Evaluation of the potential anti-alcoholism activity of chitosan grafted with gallic acid

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Abstract. In order to broaden the application of chitosan (CS) in the anti-alcoholism, gallic acid (GA) was grafted onto the CS molecular chain by 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC)-mediated coupling, and the structure of gallic acid grafted chitosan (CS-GA) was characterized by using UV-Vis spectroscopy (UV-Vis) and Fourier transform infrared spectroscopy (FTIR). The potential anti-alcoholism activity of CS-GA was preliminarily evaluated with ethanol adsorption rate and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging rate. The results showed that GA was grafted onto the CS successfully and CS-GA was obtained. CS-GA could adsorb a large amount of alcohol and reach a peak within 15 min after contact with alcohol in vitro. The DPPH radical scavenging ability of CS-GA was 57.5%, which was 2.4 times of CS. In summary, CS-GA had vigorous potential anti-alcoholism activity and is expected to be developed as a promising antidote.

1. Introduction
With the development of the economy and the increase of interpersonal communication, alcohol consumption is increasing day by day. The proportion of alcoholism in the population is increasing year by year, and the related diseases and problems caused by alcohol are becoming more and more serious [1]. For example, long-term alcoholism can cause decreased memory and learning ability, polyneuropathy, chronic gastritis, myocardial damage, and organic psychosis [2-5]. Besides, traffic accidents caused by drunk driving have always been a public health problem. Therefore, research on anti-alcoholism has attracted more and more attention.

Chitosan (CS) was a polysaccharide extracted from shrimp and crab shells. It had many excellent biological characteristics, such as biodegradability, biocompatibility, non-toxic, antibacterial, anticancer, lipid-lowering, and immune-enhancing [6, 7]. Therefore, it was widely applied in the food and pharmaceutical industries. Gallic acid (GA) was a multi-phenol compound that was widely distributed in grape, tea, gallnut, seed pod, and other plants [8]. It was found that GA had a high affinity for free radicals, which can effectively remove free radicals. Additionally, GA had specific pharmacological effects on the cardiovascular system, nervous system diseases, diabetes, and so on. GA can also protect the liver and prevent liver injury caused by some chemicals [9, 10].

In order to develop a novel anti-alcoholism candidate, CS-GA was developed by chemical modification with CS and GA as the raw materials. CS-GA was characterized by UV-Vis and FTIR, and its potential anti-alcoholism activity was preliminarily evaluated by ethanol adsorption rate and DPPH radical scavenging rate.
2. Materials and methods

2.1. Materials
CS (DD≥95%, Mw=10 kDa), GA, EDC·HCl, N-hydroxysuccinimide (NHS), DPPH, anhydrous ethanol and acetic acid purchased from Sino Pharm Chemical Reagent Co., Ltd.

2.2. Preparation of CS-GA
1.0 g of CS was added into 50 mL of DMF solution and stirred overnight at room temperature, followed by adding 1.3 g of GA. 1.4 g of EDC·HCl was dissolved in 30 mL of DMF solution, and the EDC·HCl solution was added to the reaction system within 30 minutes by using a constant pressure drop funnel. 0.4 g NHS was added to the above reaction mixture and stirred at room temperature for 48 hours. Finally, the reaction solution was dialyzed through a semi-permeable membrane (molecular weight cutoff 10 kDa) and freeze-dried to obtain CS-GA. The effective conversion rate of GA was calculated as the mass of GA in the product divided by the total amount of addition reaction and the result was 37.72%.

2.3. Characterization of CS-GA
2.3.1. UV-Vis spectroscopy. 0.005 g of CS-GA was dissolved in 50 mL of 2% acetic acid solution and diluted ten times. A UV-Vis spectrophotometer was used to establish the baseline with 2% acetic acid solution as the reference. The sample solution was scanned at a wavelength of 200~600 nm.

2.3.2. FTIR spectroscopy. The potassium bromide tablet method was used. 0.001 g of CS-GA and 0.200 g of KBr were weighed. After being pressed into thin plates, they were put into an infrared spectrometer. The testing conditions were wavenumber range of 4000~400 cm⁻¹, scanning 32 times, and the resolution was 4 cm⁻¹.

2.4. Evaluation of potential anti-alcoholism activity
2.4.1. Standard curve of ethanol. 0, 1.0, 1.5, 2.0, 2.5, and 3.0 mL of ethanol solution (6 mg/mL) and 5 mL of potassium dichromate acid solution (5%, v/v) were respectively added into 10 mL colorimetric tube. Distilled water was added to the constant volume, shaken well, heated in 90 °C water bath for 12 min, and cooled to room temperature with running water. The absorbance at 587 nm was measured by a UV-VIS spectrophotometer. The standard curve was drawn with ethanol concentration (mg/mL) as abscissa and absorbance as ordinate. As shown in Figure 1, the regression equation was as follows: y = 0.473x-0.0015, and R² = 0.9999.

![Figure 1. Standard curve of ethanol.](image-url)
Limit of detection (LOD) for ethanol was experimentally established by injecting the standard ethanol at decreasing concentrations until the analytes could not be detected (S/N = 3). Limit of quantification (LOQ) was given as the lower concentration limit of the linear ranges. And LOD and LOQ were 0.002 and 0.005 mg/mL, respectively.

2.4.2. Ethanol adsorption test. The ethanol adsorption test was carried out in 10% ethanol solution (100 mL). After 0, 5, 10, 15, 30, 60, 90, 120, 150, and 180 minutes, and the ethanol content was determined by the above method. The relationship between the amount of ethanol adsorbed by CS-GA with time and dosage was investigated.

2.5. Antioxidant properties (DPPH method)
0.1 mL of CS-GA solutions with certain gradient concentrations and 3.9 mL of DPPH ethanol solution (0.1 mmol/L) was added into the test tube, respectively. After mixing, the solution was kept away from light, and the water bath was conducted at 37 °C for one hour. The absorbance value was determined by a UV-Vis spectrophotometer at 517 nm. The results were expressed as IC50 values when absolute ethanol was used as blank control. When the DPPH scavenging effect is 50%, the concentration of the sample required is IC50. The DPPH scavenging effect was calculated as follows:

\[ \text{Scavenging effect (\%)} = (1 - \frac{A_s}{A_c}) \times 100\% \]

Where Ac and As were the absorbance values of a blank control tube and the sample tube, respectively.

2.6. Statistical analysis
All the experiments were performed in triplicate and the data were expressed as the mean ± standard deviation (SD). Statistical comparisons were performed using ANOVA analysis to demonstrate differences between groups. \( p<0.05 \) and \( p<0.01 \) were considered as statistically significant and highly significant, respectively.

3. Results and discussion
3.1. Synthesis mechanism
Because the combination of EDC·HCl and NHS is relatively safe and compatible, it is often used to catalyze the coupling reaction of amide and ester. CS-GA was prepared by the EDC coupling method, as shown in Figure 2. By adding EDC·HCl first, EDC reacted with -COOH in gallic acid to form O-acyl intermediate. Then NHS is added, and the hydroxyl group in the NHS crosslinking agent reacted with the intermediate to form a substance containing the active ester group. Finally, the substances containing active ester groups reacted with amino and hydroxyl groups of CS to form CS-GA.

![Figure 2. Synthesis mechanism of CS-GA.](image)
3.2. Structural characterization

3.2.1. UV-Vis analysis. Figure 3 shows the UV-Vis spectra of CS, GA, and CS-GA. From Figure 3 (c), CS had no visible absorption peak in the wavelength of 200-600 nm. In comparison, GA has two different absorption peaks in the wavelength of 200-600 nm, among which the absorption peak at 269 nm was more substantial, which was due to the benzene ring edge of the GA. The absorption peak was weak at 227 nm, which was attributed to the carboxyl group in the GA. The trend of absorption peaks of CS-GA (Figure 3a) and GA (Figure 3b) was similar, which indicated that the two substances had similar chromogenic functional groups. The coupling of gelatin and GA also showed similar absorption bands [11]. Compared with GA, the absorption peaks of CS-GA were red-shifted to 271 and 228 nm, respectively. Owing to the covalent linkage of GA and chitosan, the red shift was attributed to the less energy required for the electronic transitions of $\pi-\pi^*$ and n-$\pi^*$. Taken above, it indicated that GA was successfully grafted onto the CS molecular chain.

![Figure 3. UV-Vis spectra of (a) CS-GA, (b) GA, and (c) CS.](image)

3.2.2. FTIR analysis. The infrared spectra of GA, CS, and CS-GA were shown in Figure 4. The characteristic absorption peaks of GA were listed as follows. The absorption peak band of $-\text{OH}$ were from 3486 to 3276 cm$^{-1}$, C-H bond in the benzene ring was 3061 cm$^{-1}$, C=O in carboxylic acid was 1662 cm$^{-1}$, and the stretching vibration absorption peak of C=C in the benzene ring was 1607 cm$^{-1}$ and 1535 cm$^{-1}$, which were basically consistent with the reported in the literature[12]. As shown in Fig. 4b, CS had a broad and robust absorption peak at 3395 cm$^{-1}$, which was multiple absorption peaks formed by stretching vibration of $-\text{OH}$ and N-H bond in the molecule. The infrared spectrum of CS-GA was similar to that of CS, but some absorption peaks were different. Compared with the infrared spectrum of CS, the most substantial absorption peak of CS-GA was 3448 cm$^{-1}$, and the redshift was 53 cm$^{-1}$. Additionally, the absorption peak at 1034 cm$^{-1}$ of C=OH in the CS molecule disappeared. Whereas, there was a new absorption peak at 1765 cm$^{-1}$, which was assigned to the absorption peak of $-\text{COOR}$, indicating that the carboxyl group in GA reacted with the hydroxyl group in CS molecule and formed a new ester bond. Besides, CS has a sharp absorption peak at 1356 cm$^{-1}$, which corresponded to the absorption peak of N-H in primary amine. While the absorption peak of CS-GA disappeared, indicating that the nitrogen atom on the amino group of CS may also react with GA, which further indicated that CS-GA has successfully synthesized.
3.3. Evaluation of anti-alcoholism activity

3.3.1. Ethanol absorption analysis. The dynamic curves of ethanol adsorption by CS-GA with different amounts were shown in Figure 5. When the amount of CS-GA added was constant, the alcohol adsorption amount of CS-GA reached the maximum value when the contact time was 15 min, and the adsorbed alcohol would be released slowly after 15 min. Additionally, with the increase of the amount of CS-GA added, the ethanol adsorption capacity of CS-GA increased. Contacting with the same concentration of ethanol, CS-GA of 2 g reduced ethanol concentration the most by about 1.7% compared with the other two groups (0.5 and 1.5 g). These demonstrated that CS-GA had the particular alcohol-absorbing effect. It was reasonable to believe that a large number of hydroxyl and amino groups on the CS-GA conjugate formed intermolecular hydrogen bonds with ethanol. Besides, when a large amount of alcohol entered the human body in a short period, CS-GA can temporarily store a large amount of alcohol, avoiding the occurrence of acute alcoholism caused by a large amount of alcohol uptake [13-14].

![Infrared spectra of GA, CS, and CS-GA.](image1)

**Figure 4.** Infrared spectra of GA, CS, and CS-GA.

![Dynamic curves of ethanol adsorption by CS-GA.](image2)

**Figure 5.** Dynamic curves of ethanol adsorption by CS-GA.
3.3.2. Antioxidant activity. The toxic effect of alcohol on hepatocytes is damaging the hepatocyte membranes. The lipid components are over oxidized by affecting the metabolism of hepatocytes. After excessive drinking, the superoxide dismutase, malondialdehyde, glutathione peroxidase, and glutathione in the body significantly changed. The body produces a large number of oxygen free radicals, which enhance the oxidation reaction and are harmful to the tissues of the body [15-17]. Therefore, it is of considerable significance to study the antioxidant capacity of anti-alcoholism candidates for protecting the liver. The results of the antioxidant activity of CS-GA were shown in Figure 6. It can be seen that at the same concentration of 1.0×10^{-5} g/mL, the order of scavenging ability of CS, GA, and CS-GA on the DPPH radical scavenging effect was as follows: CS-GA>GA>CS. Therefore, the introduction of GA greatly enhanced the antioxidant activity of CS, even more, potent than GA. CS-GA effectively removed free radicals generated by alcohol metabolism and relieved the excessive oxidation of lipid components on the surface of liver cell membranes, thus improving the body's antioxidant capacity [18]. It was preliminarily speculated that CS-GA had a particular effect on anti-alcoholism and liver protection.

![Figure 6. DPPH radical scavenging performance of the samples.](image)

4. Conclusions
In this paper, CS-GA was prepared by the EDC coupling method. Its structure was characterized by UV-Vis and FTIR analysis, which indicated that GA was grafted onto the CS successfully. The potential anti-alcoholism activity of CS-GA was explored by alcohol adsorption and antioxidant activity. It was found that CS-GA showed excellent antioxidant performance and had a high alcohol adsorption rate, which reached more than 90%. Therefore, CS-GA is expected to become a new anti-alcoholism drug due to its excellent alcohol adsorption performance and antioxidant activity.

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