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Application of cellulase treatment in ionic liquid based enzyme-assisted extraction in combine with in-situ hydrolysis process for obtaining genipin from *Eucommia ulmoides* Olive barks

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ABSTRACT

A new approach for ionic liquid based enzyme-assisted extraction coupled with in-situ hydrolysis (ILEIH) of geniposide from *Eucommia ulmoides* Olive barks is presented, in which enzymatic hydrolysis is used in an ionic liquid aqueous medium to prepare genipin. The method relied on the use of single cellulase to the extract and hydrolyze geniposide, which is performed continuously in the same system; genipin is easy in preparation with exempting the isolation and purification of geniposide. The mechanism of ILEIH procedure was discussed in detail to illustrate the advantage of ILEIH in the extraction process. 0.5 mol/L [C6mim]Cl aqueous solution was selected as extraction solvent. The optimum conditions of 140 min treatment time, 19.81 mL/g liquid–solid ratio, 5.15 mg/mL enzyme concentration and 5.0 pH value for the ILEIH process were obtained after investigating by single factor experiments and Box-Behnken design in terms of the genipin increment. And the first-order kinetic model based on β -glucosidase in the three reaction medium were established to study their impacts on the reaction rate. The proposed ILEIH method was validated by stability, repeatability, and recovery experiments and shows reliable data in the extraction and hydrolysis process. Therefore, this proposed approach is promising for the in-situ production of genipin and should be potentially applied to the obtaining of other active aglycons.

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1. Introduction

Genipin and its glycoside, geniposide, are the main active ingredients of the barks of *Eucommia ulmoides* Oliver. The plant has been used as a traditional medicine to treat a wide variety of ailments as well as produces several health benefits, including nourishing the kidney and liver, strengthening the bone and muscle and preventing miscarriages [1]. Geniposide is thought to achieve most of its biological activities through genipin [2]. Genipin has been reported to possess a broad range of pharmacological activities, including anti-inflammatory [3], hepatoprotective [4], choleric [5], neuroprotection [6], antidepressant [7] and anticancer [8].

In addition to exhibiting wide pharmacological actions, genipin has become a promising compound used naturally as a cross-linking reagent, allowing it to form networks with important biological polymers, e.g. gelatin and chitosan applied to tissue engineering and drug carriers for enhanced delivery. Genipin exhibits a

cross-linking property comparable to chemical cross-linking agents such as glutaraldehyde, but with low cytotoxicity and that forms stable and biocompatible crosslinked products [9–11]. Moreover, as a good colorant in the food industry, genipin has specific reaction with primary amines to form blue colored adducts, which enable it to produce safe edible and heat stable pigments [12]. Because of various applications, there is an enormous demand for genipin and therefore requires a more efficient, feasible and environmentally friendly preparation process. However, the content of genipin in *E. ulmoides* is rather low; while geniposide is present a higher content [13].

At present, the preparation method for genipin relying on several steps starting from the initial extraction of geniposide from *E. ulmoides* or *Gardenia jasminoides* Ellis with organic solvent. After a series of purification processes, the purified geniposide was subjected to the hydrolysis by β -glucosidase to genipin [14,15]. However, in these methods, pure geniposide or β -glucosidase need to be purified or purchased, which leads to a complex and expensive process. Some method improvement have been made by other researchers, e.g. using different organic solvents to extract [16,17], and choosing different hydrolytic enzymes, immobilized enzymes, and further with β -glucosidase-producing strains [18,19]. To the

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best of our knowledge, however, the previous methods were based on the incontinuity process where the extraction and purification of geniposide process separated apart from the hydrolysis of the glycoside to aglycon. Besides that, the fact that geniposide is freely soluble in aqueous solution due to the solubility, and undergoes hydrolysis reaction which efficiently takes place in aqueous solution whereas genipin formed is like to release into the organic solvent. So genipin and geniposide can be not obtained in single solvent medium. Some method modifications have been made by using two-phase extraction [20]. However, these extraction techniques are discommodious, low efficient, time-consuming, target ingredient loss and involved large volumes of toxic organic solvents.

Recently, increasing attentions have been paid on the development and use of environmentally friendly preparation methods. Ionic liquids (ILs), which are composed of bulky organic cations and inorganic or organic anions, are liquid near room temperature. Ionic liquids have attracted considerable interest from researchers working in a variety of fields. Because they have many remarkable properties including negligible vapor pressure, chemical and thermal stabilities, designable structures, ease of recycling and manipulation, miscibility with water and organic solvents, and their good solubility/extractability for a wide range of organic compounds, enable many chemical reactions can be completed in a homogeneous phase. There is an increasing awareness of the need for environmental protection across numerous industries, and ionic liquids are considered in this regard to be promising alternatives to traditional solvents for the extraction of various active ingredients from plant samples such as alkaloids [21], lignans [22] and polyphenolic compounds [23]. However, the use of ionic liquids in water can lead to a reduction in the permeability for the solid plant matrix. Several researchers have attempted to resolve this permeability problem by soaking the plant samples for extended time in ionic liquid aqueous solution. Although this strategy works in some cases, very little is known about the impact of ionic liquids on the stability of these natural compounds and further research is therefore needed to understand the potential limitations of this approach [21,22,24,25].

The enzyme-assisted extraction of natural ingredients from plants has been widely investigated in terms of the advantages that it offers over conventional procedures, such as operational simplicity, high efficiency and environmental friendliness. Most of the studies in this field have used cellulase, pectinase and β -glucosidase to hydrolyze and degrade the constituents of the plant cell walls and improve the release of their intracellular ingredients. The traditional enzyme-assisted extraction process is tedious, complex, and requires high-energy consumption, thus limiting its wide application [26]. The developed method [27] of the ionic liquid-based enzyme-assisted extraction utilize activity of the cellulase in ionic liquids solution, in which a continuous process including enzyme incubation and solvent extraction was completed, even improving the poor permeability of ionic liquid solutions, allowing for the release of larger quantities of the active ingredients from the plant cells. It is worth noting that cellulase complex is formed by three major enzyme components, the endoglucanase, exoglucanase and the β -glucosidase. Moreover, cellulase is less expensive than β -glucosidase.

Based on the above the situation, we recently developed the idea to utilize ionic liquid based enzyme-assisted extraction coupled with in-situ hydrolysis method (ILEIH) for the preparation genipin from *E. ulmoides*. It was envisaged that this strategy combine enzyme-assisted extraction and in-situ hydrolysis process in ionic liquid solution to develop a direct route for the production of genipin. The proposed method would not only improve the permeability of the solvent in the cellulase-assisted extraction process, but also allow for the hydrolysis of geniposide into genipin by cel-

lulase. Cellulase is easier available, lower cost than β -glucosidase and it catalyzes the removal of sugar moiety from geniposide with high efficiency. Therefore, single cellulase was feasible for use and adequate for dual roles in one-pot production. To the best of our knowledge, however, there has been no research reported in the literature on the cellulase hydrolysis of cell walls to improve penetration and simultaneous hydrolysis of geniposide to increase yields of genipin, and use of ionic-liquid aqueous solution as a green solvent to extract genipin and geniposide.

The work described in this study represents the first reported account of an ILEIH method. The overall objective of this study was to investigate the feasibility of employing ILEIH as an efficient technique to recover genipin from *E. ulmoides*. The ILEIH method was fully optimized and the mechanism of the enhanced extraction by ILEIH was investigated by observing the impact of the process on the plant cells by scanning electron microscopy.

2. Experimental

2.1. Reagents and samples

E. ulmoides barks were bought at the medicinal materials market of Qiqihar, China. All samples were dried, milled, passed through 60 mesh stainless steel sieve. Reference compounds of genipin and geniposide were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Methanol of chromatographic grade were purchased from J & K Chemical Ltd. (Beijing, China). All ionic liquids including 1-butyl-3-methylimidazolium hydrogensulfate ([C4mim]HSO₄), 1-butyl-3-methylimidazolium chloride ([C4mim]Cl), 1-butyl-3-methylimidazolium nitrate ([C4mim]NO₃), 1-butyl-3-methylimidazolium bromide ([C4mim]Br), 1-butyl-3-methylimidazolium tetrafluoroborate ([C4mim]BF₄), 1-ethyl-3-methylimidazolium chloride ([C2mim]Cl), 1-hexyl-3-methylimidazolium chloride ([C6mim]Cl), 1-octyl-3-methylimidazolium chloride ([C8mim]Cl) and 1-decyl-3-methylimidazolium chloride ([C10mim]Cl) were bought from Monis Chem. Eng. Sci. & Tech. Co., Ltd (Shanghai, China) and used without further purification. Cellulase (EC3.2.1.4, ≥ 100 U/mg), and β -glucosidase (EC 3.2.1.21, 30 U/mg) were obtained from Imperial Jade Bio-technology Co., Ltd (Yinchuan, Ningxia, China). All the other reagents obtained from Beijing Chemical Reagents Co. (Beijing, China) were of analytical grade. Deionized water was purified by a Milli-Q water purification system from Millipore (Bedford, MA, USA).

2.2. Screening IL solutions

In this initial step, the method of ultrasonic extraction was used to evaluate the performance of ILs on the solubility of genipin and geniposide. The conditions were tested as following: 1 g of dried plant material was mixed with 10 mL of different IL solutions, and then the mixture was sonicated at 298 K for 30 min. The concentration of ILs aqueous solution was 1 M. The ultrasonic power was maintained at 50 W. After each extraction, the extract was filtrated through a 0.45 μ m filter for the subsequent HPLC analysis.

2.3. Ionic liquid based cellulase-assisted extraction coupled with in-situ hydrolysis method (ILEIH)

Cellulase was added into ILs solution to obtain a extraction solvent at defined concentrations. IL solutions were prepared with deionized water then adjusted to defined pH-values in order to obtain the proper pH of extraction solvent. 1 g of *E. ulmoides* barks powder was added to the enzyme ILs solution in an Erlenmeyer flask. The mixture was incubated under 150 rpm stirring on a multichannel magnetic stirrer with temperature

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