Tea saponins, extracted from a *Camellia oleifera* cake, were found to have a potent effect on de-alcoholic activity. To obtain highly pure tea saponins, which can better maintain the activity of alcohol dehydrogenase (ADH), this paper presents an extraction method for tea saponins using deionized water as the extraction agent and a two-stage precipitation method, including ethanol precipitation and CaO precipitation. The optimum conditions for ethanol precipitation were 95% alcohol, a duration of 1.5h and a solid/liquid ratio of 1:4; while the optimum conditions for CaO precipitation were a duration of 2h and an NH₄HCO₃/CaO ratio of 2:1. Under the optimum conditions, the content of saponins was 87.58%. The results showed that the greater the amount of tea saponins and the higher its purity, the more significant its activating effect on ADH. When the purity of tea saponins was above 75%, it activated ADH. It indicated that the de-alcoholic mechanism of tea saponins is associated with the activity of ADH. Furthermore, the study characterized the structure of tea saponins by UV absorption and Fourier Transform Infrared (FTIR) spectrometry and LC-MS.

Keywords: tea saponins; two-stage purification; ADH

1 Introduction

*Camellia oleifera* is known as a high-grade oil plant that is widely grown in China. After oil-pressing, much seed cake is left behind, containing approximately 10–18% saponins [1]. Saponins are a group of compounds which have a number of pharmacological activities including anti-inflammatory, antioxidant, anticancer, insecticidal, anthelmintic and antimicrobial [2]. Saponins from *Polygonum hydropiper* L. show high anti-angiogenic, anti-tumor, brine shrimp, and fibroblast NIH/3T3 cell line cytotoxicity [3, 4]. Saponins were observed to be the most effective showing 93.3% tumor inhibition at 1000 µg/ml with IC₅₀ values of 18.1 µg/ml by potato tumor assay. Similarly, saponins also excelled in anti-angiogenic evaluation, exhibiting 78.9% (IC₅₀ = 64.9 µg/ml) at 1000 µg/ml respectively. These studies show that crude saponins exhibited notable anti-tumor and anti-angiogenic activities. Tea saponins also have surface activities, such as emulsification, dispersion and foaming [5]. Thus, it is necessary to establish a high purity extraction and purification method for tea saponins.

There are many studies on the extraction and purification methods of tea saponins, including n-butanol extraction, recrystallization, and the use of macroporous resin [6]. However, these methods have deficiencies, such as the colour of tea saponins obtained by n-butanol extraction being too deep and the extraction resulting in more impurities. Precipitation methods have the advantages of a simple operation and are suitable for industrial production. They are widely used in the extraction and purification of polysaccharides and proteins [7]. Additionally, different types of tea saponins have shown different activities [8, 9]. Acetone precipitation and two-stage purification were compared. According to this comparison, water extraction was used as the first step. Then, a two-stage purification of tea saponins was devised and optimised. The two-stage purification includes ethanol precipitation and CaO precipitation [10]. The purpose of ethanol precipitation is to achieve an initial purification and separate some tea polysaccharides, and the purpose of the second step is to complex CaO with tea saponins and then release tea saponins using NH₄HCO₃. The activity of tea saponins purified in this way and its effect on ADH activity have not been reported. The tea saponins obtained by this purification method have an effect on ADH activity.
Chinese wine culture is a traditional culture; however excessive alcohol intake can lead to alcohol poisoning, causing symptoms such as nausea, vomiting and alcoholic liver disease [11]. Hence it is essential to study the de-alcoholic activity of tea saponins and find an effective way to maintain its activity. The saponins fraction from the seeds of the tea plant was found to show a potent protective effect against gastric mucosal lesions induced by ethanol in rats [12, 13]. However, there are no reports about the de-alcoholic mechanism of tea saponins. In vivo ethanol is first oxidized to acetaldehyde, and 80% of the ethanol is converted into acetaldehyde by ADH in the process of hepatic metabolism. Referring to the method used by Valle & Hoch in the in vitro experiments, this paper compared acetone precipitation [6] and the two-stage purification to obtain tea saponins and the effect of tea saponins on ADH activity was investigated, showing that tea saponins obtained by the two-stage purification method can dispel the effects of alcohol to some extent, providing guidance for the further study of the de-alcoholic activity mechanism of tea saponins and product development. Hereby, it is hypothesized that tea saponins reduce the absorption metabolism of alcohol by influencing ADH activity. According to some reports, tea saponin carbohydrates could inhibit the activity of ADH, and different purities of tea saponins have different effects on ADH. An additional purpose of this study was to identify the de-alcoholic mechanism of tea saponins and develop a new de-alcoholic product based on tea saponins.

2 Materials and methods

2.1 Materials

*Camellia oleifera* cake was provided by Anhui Shanmei biotechnology Co., Ltd (Anhui, China), which was crushed to 40 mesh before leaching. Tea saponins (96% purity) were purchased from Shanghai Fortune Biotechnology Co., Ltd. (Shanghai, China).

2.2 Chemicals

Ethanol (AR) and Vanillin (AR) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methanol and acetonitrile (both HPLC-grade) were obtained from Merck Chemicals (Shanghai, China). Concentrated sulfuric acid, calcium oxide, ammonium bicarbonate and et.al. were obtained from Sinopharm and were of analytical grade unless otherwise stated.

2.3 Tea saponins extraction and purification procedure

This is a flow chart of tea saponins extraction and purification procedure. First, water extraction was used to leach tea saponins from seed cakes. To remove the impurities, a certain amount of ethanol was added to the...
initially purified liquid. Here, the purified extracted liquid was obtained after centrifugation. A certain amount of precipitation agent (CaO) was added to the purified extracted liquid to obtain the saponins precipitate after centrifugation. Then, \( \text{NH}_4\text{HCO}_3 \), the tea saponins release agent, was added to the saponins precipitate. To determine the appropriate conditions under which saponins was sufficiently prepared to exhibit a positive effect on ADH and have good yield and purification, single factor experiments and orthogonal experiments were studied.

2.4 Effect of tea saponins on ADH activity

2.4.1 Comparison of acetone precipitation and the two-stage purification

Two different saponins samples were separately obtained by acetone precipitation and the two-stage purification. The conditions of acetone precipitation were as follows: 80% ethanol for dissolving, a ratio of solid to liquid of 1:20, acetone used as the precipitation solvent, and a volume ratio of ethanol to acetone of 1:2.

2.4.2 Effect of purity and amount of tea saponins on ADH activity

In vitro experiments using an improved Valle & Hoch method were applied to determine the activity of ADH.

Sodium pyrophosphate buffer (1.5 mL), 27 mmol/L oxidized coenzyme I (NAD\(^+\)) (1.0 mL), 11.5% ethanol solution (v/v) (0.5 mL), and 1 mg/mL tea saponins (0.1 mL) solution were added to a test tube. Then, the tube was placed in a 25°C water bath and incubated for 5 min. Then, 0.1 mL of ADH (0.25 U/mL) were added. The absorbance was measured every minute at 340 nm.

\[
R = \frac{(E_1 - E_0)}{E_0} \times 100\%
\]

where
- \( R \) represents the activation rate of ADH (%)
- \( E_1 \) represents the enzyme activity in the tea saponins solution (U/mg)
- \( E_0 \) represents the enzyme activity in a blank solution (U/mg)

Determination of tea saponins content by vanillin sulfuric acid colorimetric method described by He et al. [14]

2.4.3 Standard curve

In total, 0.100 g of 96% tea saponins powder of high purity was accurately weighed and dissolved in 100 mL of 80% ethanol. Six 10 mL volumetric flasks were numbered 1 to 6. To flasks No. 2–6 were separately added 0.1, 0.2, 0.3, 0.4, 0.5 mL of the pure tea saponins solution. Then, to flasks No. 1-5 were separately added 0.5, 0.4, 0.3, 0.2, 0.1 mL of distilled water. 0.5 mL vanillin and 5 mL 77% sulfuric acid solution were sequentially added to each flask. All the flasks were maintained in a 60°C constant temperature water bath for 20 min, then immediately placed into an ice-water bath and incubated for 10 min, and finally placed at room temperature for 10 min. Tea saponins absorbance was determined at 550 nm, which is the maximum absorption wavelength. A standard curve was obtained with tea saponins concentration (g/L) as the abscissa and absorbance (A) as the vertical axis.

Standard curve equation for tea saponins:

\[
A = 9.85x + 0.0716
\]

Combined with the standard curve, tea saponins content is calculated using the following equation:

\[
\text{Content of tea saponins} = \frac{(A - 0.0716) \times V}{9.85 \times m} \times 100\%
\]

where
- \( A \) represents absorbance
- \( V \) represents the volume of the solution (mL)
- \( m \) represents the quality of the sample (mg)

Saponins release rate % = \( \frac{m_1}{m_2} \times 100\%
\]

where
- \( m_1 \) represents the quality of the tea saponins released by \( \text{NH}_4\text{HCO}_3 \)
- \( m_2 \) represents the quality of the CaO complex with tea saponins

2.5 FTIR Analysis

Fourier Transform Infrared (FT-IR) spectra were recorded using a Nicolet 5700 Infrared spectrometer (Thermo Electron Corporation, New York, New York State, United States). KBr pellets of solid samples were prepared from mixtures of KBr and the samples. Infrared scanning was performed between 400 cm\(^{-1}\) and 4000 cm\(^{-1}\) at room temperature.
2.6 HPLC Analysis

HPLC analysis was performed with an Agilent (USA) 1200 Series system equipped with a binary pump (G1312A), a thermostatic column compartment (G1316A), and a diode-array detector (DAD; G4212B). Chromatographic (HPLC) separation was carried out with a Zorbax Eclipse Plus C18 column (250 mm × 4.6 mm, 5 µm, Agilent) and an acetonitrile–water mobile phase as the eluent (Gradient elution conditions are as follows: acetonitrile: H2O=20:80 to 100:0 over 20 min and then back to 20:80 over 5 min) [5, 15] at a flow rate of 1.0 mL·min⁻¹ and 30°C. Detection and quantification were performed at 215 nm.

2.7 MS Analysis

MS analysis was performed with ACQUITY LC LCT Premier XE (Waters, United States). Detection was performed in the full scan mode from m/z 500 to 5000. The capillary voltage was set at 2.5 kV. The ion source temperature and desolvation temperature were optimized at 120°C and 450°C, respectively. The flow rates of desolvation gas and gone gas were set at 600 L/h and 50 L/h, respectively. Argon was the collision gas.

3 Results and Discussion

3.1 Optimization of ethanol precipitation conditions for tea saponins

Using single factor experiments (Tab. 1) and orthogonal experiments (Tab. 2), the most appropriate ethanol precipitation conditions were determined, which were 95% ethanol, a precipitation reaction time of 1.5 h, and a concentrated liquid to ethanol ratio of 1:4. Under these conditions, the purity of tea saponins was 58.98%, and crude tea polysaccharides were obtained in this step.

3.2 Analysis of Single Factor Experiments for CaO precipitation

The effects of CaO precipitation time on the degree of yield of tea saponins are shown in Fig. 1. Too long or too short precipitation times would be unsuitable; thus, the experiments were started from 0.5 h and ended at 3 h. A suitable CaO precipitation time of 2 h was found, as indicated in Fig. 1. The effect of the transition time on the degree of yield of tea saponins is shown in Fig. 2. The transition process was a way to release the saponins from...
the complex with CaO. A suitable transition time of 1 h is shown in Fig. 2. The effect of the amount of NH$_4$HCO$_3$ on the degree of yield of tea saponins is shown in Fig. 3. A suitable amount of NH$_4$HCO$_3$ was found in Fig. 3 as 2 times the amount of CaO. The saponins release rate was nearly 75%. In summary, the optimum conditions were 2 h for the CaO precipitation time, a 1 h transition time and a 2:1 ratio of NH$_4$HCO$_3$/CaO. Under the optimum conditions, the content of saponins in the product was 87.58%.

3.3 Effect of different purities/amount of tea saponins on the activation rate of ADH

The effect of tea saponins purity on the degree of ADH activity is shown in Fig. 4. When the purity of tea saponins was above 75%, ADH was activated. However, when the purity of tea saponins was below 65%, ADH was inhibited. The reason is probably the impurities influencing ADH when the purity of tea saponins is below 65%. Swati B. Jadhav et al. found that “All the carbohydrates inhibited the enzyme”. The oil-tea cake contains many carbohydrates; thus, when the purity of tea saponins is below 65%, the carbohydrate impurities possibly inhibit the activity of ADH [16]. The effect of the tea saponins amount on the degree of ADH activity is shown in Fig. 5. As the amount of tea saponins increased, tea saponins activation of ADH was more significant. Hence, tea saponins content and the ADH activation rate are positively correlated in a certain range.

3.4 Comparison of acetone precipitation and two-stage purification

The results showed that tea saponins obtained by acetone precipitation had no effect on the activity of ADH. However, the tea saponins obtained by the two-stage purification had an effect on the activity of ADH. Therefore, the two-stage purification was chosen.
3.5 UV analysis

As shown in Fig. 6, tea saponins had a maximum absorption peak at 215 nm, which was similar to other reports [15]. This wavelength can be used for the structure identification of tea saponins.

3.6 FTIR analysis

The FTIR spectra of tea saponins was similar to those of saponins that other researchers have studied. A hydroxyl stretching vibration was noted at approximately 3500 cm\(^{-1}\). At 2942 cm\(^{-1}\), a methyl, methylene stretching vibration was observed. No absorption at approximately 2500 cm\(^{-1}\) ~ 1900 cm\(^{-1}\) indicates that there was no accumulation of double bonds and triple bonds. At 1717 cm\(^{-1}\) ~ 1616 cm\(^{-1}\), a carbonyl group stretching vibration peak was observed. At 1419 cm\(^{-1}\) and 1375 cm\(^{-1}\), the antisymmetric deformation vibration peak \(-\text{CH}^{-}\) and the symmetric deformation vibration peak were observed, respectively. At 1261 cm\(^{-1}\), a bending vibration peak within the C-O-H plane was noted. At 1076 cm\(^{-1}\), the spectrum has a C-O-C stretching vibration peak (Fig. 7).

3.7 LC-MS analysis

The image in Fig. 8 was obtained with 1 mg·mL\(^{-1}\) tea saponins. It shows the peak time of tea saponins as 8 min. Compared with other conditions, the chromatogram peak time is shorter, and the stability is better under the gradient elution conditions. In the negative-ion TOF-MS for tea saponins, quasi-molecular ion peaks were observed at m/z 1189[M-H]\(^{-}\), 1219[M-H]\(^{-}\) and 1231[M-H]\(^{-}\) (Fig. 9). Thus, the molecular formula for tea saponins may be either \(C_{57}H_{90}O_{26}\), \(C_{58}H_{92}O_{27}\) [17] or \(C_{59}H_{92}O_{27}\) [18]. There may be different molecular ion peaks for the tea saponins monomer because the tea saponins replacement group species is numerous; thus, purified tea saponins has several specific monomer structures. The relative molecular mass of the tea saponins we obtained was
between 1100 and 1300, which is basically identical with the theory inferential value and the literature reported values [15].

4 Conclusion

Tea saponins were purified by two-stage purification, and the content of saponins in the product was 87.58%. Compared to other ways of purifying tea saponins, the precipitation method has the advantages of a simple operation and is suitable for industrial production, and it shows positive effects on the activity of ADH while other processes, such as acetone precipitation, have no ADH effect. Moreover, the de-alcoholic mechanism of tea saponins is associated with the activity of ADH. In this study, the greater the amount of tea saponins, the more significant its activating effect on ADH. Additionally, the higher the purity of tea saponins, the more significant the activating effect on ADH and the ADH activity reached greater than 50% when the tea saponins purity reached 75%. Tea saponins obtained by the two-stage purification method has a high therapy effect.

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