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Antimicrobial and Preservative Effects of *Codonopsis Pilosula* Extract and Identification of its Active Ingredients

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Abstract: *Codonopsis pilosula* (*C. pilosula*), *Astragalus membranaceus* (*A. membranaceus*) and *Angelica sinensis* (*A. sinensis*) are three common medicinal and dietary tonifying herbs. In this research, the antimicrobial effects of their aqueous and ethanol extracts were analyzed by using the filter paper diffusion method and microdilution method. The results showed that the aqueous extracts of these three medicinal herbs had no significant inhibitory effect on the growth of four common food-contaminated microorganisms: *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Candida albicans* (*C. albicans*) and *Saccharomyces cerevisiae* (*S. cerevisiae*). Only the *C. pilosula* ethanol extract (denoted as CEE) could significantly inhibit the growth of *S. aureus*. The antimicrobial and preservative effects of CEE were investigated by using carrot juice as a model. It was found that CEE exhibited significant synergistic antimicrobial and preservative effects with the chemical preservative benzoic acid. When benzoic acid was reduced to half of its conventional food preservative dosage, the combined addition could completely inhibit the growth of *S. aureus* within 6 d. CEE was further analyzed by high-performance liquid chromatography (HPLC) and primary mass spectrometry (MS1), and lobetyolin was preliminarily identified as the main active ingredient. The minimum inhibitory concentration (MIC) of lobetyolin was determined to be 40 µg/mL when used alone. The cell counting kit-8 (CCK-8) assay showed that both CEE and lobetyolin exhibited low cytotoxicity on human normal liver QSG-7701 cells at the preservative dosage, suggesting promising potential for developing safe and effective food preservatives from traditional Chinese medicinal herbs.

Keywords: *Codonopsis pilosula* (*C. pilosula*); ethanol extract; antimicrobial effect; bio-preservative; lobetyolin

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0 Introduction

During the process of food production, processing

and consumption, factors such as environment and temperature pose a serious threat to food safety, easily leading to food spoilage and decay. Therefore, adding an appropriate number of preservatives to extend the shelf life of food and improve food safety has become a consensus in the food processing industry. At present, most preservatives used in food production and processing worldwide are still chemical compounds. Although chemical preservatives are efficient and cost-effective, studies have shown that their long-term use may bring some safety risks, such as carcinogenic and teratogenic effects^[1-4]. Meanwhile, as society progresses, public awareness of physical health and food safety continues to grow. To meet evolving demands, the food industry is actively developing safe and efficient natural preservatives^[5-7].

China has abundant medicinal plant resources, and most traditional Chinese herbs have medicinal value in regulating the spleen and stomach, enhancing physical fitness, and preventing and treating diseases. Some medicinal and dietary tonifying herbs are often used as flavor enhancers and antimicrobial preservatives in food. The Chinese Medicine Dictionary includes over 2 000 types of Chinese medicinal herbs with antimicrobial and preservative effects^[8-10]. Existing research has confirmed that extracts from nearly 100 Chinese medicinal herbs have antimicrobial and preservative effects, which can effectively prevent the decay and deterioration of fruits and vegetables^[10-13]. *Codonopsis pilosula* (*C. pilosula*), *Astragalus membranaceus* (*A. membranaceus*) and *Angelica sinensis* (*A. sinensis*) are all bulk medicinal herbs, which are widely used in traditional Chinese patent medicines and simple preparation prescriptions. The extracts of these three medicinal herbs have complex components and exhibit health functions such as tonifying qi, generating saliva and nourishing blood^[14-17]. However, their antimicrobial and preservative effects are rarely reported. This work aims to systematically study their antimicrobial and preservative effects and identify the active ingredients. It is expected to provide new directions for the development and utilization of unique Chinese

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herbal resources and promote the development and application of new preservatives that combine nutritional health with antimicrobial and preservative properties.

1 Materials and Methods

1.1 Materials

Four microorganisms, *E. coli*, *S. aureus*, *C. albicans* and *S. cerevisiae*, were all cultured in the Microbiology Laboratory of Donghua University (Shanghai, China); human normal liver QSG-7701 cell line was purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China); three medicinal herbs, *C. pilosula*, *A. membranaceus* and *A. sinensis*, were purchased from a pharmacy in Songjiang District (Shanghai, China); benzoic acid was purchased from China National Pharmaceutical Chemical Reagent Co., Ltd. (Shanghai, China).

1.2 Preparation of medicinal herb extracts

C. pilosula, *A. membranaceus* and *A. sinensis*

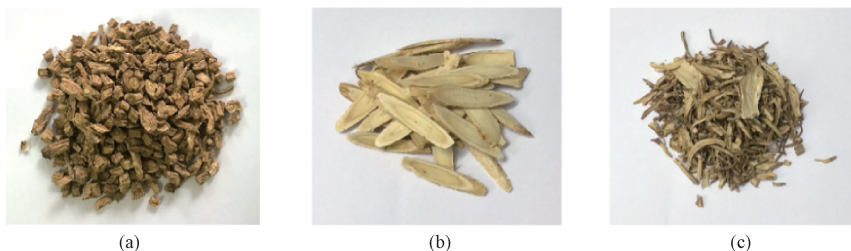


Fig. 1 Samples of three medicinal herbs; (a) *C. pilosula*; (b) *A. membranaceus*; (c) *A. sinensis*

1.3 Sensitivity testing of medicinal herb extracts

The sensitivity of four microorganisms (*E. coli*, *S. aureus*, *C. albicans* and *S. cerevisiae*) to medicinal herb extracts was measured by using the strain growth rate inhibition method. Four microorganisms were resuspended in fresh culture media, respectively (Luria-Bertani (LB) medium for *E. coli* and *S. aureus*; yeast extract-peptone-dextrose (YPED) medium for *C. albicans* and *S. cerevisiae*). Then the resuspended microbial suspensions were diluted with the sterile culture medium to an optical density at a wavelength of 600 nm (OD_{600nm}) of 0.1. The medicinal herb aqueous or ethanol extracts were added to the cultures, respectively, and an equal volume of deionized water was added to the culture to serve as the control group. The cultures were incubated at appropriate temperatures (37 °C for *E. coli* and *S. aureus*; 30 °C for *C. albicans* and *S. cerevisiae*). The antimicrobial effect of the medicinal herb extracts was evaluated by measuring the growth of four microorganisms after 18 h. Each experiment was performed in triplicate. The antimicrobial effect r_b was calculated as

$$r_b = (F_1 - F_2) / F_1 \times 100\%, \quad (1)$$

where F_1 denotes the OD_{600nm} of the control group; F_2 denotes the OD_{600nm} of the medicinal herb extract-treated group. The antimicrobial effect is categorized as follows:

(Fig. 1) were ground into powder by using a Chinese medicine grinder and dried for storage.

The aqueous extracts of these three medicinal herbs were prepared as follows. A total of 6 g of dry plant powder was soaked in 100 mL of deionized water for 30 min. The mixture was then heated and boiled for extraction. The extraction process was repeated three times (2 h per extraction), with filtration after each step. The combined filtrate was concentrated at a reduced pressure by using a rotary evaporator at 50 °C to obtain a crude extract. The concentrated extract was reconstituted in deionized water and sterilized by filtration through a 0.22 μm microporous membrane to yield the final aqueous extract (stock solution).

The ethanol extracts of these three medicinal herbs were prepared following the same procedure as the aqueous extracts, except that the extraction solvent was replaced with 80% ethanol (80 mL absolute ethanol plus 20 mL deionized water).

when $r_b < 10\%$, it is marked as “-”, indicating no antimicrobial effect; when $10\% \leq r_b < 20\%$, it is marked as “+”, indicating weak antimicrobial effect; when $20\% \leq r_b < 50\%$, it is marked as “++”, indicating strong antimicrobial effect; when $r_b \geq 50\%$, it is marked as “+++”, indicating complete antimicrobial effect.

1.4 Confirmation of antimicrobial effect of *C. pilosula* extract

The antimicrobial effect of the *C. pilosula* extract was determined by the filter paper diffusion method. Firstly, the experimental filter paper discs were prepared by punching highly absorbent filter paper into uniformly sized rounds, which were then wrapped in the tissue and autoclaved at 121 °C for 20 min. *S. aureus* was cultured in beef extract peptone medium (pH 7.0–7.2). The medium was sterilized, cooled to about 50 °C, poured into the sterilized petri dish, and allowed to solidify. For sample application, 5 μL of the *C. pilosula* extract was dropped onto the sterilized filter paper, while controls included 80% ethanol with sterile water as the blank control and 4 μL of ampicillin solution as the positive control. The disks were evenly soaked, air-dried and then placed onto the inoculated agar plates, which had been prepared by spreading 0.2 mL of *S. aureus* suspension onto the solidified medium. The plates were incubated at 37 °C for 24 h, after which the antimicrobial effect was

evaluated by measuring the diameter of the inhibition zones surrounding the disks.

1.5 Analysis of antimicrobial and preservative effects of CEE

By using the carrot juice model^[18], the combined antimicrobial and preservative effects of CEE and benzoic acid were studied. The carrot juice was extracted by using a wall-breaking machine, sterilized, filtered and adjusted to a pH value of 6.0 to ensure optimal growth conditions for the experimental microorganisms. The prepared carrot juice was then used to create a microbial suspension with a colony counts of 10^4 CFU/mL (CFU is short for colony forming unit). The experiment was designed with several groups. A blank control group consisted of 1.8 mL of microbial suspension and 200 μ L of liquid culture medium. An ethanol control group consisted of 1.8 mL of microbial suspension and 200 μ L of 40% ethanol. Two experimental groups were set up, namely a separate additive group and a combined additive group. In the separate additive group, 1.8 mL of microbial suspension was combined with 100 μ L of 40% ethanol and either 100 μ L of CEE or 100 μ L of benzoic acid solution. The combined additive group involved mixing 1.8 mL microbial suspension with 100 μ L of CEE and 100 μ L of benzoic acid solution. After thorough mixing, all samples were incubated at 25 °C. At designated time intervals (1–6 d), samples were taken, plated onto a solid culture medium, and incubated at 37 °C for 8 h before microbial colonies were counted. Each experimental condition was performed in triplicate to ensure reproducibility.

1.6 Analysis and identification of antimicrobial active ingredients in CEE

Chromatographic conditions were as follows. The chromatographic separation was carried out on an Agilent Eclipse XD-C18 chromatography column (150 mm \times 4.6 mm, 5 μ m) by using a high-performance liquid chromatography (HPLC) instrument (LC-20A HT, Shimadzu, Japan). The mobile phase was a binary eluent of acetonitrile (A) and 0.2% (volume fraction) phosphoric acid (B), with the following gradient program: 0–10 min, 5%–8% (volume fraction) A; 10–35 min, 8%–20% A; 35–50 min, 20%–30% A; 50–60 min, 30%–50% A; 60–65 min, 50%–80% A. The injection volume was 10 μ L. The flow rate was 0.8 mL/min, and the detection wavelength was 220 nm^[19].

Primary mass spectrometry (MS1) conditions were as follows. The main instrument was the TSQ Quantis™ triple quadrupole mass spectrometer (TSQ02, Thermo Fisher Scientific, USA). In the positive ion mode of electric spray, nitrogen was used as sheath gas and auxiliary gas, and the flow rate was 45 L/min and 10 L/min, respectively. The capillary temperature was 320 °C, and the ion source temperature was 310 °C. The spray voltage was 3 kV. The high-resolution mass spectra were acquired at a mass-to-charge ratio (m/z) range of

150–2 000 and a resolution of 70 000^[20].

1.7 Determination of minimum inhibitory concentration (MIC) of lobetyolin

The MIC of lobetyolin was obtained by the microdilution method. A serial dilution of lobetyolin was prepared by adding 1.9 mL of the sterile liquid medium to each of six test tubes. Then, 100 μ L of lobetyolin solution was added to the first tube and mixed. Subsequently, 100 μ L of the mixture was transferred to the second test tube and mixed, and the process was repeated up to the sixth tube. After mixing, 100 μ L of the mixture from the sixth tube was discarded to establish the concentration gradient. Then, 100 μ L of microbial suspension was added to each of the six (1–6) tubes as the experimental group. The 7th test tube was set as the positive control, with 1.9 mL of sterile liquid medium and 100 μ L of experimental microbial suspension, to observe the growth status of the microorganisms. The 8th test tube was a negative control, with 1.9 mL of sterile liquid medium and 100 μ L of lobetyolin solution. The mixed test tubes were incubated in a shaking bed at 37 °C for 24 h, and the turbidity changes of the microbial suspension in each test tube were observed, with three replicates per group.

1.8 Cytotoxicity determination of CEE

Human normal liver QSG-7701 cells were commercially obtained and cultured in a Dulbecco's modified Eagle medium (DMEM) containing a final mass fraction of 1% dual antibiotic mixture (penicillin and streptomycin) and 10% fetal bovine serum at 37 °C in a 5% (volume fraction) CO₂ incubator. After digestion and cell counting, the well-growing cells were seeded with a cell counts of 10^5 CFU/mL and cultured in a cell culture incubator containing 5% (volume fraction) CO₂ at 37 °C. After the cells were adherent, CEE, lobetyolin, 5-fluorouracil (positive control), vitamin C (negative control), and 80% ethanol (blank control) were added, respectively. After incubation for 24 h, 10 μ L of CCK-8 reagent was added to each well and then incubated for about 1 h. The absorbance was measured at 450 nm with a microplate reader (Bio-Rad, CA, USA). The cell viability r_{cs} was calculated according to

$$r_{cs} = [(A_3 - A_1) / (A_2 - A_1)] \times 100\%, \quad (2)$$

where A_1 , A_2 and A_3 denote the absorbance of the background, the blank control cells and the treated cells, respectively.

1.9 Statistical analysis

All experiments were performed at least three times. Student's t -test was used for statistical analysis. $P < 0.05$ means a statistically significant difference. The asterisk was used to denote significance levels: * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** denotes $P < 0.001$.

2 Results and Discussion

2.1 Antimicrobial effects of *C. pilosula*, *A. membranaceus* and *A. sinensis* extracts

Aqueous extracts and ethanol extracts of three medicinal herbs were subjected to antimicrobial experiments. The inhibitory effects of different extracts on four common microorganisms (*E. coli*, *S. aureus*, *C. albicans* and *S. cerevisiae*) were expressed by using

the antimicrobial effect r_b . The results are shown in Table 1. Ampicillin (50 $\mu\text{g}/\text{mL}$) and an aminoglycoside antibiotic, G_{418} (50 $\mu\text{g}/\text{mL}$), were used as positive controls to inhibit microbial growth. Aqueous extracts of three medicinal herbs have no significant antimicrobial effect on all four tested microorganisms ($r_b < 10\%$). Among the three ethanol extracts, CEE shows a significant antimicrobial effect on *S. aureus* ($r_b > 20\%$), while the other two ethanol extracts have no significant antimicrobial effect on the tested microorganisms.

Table 1 Antimicrobial effect of three medicinal herb extracts

Microorganism	Antimicrobial effect							
	CAE	CEE	AAE	AEE	SAE	SEE	Ampicillin	G_{418}
<i>E. coli</i>	-	-	-	-	-	-	+++	+
<i>S. aureus</i>	-	++	-	-	-	-	++	+
<i>C. albicans</i>	-	-	-	-	-	-	-	++
<i>S. cerevisiae</i>	-	-	-	-	-	-	-	+++

Note: CAE, AAE, AEE, SAE and SEE represent *C. pilosula* aqueous extract, *A. membranaceus* aqueous extract, *A. membranaceus* ethanol extract, *A. sinensis* aqueous extract and *A. sinensis* ethanol extract, respectively.

2.2 Antimicrobial effects of CAE and CEE

The antimicrobial effects of CAE and CEE on *S. aureus* were further tested by using the filter paper diffusion method. The results are shown in Fig. 2 (a). The diagram of sample mass concentrations and location patterns used in the antimicrobial experiments is presented in Fig. 2 (b). H_2O and 80% ethanol were used as

negative controls, and ampicillin was used as a positive control. As shown in Fig. 2, CAE has no significant effect on the growth of *S. aureus*, which is consistent with the above results. At the same time, CEE can significantly inhibit the growth of *S. aureus*, and the inhibitory effect becomes more significant with the increase of its mass concentration.

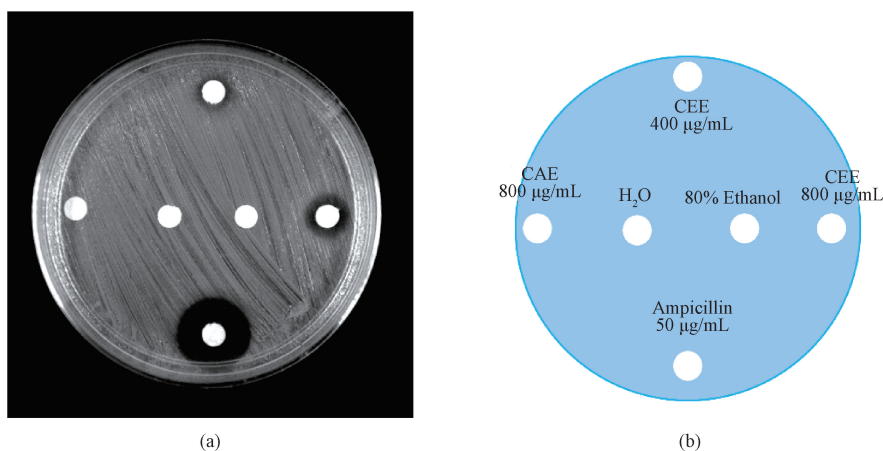


Fig. 2 Antimicrobial results; (a) antimicrobial effects of CAE and CEE on *S. aureus*; (b) diagram of sample mass concentrations and location patterns used in antimicrobial experiments

2.3 Antimicrobial and preservative effects of CEE on carrot juice

CEE and benzoic acid were added alone or in combination in carrot juice, and cultured at 25 $^{\circ}\text{C}$ for 6 d to test their antimicrobial effects on low colony counts (10^4 CFU/mL) of *S. aureus*. The results are shown in Table 2. The *S. aureus* colony counts are expressed in logarithmic units, and the quantitative antimicrobial effects are expressed as mean \pm standard deviation of the

logarithmic colony counts. The addition of benzoic acid and CEE alone has a certain degree of antimicrobial effect, and the antimicrobial effect of CEE is significantly higher than that of benzoic acid within 3 d. The combined addition of 0.010% (mass fraction) benzoic acid (1/4 of MIC alone) and 0.005% (mass fraction) CEE (1/2 of MIC alone) completely inhibits the growth of *S. aureus* for 2–3 d, demonstrating a strong combined antimicrobial effect.

Table 2 Antimicrobial effects of CEE and benzoic acid on *S. aureus* in carrot juice

Benzoic acid mass fraction/%	CEE mass fraction/%	Quantitative antimicrobial effect					
		1 d	2 d	3 d	4 d	5 d	6 d
0	0	3.98±0.11	7.09±0.07	8.51±0.17	9.67±0.21	9.85±0.45	9.57±0.47
0.010	0	3.91±0.09	6.23±0.13	7.64±0.34	8.58±0.32	9.36±0.20	9.66±0.15
0	0.005	4.03±0.04	4.64±0.12	7.31±0.51	8.79±0.27	9.01±0.27	9.50±0.31
0.010	0.005	4.01±0.30	0.00±0.00	0.00±0.00	1.01±0.18	1.52±0.29	3.81±0.34

2.4 Analysis and identification of active ingredients in CAE and CEE

The differences in chemical composition between CAE and CEE were detected by using HPLC. It can be seen from Figs. 3 (a) and 3 (b) that CEE and CAE exhibit similar 1, 2 and 3 peaks at a retention time of 2–5 min, indicating the presence of some similar substances in CEE and CAE. However, only CEE

shows a characteristic peak 4 at a retention time of about 14 min. It is speculated that this component is an active substance with antimicrobial effects. According to MS1 identification, as shown in Fig. 3 (c), the ion peak m/z in the positive ion mode is 419.1677 [$M + Na$]⁺, indicating that the substance is lobetyolin with a relative molecular mass of 396 and a molecular formula of $C_{20}H_{28}O_8$.

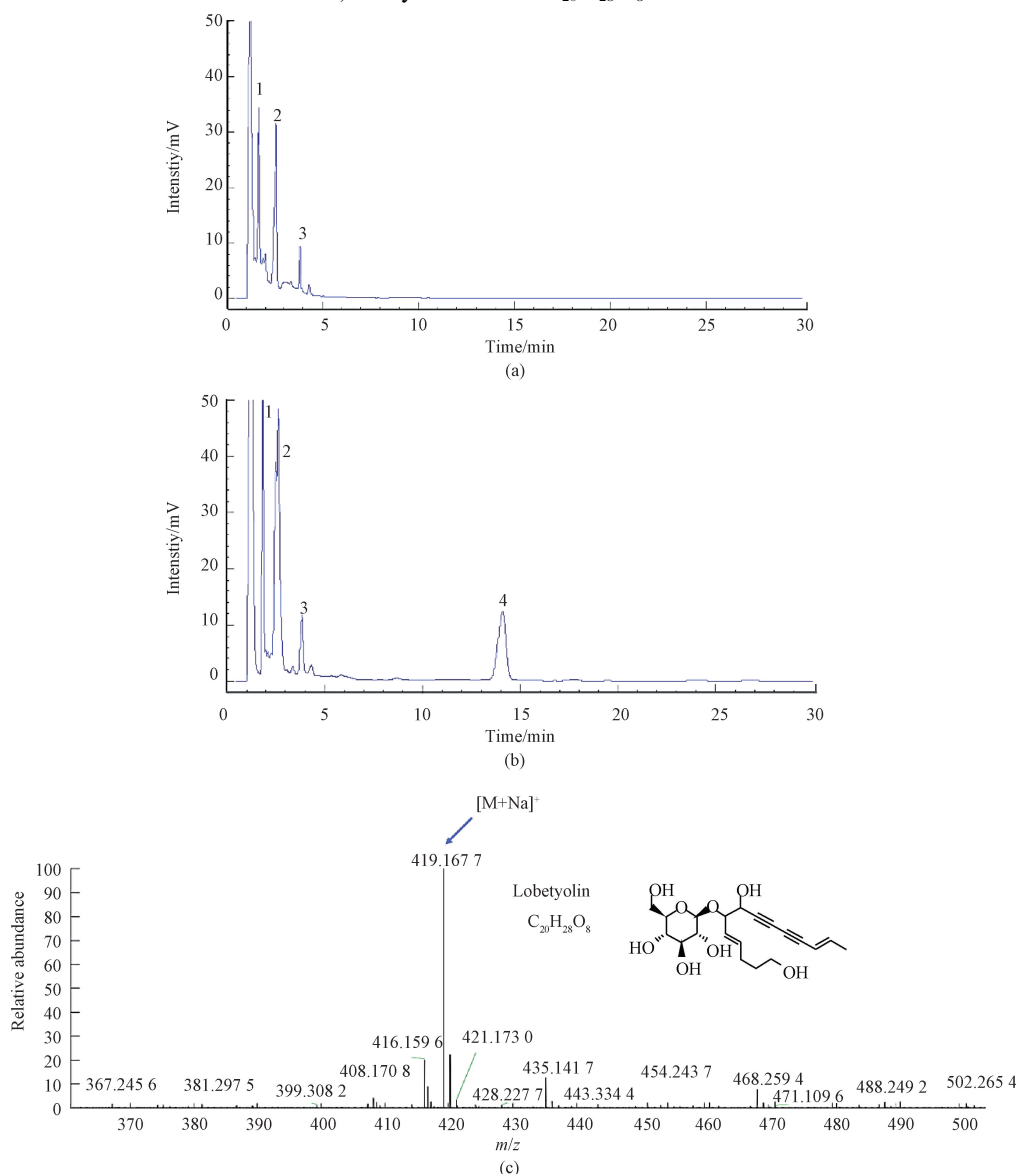


Fig. 3 HPLC analysis and MS1 identification of antimicrobial active ingredients; (a) HPLC analysis of CAE; (b) HPLC analysis of CEE; (c) MS1 identification of antimicrobial active ingredients in CEE

2.5 MIC of lobetyolin

According to Table 3, the MIC of lobetyolin on *S. aureus* in LB medium is 40 $\mu\text{g}/\text{mL}$. When the mass concentration of lobetyolin exceeds 40 $\mu\text{g}/\text{mL}$, it completely inhibits the growth of *S. aureus*. It partially inhibits microbial growth at a mass concentration of 20 $\mu\text{g}/\text{mL}$. As the mass concentration of lobetyolin decreases, its inhibitory effect weakens.

Table 3 MIC of lobetyolin on *S. aureus*

Test tube number	Lobetyolin mass concentration/ $(\mu\text{g}/\text{mL})$	Antimicrobial effect
1	160	+++
2	80	+++
3	40	+++
4	20	++
5	10	+
6	5	-
7	0 (Positive control)	-
8	Negative control	-

2.6 Cytotoxicity assessment of CEE and lobetyolin

As shown in Fig. 4, after treating QSG-7701 cells with different mass concentrations of CEE (a: 25 $\mu\text{g}/\text{mL}$ and b: 50 $\mu\text{g}/\text{mL}$) or lobetyolin (c: 5 $\mu\text{g}/\text{mL}$) for 24 h, the cell viabilities are all higher than 87%. The positive control, 5-fluorouracil (d: 40 $\mu\text{g}/\text{mL}$), significantly reduces cell viability ($P < 0.001$). Vitamin C (e: 100 $\mu\text{g}/\text{mL}$), as a negative control, has no significant effect on the cell viability. Therefore, it is indicated that CEE and lobetyolin have very little cytotoxicity on QSG-7701 cells.

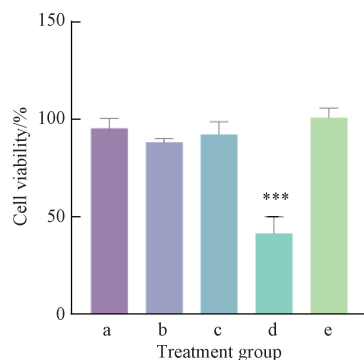


Fig. 4 Cytotoxicity of CEE and lobetyolin on QSG-7701 cells

3 Conclusions

In this study, we used both water and ethanol to extract the effective ingredients of three Chinese medicinal herbs (*C. pilosula*, *A. membranaceus* and *A. sinensis*) and carried out antimicrobial experiments on four common microorganisms (*E. coli*, *S. aureus*,

S. cerevisiae and *C. albicans*). The results showed that only CEE exhibited significant antimicrobial effect on *S. aureus*. CEE showed good antimicrobial activity in both LB medium and carrot juice models, and exhibited synergistic antimicrobial effect with benzoic acid. The growth of *S. aureus* could be inhibited within 6 d, even when the mass fraction of benzoic acid was reduced by more than half. CEE was further analyzed by HPLC and MS1, and lobetyolin was identified as the main active ingredient. Its MIC on *S. aureus* was 40 $\mu\text{g}/\text{mL}$. The results of the cytotoxicity experiment showed that CEE and lobetyolin had no significant toxic side effects on the human normal liver QSG-7701 cells. Our findings demonstrate that CEE has a potential application in food preservation.

In the context of the era of great health, with the continuous improvement of people's health awareness, the demand for natural and safe health foods continues to increase. The development of new preservatives that integrate nutritional health, antimicrobial and preservative properties by using Chinese medicinal herb *C. pilosula* as raw material has broad prospects. Meanwhile, with the support of the country for the traditional Chinese medicine industry, traditional Chinese medicinal herbs and their affiliated industries will receive more attention and development^[21-22].

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党参提取物抑菌保鲜作用及其活性成分的鉴定

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摘 要: 党参、黄芪和当归是 3 种常见的药食两用补益类中药。该研究采用滤纸片扩散法和微量稀释法对这 3 种中药的水提取物和乙醇提取物的抑菌作用进行分析。结果表明, 3 种中药的水提取物对 4 种常见的食品污染微生物(大肠埃希菌、金黄色葡萄球菌、白念珠菌、酵母菌)的生长均无明显的抑制作用, 仅党参的乙醇提取物能显著抑制金黄色葡萄球菌的生长。研究发现, 以胡萝卜汁为模型时, 党参的乙醇提取物与化学防腐剂苯甲酸联用具有显著的协同抑菌保鲜效果。当苯甲酸用量减至常规食品保鲜剂量的一半时, 该复合保鲜体系能在 6 d 内有效抑制金黄色葡萄球菌的生长。采用高效液相色谱法和质谱法分析了党参的乙醇提取物, 并初步鉴定党参炔苷为其主要活性成分。党参炔苷单独使用时, 最小抑菌质量浓度为 40 $\mu\text{g}/\text{mL}$ 。采用细胞计数试剂盒-8 检测细胞活力, 结果表明, 在保鲜剂量范围内, 党参的乙醇提取物及党参炔苷对人体正常肝脏细胞 QSG-7701 无明显细胞毒性, 这表明利用传统中药开发安全有效的食品保鲜剂具有良好前景。

关键词: 党参; 乙醇提取物; 抑菌作用; 生物保鲜剂; 党参炔苷