. Subsequently, the one of four crude samples of the highest contents of UA and OA was taken as the extraction samples.

3.4. Statistical analysis

All experiments were repeated three times and datas were expressed as mean values ± standard deviations (sd). Statistical analyzes were performed using Excel (Microsoft Office Excel, 2003). Data were statistically analysed using one-way ANOVA. The confidence limits used in this study were based on 95% ($P < 0.05$).

4. Conclusions

After the isolation of exo-sarcocarps from whole fruit, an ultrasound-assisted solvent extraction method was developed for the extraction of OA and UA from the exo-sarcocarps. The extraction yields of OA and UA from the exo-sarcocarps were higher than yields from whole fruits using two different extraction methods. The effects of several experimental parameters on the extraction yields of OA and UA by UA-SE were evaluated, and the extraction efficiency from exo-sarcocarps were more significant than their influence on extraction efficiency from whole fruits. Under ultrasonic irradiation conditions, the yields of OA and UA from exo-sarcocarps were 24.34 ± 2.09 mg/g, and 7.82 ± 0.09 mg/g, respectively. These yields were approximately four times higher than yields of OA and UA of whole fruits. The crude extracts obtained in this work could be used as components for the formulation of medications or could undergo further purification to generate purified OA and UA. The results reported in this work should facilitate development of less organic solvent, high efficiency and low-cost OA and UA extraction methods from fruits. The method has some shortcomings, for example, the separation method is limited to the fruit that can be separated, and the separation process requires a large amount of water, etc. These need to be further improved and optimized for subsequent industrial extraction.

Declarations

Author contribution statement

Xiao-Ning Dong: Conceived and designed the experiments; Wrote the paper.

Qiang Zhao: Performed the experiments; Analyzed and interpreted the data.

Xiao-Feng Wang: Performed the experiments; Analyzed and interpreted the data, Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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