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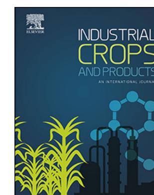
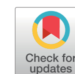


Enhanced hypericin extraction from *Hypericum perforatum* L. by coupling microwave with enzyme-assisted strategy



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ABSTRACT

Hypericin is considered to be the most biologically active substance in the crude extract of *Hypericum perforatum* L. (also known as St. John's wort) and has a wide range of pharmacological effects. In this study, a high resolution high performance liquid chromatography method for determining hypericin was established by comparing different chromatographic conditions. Xylanase-assisted extraction and microwave-assisted extraction can improve the extraction yield of hypericin significantly. And the coupling strategy between two methods resulted in a significant difference on the extraction yield. Microwave-assisted extraction after xylanase-assisted extraction was found to be the most efficient strategy for extracting hypericin. The yield was $0.319 \pm 0.006\ \mathrm{mg\ g^{-1}}$, which was a 209.7% increase over unassisted extraction. The combination of enzyme assisted extraction followed by microwave assisted extraction can be more commonly applied to improve extraction efficiency of bioactive compounds from plants.

1. Introduction

Hypericum perforatum L., also known as *Hypericum perforatum*, Shangtian Ladder and St. John's wort, is a perennial herb that belongs to genus *Hypericum* plant (Li and Wang, 2010). As a medicinal plant, it has been well studied, and the pharmacological activity that has been recorded provides evidence of antidepressant, antiviral and antibacterial effects (Barnes et al., 2001). *Hypericum perforatum* is widely used in pharmaceutical and food industries. It is rated by the European Commission as a natural source of food flavoring (Category 5) (Cai et al., 2015). The herb has already become one of the best-selling botanicals in North America and Europe.

Hypericum perforatum contains many biologically active substances such as phloroglucinol, flavonoids, and naphthoquinones, of which hypericin is the main compound (Abdelhadi et al., 2015). Hypericin is a natural, rigid, and polycyclic aromatic compound which is considered to be the most biologically active substance in the *Hypericum perforatum* extract (Huang et al., 2014). Previous research has indicated the usefulness of hypericin in a variety of medical applications such as burns, mild depression, and anxiety (Marrelli et al., 2014), and also showed for anti-depression, antibacterial, anti-viral, and anti-tumor activities (Crockett et al., 2008). Hypericin can also be used as a natural and effective photosensitizer for cancer treatment, because of its strong photosensitivity (Jendzelovsky et al., 2019).

The hypericin content in *Hypericum perforatum* is low and often accompanied with structurally similar substances. Ethanol extraction is the most common extraction method of hypericin as it has a polyphenolic hydroxyl structure and a relatively high polarity (Huang et al., 2014). The yield of hypericin was $35\ \mathrm{mg\ kg^{-1}}$ by using Soxhlet extraction with ethanol from the raw material *Hypericum perforatum* which was collected at the time of flowering (Cossuta et al., 2012). The ethanol extraction is time-consuming (16 h) and results in a low hypericin yield. In order to improve the efficiency of hypericin extraction several approaches have been used. The combination of integrated expanded bed adsorption chromatography and countercurrent chromatography was utilized for the simultaneous extraction and purification of hypericin, which resulted in 2.2 mg of hypericin (purity of 95.0%) from 40 mg of crude sample (Cai et al., 2015). Another reported approach made use of a molecularly imprinted polymer to extract hypericin from *Hypericum perforatum* with a recovery rate of 82.3% (Li et al., 2014). Although a large number of studies have been carried out on the original plant cultivation of the source of hypericin, the chemical composition and pharmacology of hypericin, and clinical research using this compound, not many studies to improve its extraction and

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Table 1
Different chromatographic conditions for determining hypericin.

Methods	Mobile phase	Column temperature (°C)	Detection wavelength (nm)
A	Ethylacetate, 15.6 g L^{-1} sodium dihydrogen phosphate (pH = 2 with phosphoric acid) and methanol, volume ratio of 39:41:160 [a]	40	590
B	Methanol, acetonitrile, water and 3% aqueous phosphoric acid, volume ratio of 45:50:4.5:0.5 with triethylamine adjusted to pH 6 [b]	25	590
C	Methanol, ethyl acetate and phosphate buffer (pH = 2, 0.1 mol L^{-1}), volume ratio of 60:20:20 [c]	25	590
D	Methanol, acetonitrile, 0.1 mol L^{-1} sodium dihydrogen phosphate solution, volume ratio of 200:300:100	40	284
E	Methanol, acetonitrile, 0.1 mol L^{-1} sodium dihydrogen phosphate solution, volume ratio of 200:300:100	40	590

[a] From Ozkan et al. (2018).
[b] From Li and Wang (2010).
[c] From Wang (2010).

preparation process have been undertaken.

Enzyme-assisted extraction is a gentle, efficient and eco-friendly method which is used to extract various compounds (Liu et al., 2014). Most bioactive components exist in side cells, and only a small extent in the intercellular space. Enzymes improve the extraction process by hydrolyzing plant cell walls and promoting cell disintegration (Maric et al., 2018). Compared with conventional extraction technology (solvent extraction or solid-liquid extraction), enzyme-assisted extraction can be accomplished using a low enzyme concentration, and generates less waste water and requires lower temperatures thereby reducing energy consumption (Rosello-Soto et al., 2016; Wikiera et al., 2016). Hence, this method is getting more attention given the need for eco-friendly extraction technologies (Puri et al., 2012). Sekhon et al. (2018) indicated that soybean [*Glycine max* (L.) Merr.] enzyme-assisted extraction of corn (*Zea mays* L.)-soybean *co*-products can effectively reduce cellulose and fat content and increase the protein content. Poojary et al. (2017) found that enzyme-assisted extraction of umami and total free amino acids in mushrooms was 20 times more efficient than conventional HCl extraction.

As an unconventional clean energy source, microwave has received increasing attention in the field of coupling extraction technology (Rokhati et al., 2018). Microwave energy is transmitted through the interaction of polar components, which is converted into heat by electromagnetic energy (Acierno et al., 2004). The unique heating method of microwaves can greatly reduce the reaction time and increase the yield (Kappe, 2004). Rokhati et al. (2018) reported a significantly reduced process time for cellulose hydrolysis by chitosan using microwave-assisted technology as compared with shaker incubator. Kumar et al. (2018) reported a reduced extraction time for papaya seed oil was extracted by microwave as compared with conventional mechanical extraction methods. Punegov et al. (2015) reported the microwave-assisted extraction of hypericin and pseudohypericin from *Hypericum perforatum*, and found that microwave activation can reduce the duration of the total extraction study of raw plant material ten times.

The objective of the present study was to improve the hypericin extraction yield from *Hypericum perforatum* by coupling microwave with enzyme-assisted strategy. The determining method of hypericin based on the high performance liquid chromatography (HPLC) was established. The different coupling strategies to combing microwave-assisted extraction and enzyme-assisted extraction were investigated, and the effects of the operating conditions on the extraction yield of hypericin were analyzed in detail.

2. Experimental

2.1. Materials

Chinese herbal medicine of *Hypericum perforatum* was obtained from Guangxi and placed into a crusher to through the 40 mesh sieve. The crude extract of *Hypericum perforatum* was supplied by Xi'an Kailai Biological Engineering Co., Ltd. and used without any purification. Hypericin standard with the purity > 98% was purchased from Shanghai Shidande Standard Technology Service Co., Ltd. Ultra-pure water was prepared by a CLW-K20 purification system (Chongqing, China). HPLC grade solvents (methanol and acetonitrile) and analytical grade reagents (sodium dihydrogen phosphate, phosphate, 95% ethanol and ethyl acetate) were purchased from Honeywell Trading (Shanghai) Co., Ltd. Xylanase (6000 U mg^{-1}), cellulase (50 U mg^{-1}), pectinase (500 U mg^{-1}), and complex protease (120 U mg^{-1}) were obtained from Shanghai Yuanye Biological Technology Co., Ltd.

2.2. Analysis of hypericin by HPLC

2.2.1. Preparation of the standard and sample solution

5.0 mg hypericin standard was weighed into a 50 mL brown volumetric flask, and dissolved in methanol to prepare a standard solution with a concentration of 100 mg L^{-1}.

The crude extract of *Hypericum perforatum* (0.15 g) was dissolved in methanol (about of 40 m L) using a brown volumetric flask (50 m L) with ultrasonic extraction for 30 min. Subsequently the mixture was placed at room temperature and methanol was added to the mark. The resulting sample solution was filtered through a 0.22 μm microporous membrane and placed in a brown sample vial.

The extract obtained by extracting under the adopted conditions in this study was freeze-dried to obtain hypericin-rich *Hypericum perforatum* extract. The extract solution for determining hypericin was prepared according to the method of preparing the sample solution.

2.2.2. Different chromatographic conditions for determining hypericin

Different chromatographic conditions were used for HPLC analysis of hypericin, as shown in Table 1. All measurements were performed by using a Kromasil C18 column (250 × 4.6 mm, particle size 5 μm), and 10 μ L was injected into a LC-2030C 3D HPLC system (Shimadzu, Kyoto, Japan). All mobile phase were filtered with a 0.22 μm membrane filter before using and the flow rate was 1.0 m L min^{-1}. Each standard or sample solution was detected by five methods and injected three times.

2.3. Enzyme-assisted extraction of hypericin

2.3.1. Comparison of different enzyme

Hypericin was extracted from *Hypericum perforatum* with the assistance of enzymes and analyzed quantitatively using the HPLC. Xylanase, pectinase, cellulase, complex protease (2.0% of the weight of the herb) and a combination of two enzymes were used to assist extraction of hypericin, respectively. Firstly, 40 m L of ultra-pure water was added into a beaker containing 5 g of *Hypericum perforatum*. The *Hypericum perforatum* suspension was soaked at 60 °C for 30 min, and subsequently the mixture was adjusted to pH 4.6 with 0.1 mol L^{-1} HCl. The enzyme was dissolved in moderate amount of ultra-pure water, and activated at 40 °C for 10 min. The activated solution was added to *Hypericum perforatum* soaking solution that has been adjusted to the

temperature and pH. Then mixture was incubated at 50 °C for 5 h. After that, mixture was incubated at 85 °C for 5 min to inactive the enzymes. The mixture was filtered under vacuum, and the filter residue was recovered and extracted with 30 mL of 95% ethanol under reflux at 85 °C for 2.5 h. The extract was filtered immediately, and the filtrate was collected to obtain the final extract. The extraction yield of hypericin ($mg\ g^{-1}$) was calculated per gram (dry weight) *Hypericum perforatum*

2.3.2. Influences of several factors on the extraction of hypericin with xylanase

Based on the results of section 2.3.1, xylanase was applied in the subsequent experiment. Aliquots containing 0.05 g, 0.075 g, 0.10 g, 0.15 g, and 0.20 g (1.0%, 1.5%, 2.0%, 3.0%, and 4.0% of the weight of the herb) of xylanase were accurately weighed and activated as described in section 2.3.1. The activation solution was added into the *Hypericum perforatum* soaking solution with pH 4.6. The enzymatic extraction was carried out in a water bath at 50 °C for 5 h. The effect of pH was studied with an enzyme concentration of 0.1 g (2.0%), an enzymatic hydrolysis temperature of 50 °C and an enzymatic hydrolysis time of 5 h, and the pH was controlled at 4.0, 4.4, 4.6, 4.8 and 5.0, respectively. The enzymatic hydrolysis time was changed from 3 to 9 h, in order to study the effect of extraction time. Finally, the enzymatic hydrolysis temperature from 35 to 55 °C was investigated to explore the effect of temperature on the enzyme-assisted extraction.

2.4. Microwave-assisted extraction of hypericin

Hypericum perforatum (5 g) was accurately weighed, and 40 m L of ultrapure water was added. Subsequently the mixture was soaked for 30 min at 60 °C. After that, the *Hypericum perforatum* soaking solution was placed into a microwave-assisted flask (100 m L) of the MAS-II microwave synthesis extraction system (Shanghai, China) for extraction at the temperature of 50 °C, the microwave time of 9 min, and the microwave power of 350 W. Finally, after microwave-assisted extraction, vacuum filtration was performed to recover the filter residue for reflux extraction to obtain a final extract. The selection of various parameters was based on the results of Section 2.5.3.

2.5. Microwave with enzyme-assisted extraction

Different coupling strategies of microwave and xylanase were used to assist the extraction of hypericin from *Hypericum perforatum*. The coupling strategies were as described below. The other steps were performed according to Section 2.3.2 using the following conditions for enzyme-assisted extraction, 0.1 g (2.0%) xylanase, pH 4.6, temperature of 45 °C, and 7 h hydrolysis time.

2.5.1. Microwave-assisted extraction before enzyme-assisted extraction

The *Hypericum perforatum* suspension incubated at 60 °C for 30 min was placed into a microwave equipped flask (100 m L) for extraction with the following conditions: 50 °C microwave temperature, 350 W microwave power, and 9 min microwave time. Then the mixture was transferred into a beaker and the activated enzyme solution was added to continue the enzyme-assisted extraction as described in Section 2.3.

2.5.2. Simultaneous microwave and enzyme-assisted extraction

The *Hypericum perforatum* suspension was added to the enzyme activation solution after 30 min incubation at 60 °C. The mixture was placed into a 100 mL flask for microwave extraction with the following conditions: 50 °C microwave temperature, 350 W microwave power, and 9 min microwave time. Subsequently the mixture was transferred to a water bath for continued enzymatic hydrolysis as described in Section 2.3.

2.5.3. Microwave-assisted extraction after enzyme-assisted extraction

After the enzyme-assisted extraction, the mixture of the enzymatic hydrolysis was transferred into a flask (100 m L) for the microwave extraction according to the follow conditions: 50 °C microwave temperature, 350 W microwave power, and 9 min microwave time.

The three main factors of microwave-assisted extraction temperature, time and power were investigated. Microwave time of 3, 6, 9, 12, 15, and 18 min were selected to investigate the effect of time on the extraction yield. Microwave-assisted extraction was observed at different power from 250 to 850 W. Furthermore, the effect of temperature was tested at the range of 30 to 80 °C.

3. Results and discussion

3.1. Determination of hypericin by HPLC

Although there are many HPLC methods for detecting hypericin, the reported detection conditions have significant differences resulting in different detection efficiencies (Li and Wang, 2010; Ozkan et al., 2018; Wang, 2010), because the mobile phase, pH and other detection conditions affect the separation between the components. The purchased crude extract of *Hypericum perforatum* and hypericin standard were applied to optimize the chromatographic conditions for the determination of hypericin, as shown in Fig. 1. For the different detection conditions, all experiments were performed three repeated independent experimental runs. The resolution of hypericin for the method A, B, C, D and E in the chromatogram were 8.043 ± 0.030, 10.241 ± 0.460, 9.841 ± 0.043, 2.008 ± 0.004 and 13.926 ± 0.035, respectively. The resolution of the method E between the target (hypericin) and the adjacent peak was higher than that of the other methods. The ratio of the organic phase in the mobile phase affects the chromatographic peak resolution and retention time. When 284 nm was used as the detection wavelength in the method D, the peak area response of the hypericin peak was too small comparing with the peak area response of the other compounds. And a few compounds can be detected at 284 nm (Liu et al., 2000).

In summary, the HPLC method E was established to measure hypericin. The retention time of hypericin was 18.646 min. The calibration curve for hypericin showed a good linearity in the range of 4-14 $mg\ L^{-1}$ hypericin. And the calibration curve of hypericin was $y = 3548.9\ x - 17878$, $r^2 = 0.9986$, where y is the peak area and x is the concentration of hypericin ($mg\ L^{-1}$).

3.2. The effect of enzyme-assisted extraction on the extraction yield

3.2.1. The effect of enzyme type on the extraction yield

The effect of enzyme-assisted extraction on the extraction yield depends on the enzyme type, reaction time and enzyme concentration (or enzyme amount), temperature and pH of the plant material, and other physical parameters that affect enzyme activity (Poojary et al., 2017). The extraction results using different enzymes, with the extraction yield of hypericin as the evaluation index, are shown in Fig. 2. The extraction yield of hypericin was 0.103 ± 0.004 $mg\ g^{-1}$ when the extraction was carried out without any enzyme. That is almost triple the yield comparing with the reference (Cossuta et al., 2012). Enzymatic extraction using xylanase, complex protease and a combination of pectinase and cellulase resulted in a higher extraction efficiency. Fig. 2 shows that xylanase was the best choice for the extraction of hypericin with an extraction yield of hypericin of 0.165 ± 0.005 $mg\ g^{-1}$. The plant cell wall is mainly composed of cellulose, hemicellulose and lignin (Chow and Ting, 2019). Hemicellulose is a heteropolymer of which the main component is xylan, a complex polysaccharide with a precise composition that depends on the plant species and the tissue source (Naidu et al., 2018). Xylanase is a combinatorial enzyme, and synergic effect between enzymes molecules enhances the sensitivity of heteropolyxylan to the endoxylanase. Xylan can be completely hydrolyzed by a endo-1,4-β-xylanase, which cleaves the xylan backbone into smaller xylooligosaccharides, causing cell wall rupture (Bajpai, 2009).

Fig. 1. Effect of different chromatographic conditions on the determining hypericin. (a) The chromatogram of the crude extract for method A. (b) The chromatogram of the crude extract for method B. (c) The chromatogram of the crude extract for method C. (d) The chromatogram of the crude extract for method D. (e) The chromatogram of the crude extract for method E. (f) The chromatogram of hypericin standard for method E.

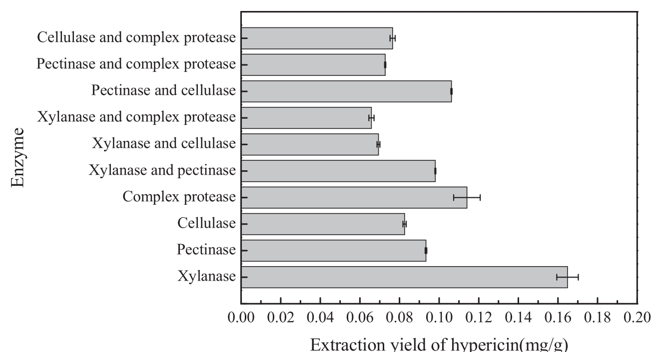

Fig. 2. Effect of different enzymes on the extraction yield of hypericin. Conditions: enzyme concentration of 0.1 g (2.0%), time of 5 h, pH 4.6 and temperature of 50 °C.

Therefore, the rupture of the plant cell wall is accelerated by xylanase. The mentioned combinations of the two enzymes are not as effective as the xylanase alone, probably because the two enzymes compete for contact with the substrate surface and thus inhibit each other. Xylanase was selected as the optimal enzyme for the enzyme-assisted extraction of hypericin.

3.2.2. The effect of enzyme concentration and pH on the extraction yield

The effect of different concentrations of enzyme and pH on hypericin extraction are given in Fig. 3. With the increasing xylanase concentration, the extraction yield of hypericin reached $0.164 \pm 0.003\ \mathrm{mg\ g^{-1}}$ at 2.0% enzyme load (Fig. 3a). The extraction yield of hypericin decreased with increasing enzyme concentration above 2.0%. Higher enzyme loading may have resulted in faster total hydrolysis and end product inhibition of xylanase (Li et al., 2012). The excess enzyme could no longer effectively bind to the substance when the enzyme concentration is too high, and may block the binding of part of the enzyme, resulting in waste of the enzyme (Fu et al., 2018).

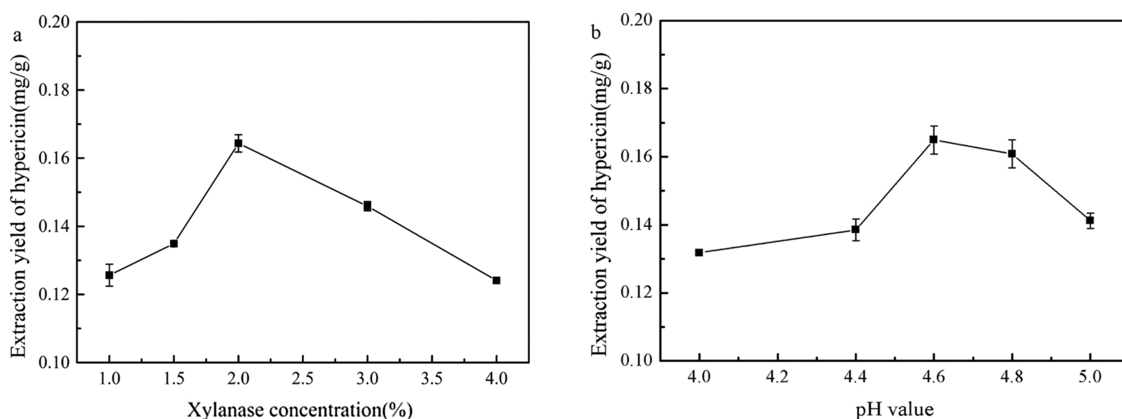

Fig. 3. Effect of xylanase concentration (a) and pH (b) on the extraction yield of hypericin. (a) Conditions: time of 5 h, pH 4.6 and temperature of 50 °C. (b) Conditions: xylanase concentration of 0.1 g (2.0%), time of 5 h and temperature of 50 °C.

Xylanase has been reported to be stable in the pH range of 4.0-8.0 (Silva et al., 2015), and pH 4.0-5.0 was investigated in this study. Fig. 3b shows that the extraction yield of hypericin was pH dependent, and reached a maximum value at pH 4.6. pH does not only affect the enzyme activity, but also affects the solubility of hypericin. pH changes can stop enzyme activity by denaturing (altering) the three-dimensional shape of the enzyme by breaking ionic and hydrogen bonds (Li et al., 2012). Hypericin generally forms hypericin aggregates in water and biological media. However, the solubility of hypericin can be improved by deprotonation of phenolic groups located in the bay and surrounding locations (de Andrade et al., 2017). And the change of pH value affects the deprotonation of hypericin (Keša and Antalík, 2017).

3.2.3. The effect of enzymatic hydrolysis time and temperature on the extraction yield

Fig. 4a shows that an increasing extraction yield of hypericin was observed during the initial hydrolysis stage (5 h). The lowest extraction yield of hypericin was observed at 6 h, possibly due to the oxidization of hypericin (Wang et al., 2014). It was likely that the oxidization rate of hypericin was already higher than the release rate of hypericin with xylanase at 6 h. After 6 h, the oxidization rate of hypericin remained steady. However, the release rate of hypericin increased with increasing time. The xylanase-assisted extraction reached a maximum value of 0.220 ± 0.005 mg g^{-1} at 7 h. The rupture of the cell wall of *Hypericum perforatum* reached its maximum level due to product inhibition. After that, decreasing yield in the following 1 h was observed. The yield of hypericin seemed to be constant after 8 h of hydrolysis. This demonstrates that an incubation period of 7 h was ideal for the xylanase catalyzed hydrolysis of *Hypericum perforatum* cell walls.

Different enzymes have their own optimum temperature, and the enzyme activity is the highest in the optimal temperature range. As shown in Fig. 4b, the optimum temperature of xylanase-assisted extraction was 45 °C. This is similar to the result of Abd El Aty et al. (2018) who showed that xylanase has a temperature optimum of 50 °C. The number of activated molecules and the rate of the enzyme reaction increase with the increasing temperature. At temperatures above this optimum temperature the enzyme is gradually inactivated resulting in a lower reaction rate. The utilization of xylanase can significantly improve the extraction yield of hypericin (0.236 ± 0.003 mg g^{-1}) which is 129.1% higher than the extraction without enzyme. The conditions were enzyme concentration of 2.0%, pH 4.6 and enzymatic hydrolysis at 45 °C for 7 h.

3.3. The effect of microwave-assisted extraction

Microwave-assisted extraction without any enzyme resulted in a hypericin extraction yield of 0.276 ± 0.002 mg g^{-1}, which was higher than the yield with xylanase-assisted extraction (0.236 ± 0.003 mg g^{-1}). The time required for microwave-assisted extraction was shorter than the enzyme-assisted extraction. Microwave heating can quickly increase the internal temperature of the object, accelerate the cell wall disruption and dissolution, and therefore achieve enhanced extraction efficiency. Microwave-assisted extraction was found to significantly improve the extraction yield of hypericin as compared with the unassisted extraction.

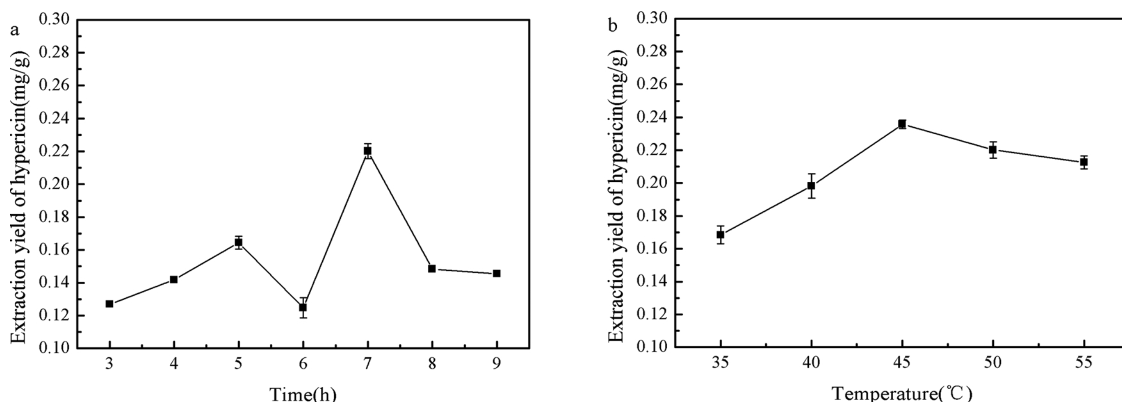

Fig. 4. Effect of enzymatic time (a) and temperature (b) on the extraction yield of hypericin. (a) Conditions: xylanase concentration of 0.1 g (2.0%), pH 4.6 and temperature of 50 °C. (b) Conditions: xylanase concentration of 0.1 g (2.0%), time of 7 h and pH 4.6.

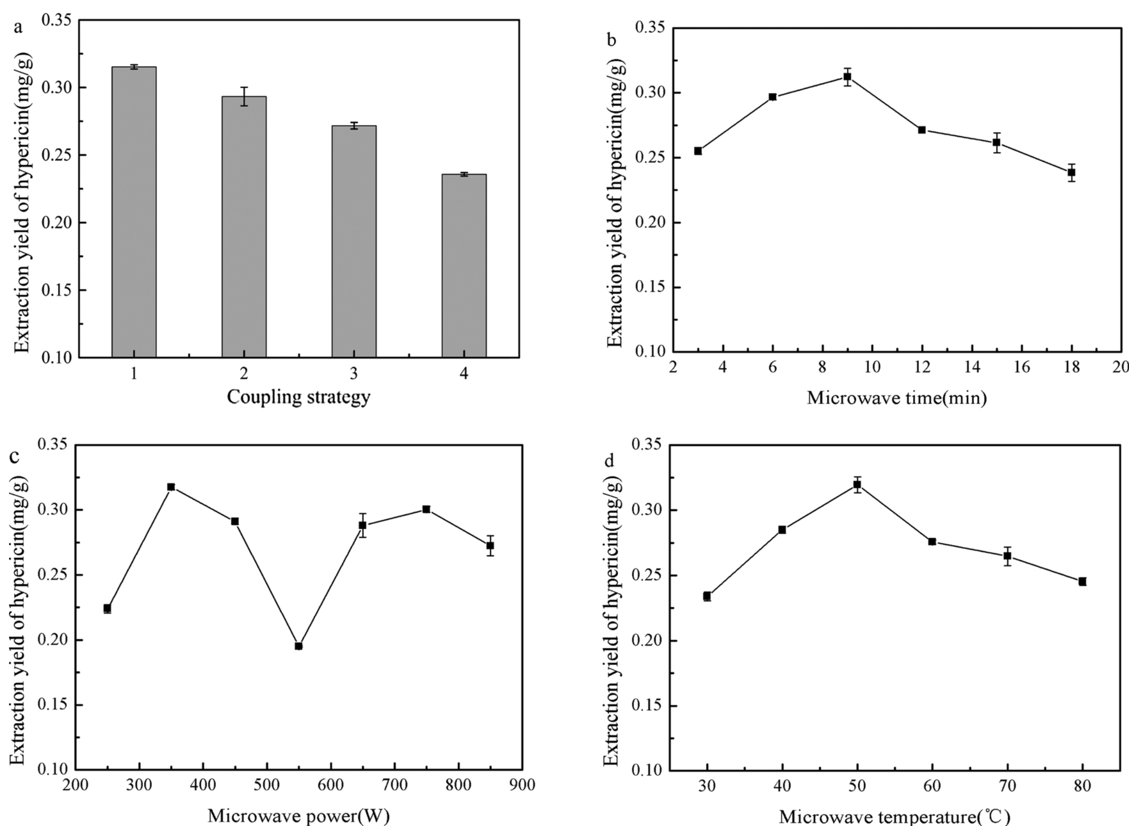

Fig. 5. Effect of microwave with xylanase-assisted strategy on the extraction yield of hypericin. (a) Different coupling strategies. 1, microwave-assisted extraction after xylanase-assisted extraction. 2, microwave-assisted extraction before xylanase-assisted extraction. 3, simultaneous microwave and xylanase-assisted extraction. 4, xylanase-assisted extraction. Conditions for xylanase-assisted extraction: xylanase concentration of 0.1 g (2.0%), pH 4.6, temperature of 45 °C, time of 7 h. Conditions for microwave-assisted extraction: 350 W microwave power, 50 °C microwave temperature, 9 min microwave time. (b) Microwave time. Conditions: 350 W microwave power, 50 °C microwave temperature. (c) Microwave power. Conditions: 50 °C microwave temperature, 9 min microwave time. (d) Microwave temperature. Conditions: 350 W microwave power, 9 min microwave time.

3.4. Microwave and enzyme coupling extraction of hypericin

3.4.1. Determination of the coupling strategy

Based on the above results, microwave-assisted extraction and xylanase-assisted extraction each enhance the extraction yield of hypericin significantly. Different coupling strategies between both methods were investigated to achieve an even higher extraction yield of hypericin. As shown in Fig. 5a, microwave-assisted extraction after xylanase-assisted extraction was the best strategy, microwave-assisted extraction before xylanase-assisted extraction was second, and microwave and simultaneous xylanase-assisted extraction was the worst. Using the best strategy, microwave-assisted extraction after xylanase-assisted extraction, the extraction yield of hypericin ($0.315 \pm 0.002\,mg\,g^{-1}$) increased by 33.5% compared with only xylanase-assisted extraction and 11.4% compared with only microwave-assisted extraction. Xylanase was used to hydrolyze matrix of the cell wall of *Hypericum perforatum*, thereby increasing cell permeability (Maric et al., 2018). Subsequently microwave irradiation caused ionic conduction and dipoles rotation of water molecules and polar cellular components (Wang et al., 2016). These two combined mechanisms create synergy and promotes the release of energy resulting in prompt heating. The internal heating promotes both rapid material damage and the increased diffusion of hypericin to the cooler solvent (Maric et al., 2018). The combination of xylanase and microwave irradiation provided a fast and efficient hypericin extraction. However, the use of enzyme and microwave irradiation at the same time may disrupt the enzyme structure (thermal unfolding) and reduce the enzyme activity, thereby reducing the overall extraction yield.

3.4.2. The effect of microwave conditions on the microwave-assisted extraction after xylanase-assisted extraction

The method of microwave-assisted extraction after xylanase-assisted extraction was applied to extract hypericin from *Hypericum perforatum*. Microwave power, microwave time and microwave temperature all affect the extraction yield of hypericin. The time required for microwave-assisted extraction is usually shorter. Even at low power and low temperatures, excessive exposure to microwave radiation can result in a change in the chemical structure of the active compound and a lower extraction yield, so the microwave time is usually kept between a few and circa 30 min (Kappe, 2004). As shown in Fig. 5b, the extraction yield of hypericin gradually increased by applying microwave times from 3 min to 9 min, and reached the highest level of $0.312 \pm 0.007\,mg\,g^{-1}$ at 9 min. Shorter and longer microwave times resulted in a lower extraction yield. This phenomenon may be attributed to the fact that when the microwave time is too short, the microwave energy is not enough to change the cell structure, and the hypericin cannot be effectively released. However, the active ingredient may be destroyed at longer microwave times resulting in the release of excessive impurities (Fu et al., 2018). The extraction yield was greatly improved and the extraction time required was significantly shortened compared with the conventional solvent extraction.

The effect of microwave power on the extraction yield of hypericin is shown in Fig. 5c. With the increase in microwave power from 250 to 350 W, the yield was increased. At higher microwave power (350–550 W) the yield decreased. With the gradual increase in power, *Hypericum perforatum* cell membrane is ruptured by strong internal pressure and intermolecular frictional force resulting in hypericin release. However, at higher powers the pH may drop due to the

volatilization of water (Fu et al., 2018). The instability of hypericin is more severe in the lower pH range as in the higher pH range (Ang et al., 2004). The degradation rate of hypericin would then exceed the release rate of hypericin. This resulted in a lower yield of hypericin which reached a minimum at 550 W. Surprisingly, the yield increased by applying microwave powers from 550 to 750 W. The higher release rate of hypericin was obtained with increasing power. At microwave powers above 750 W hypericin may undergo thermal oxidization (Chan et al., 2011; Shang et al., 2016; Wang et al., 2014). Therefore, taking the energy consumption and production efficiency into account, 350 W was chosen as the microwave power.

The effect of microwave temperature on the extraction yield of hypericin is shown in Fig. 5d. The extraction yield of hypericin reached the highest level of $0.319 \pm 0.006\,mg\,g^{-1}$ at 50 °C, which is an improvement of 209.7% over the conventional solvent extraction. Hypericin is a heat sensitive substance and can be easily oxidized or decomposed. A lower temperature will help to avoid oxidative deterioration of hypericin. As studied by Wang et al. (2014) hypericin is unstable under high temperature conditions. And the content of hypericin that could be extracted using the novel strategy was found to be 0.84% of the *Hypericum perforatum* crude extract.

4. Conclusions

A HPLC method with a high resolution of 13.926 ± 0.035 for the determination of hypericin was established. Xylanase-assisted extraction, microwave-assisted extraction and the combination of two methods were found to significantly enhance the extraction yield of hypericin. Microwave-assisted extraction after xylanase-assisted extraction was successfully applied to extract hypericin from *Hypericum perforatum*, and the factors of extraction process were investigated. The extraction yield of hypericin was $0.319 \pm 0.006\,mg\,g^{-1}$, which was an increase of 209.7% compared with the conventional solvent extraction. Moreover, the combination of the two extraction methods was superior over each method individually. The combination of the two extraction methods in the right order is a feasible strategy for the extraction of natural products from plant materials. Hypericin, as a natural bioactive substance with a wide range of pharmacological effects, should be further studied to find more efficient extraction and purification strategies.

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