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Original Article

Chemical components in cultivated *Cordyceps sinensis* and their effects on fibrosis

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ABSTRACT

Objective: Cultivated *Cordyceps sinensis* powder has been used as clinical drug and healthy food to nourish the lung and kidney, which solves the problem of serious shortage of wild *C. sinensis*. This study aims to explore the chemical components and compared their anti-fibrotic effects in cultivated *C. sinensis*.
Methods: Nucleosides, sterols and polysaccharides were separated and purified from cultivated *C. sinensis*, and analyzed by high performance liquid chromatography, gas chromatography-mass spectrometry and chemical chromogenic methods, respectively. In high glucose-induced rat mesangial cell models, fibronectin and type 1 collagen were used as evaluation indicators.
Results: There were 10 kinds of nucleosides and one sterol in cultivated *C. sinensis*. The contents of nucleosides, sterols and polysaccharides in the cultivated *C. sinensis* were close to 2%, 0.55% and 4.4%, respectively. Furthermore, nucleoside, sterol and polysaccharide components exhibited varying degrees of anti-fibrotic activity. The nucleoside components and sterol components inhibited the expression of extracellular matrix more effectively in the three main components.
Conclusion: Cultivated *C. sinensis* remains the similar compounds with the wild *C. sinensis*, and nucleosides and sterols may be the main active substances that contribute to its anti-fibrotic effects. The project of this study may provide valuable information on further optimization of more effective remedies with few side effects based on cultivated *C. sinensis*.

1. Introduction

Cordyceps sinensis (Berk.) Sacc., a specious tonic food and traditional Chinese medicine (TCM), has been used for thousands of years to prevent and cure human diseases (Chen, Chen, Pan, Chang, & Huang, 2017). A lot of studies have demonstrated that the chemical compounds extracted from *C. sinensis* have various pharmacological actions, such as nephroprotective, hepatoprotective, immunomodulatory, anti-ageing, anti-inflammation, antioxidant and anti-apoptosis propeties (Yue, Ye, Zhou, Sun, & Lin, 2013).

C. sinensis is rich in nucleosides, sterols, polysaccharides, proteins and amino acids (Liu et al., 2016). The nucleosides are recognized as the main bioactive components in *C. sinensis*, and are used as chemical markers for quality control of *C. sinensis* (Shi et al., 2020; Zong et al., 2015). The previous studies showed that nucleosides suppressed inflammation in the progress of chronic obstructive pulmonary disease (Sun et al., 2018b), inhibited the epithelial mesenchymal transition and protected against renal fibrosis in diabetic nephropathy (Dong, Sun, Wei, Li, & Zhao, 2019b). Sterols are also very important bio-functional components in fungi, and were separated or identified from *C. sinensis* by many researchers (Chen et al., 2021; Siwulski et al., 2019; Sun et al., 2017; Zhao et al., 2020). Ergosterol, a principal sterol, has been used as a useful chemical marker for evaluating the quality of *C. sinensis* (Yuan,

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Wang, Liu, Kuang, & Zhao, 2007). Numerous studies have summarized the sterols/ergosterol pharmacological activities, such as anti-tumor, anti-inflammation, anti-fibrosis, anti-oxidation and regulation of immunity (Dong, Sun, Wei, Li, & Zhao, 2019a; Heroor, Beknal, Mahurkar, Hiremath, & Inamdar, 2020; Sun et al., 2020; Tai, Choong, Lin, Shi, & Tai, 2016; Wu et al., 2018; Xu et al., 2018a; Zhang, Xu, Li, & Wang, 2015). What's more, polysaccharides have been reported to have immunomodulatory, anti-tumor, anti-proliferative and anti-oxidative effects (Bi et al., 2020; Liu, Zhu, Liu, & Sun, 2019; Zhang et al., 2020). Pilot studies have extracted and revealed the structure of polysaccharides as well as their underlying molecular mechanism (He et al., 2020; Sun et al., 2018a). The protein and amino acid components of *C. sinensis* contribute to reducing blood pressure and alleviating bone marrow suppression (Chiou, Chang, Chou, & Chen, 2000; Zhang et al., 2022).

Fibrosis is characterized by an excessive accumulation of the extracellular matrix (ECM) as a response to different types of tissue injuries, leading to organ dysfunction (Panizo et al., 2021). Fibrosis is a key factor in the development and progression of respiratory disease (especially lung disease) and kidney disease and can be associated with high morbidity and mortality (Djudjaj & Boor, 2019; Richeldi, Collard, & Jones, 2017). *C. sinensis* exhibits good anti-fibrotic activity in the treatment of lung and kidney diseases (Du et al., 2015; Yang et al., 2018). Nowadays, cultivated *C. sinensis* powder has been used as clinical drug (like Jinshuibao Capsule and Bailing Capsule) to cure chronic respiratory disease and nephropathy, which solves the problem of serious shortage of wild *C. sinensis*. However, the compounds in cultivated *C. sinensis* powder were few to report. According to the content of each component in *C. sinensis* as well as the correlation between their biological activities and fibrosis, this project focuses on separating and purifying nucleosides, sterols and polysaccharides components to preliminarily evaluate their anti-fibrotic activity. The protective of this research lays the foundation for studying the material basis of cultivated *C. sinensis*.

2. Materials and methods

2.1. Materials

The dried cultivated *C. sinensis* powder was a gift from Hangzhou Zhongmei East Pharmaceutical Co. (Hangzhou, China; Lot: 1602705132). All of nucleoside and ergosterol standards were purchased from Aladdin Regents Co. (Shanghai, China; Lot: cytidine J1415013, adenine B1629442, guanine E1715168, uracil B1619157, hypoxanthine G1725018, uridine D1601165, adenosine B1624001, 2′-deoxyadenosine E1512027, guanosine L1510026, thymidine J1424056, ergosterol K1413056). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Biological Industries (Beit Haemek, Israel). Penicillin and streptomycin were purchased from Solarbio Biotechnology (Beijing, China). Fibronectin antibody was obtained from Abcam (Cambridge, UK). Collagen I antibody was provided by R&D system (Minneapolis, MN, USA). All other chemicals were of analytical grade from Guo-Yao Chemical Regent Company (Shanghai, China).

2.2. Equipments

High performance liquid chromatography (HPLC) and gas chromatography-mass spectrometer (GC–MS) were of Agilent Technology Company (Kalifornien, USA). Rotary evaporator (N-1100S-WB) was of EYELA Co. (Tokyo, Japan). Milli-Q Reference (Millipore Co., Ltd., New York, USA) was used for distilled water. Freeze dryer (ALPHA 1–2 LD plus) was of Marin Christ (Osterode, Germany). Vacuum drying oven was of EYELA Co. (Tokyo, Japan).

2.3. Cell culture

Rat mesangial cells (RMCs) were purchased from American Type Culture Collection (Manassas, VA, USA). RMCs were cultured in DMEM supplemented with 100 U/mL of penicillin G, 100 μg/mL of streptomycin, and 10% (volume percent) inactivated FBS. Cells were grown at 37 °C in a humidified 5% CO_2 incubator.

2.4. Detection of nucleosides in cultivated C. sinensis

2.4.1. Extraction of nucleosides

The dried cultivated *C. sinensis* powder was mixed with distilled water (1:10 ratio of raw material to water) in a conical flask (500 mL) and ultrasound-assisted (ultrasonic power 100 W) extracted three times for 1 h. Then, the merged suspension was centrifuged at 1000 r/min, concentrated using a rotary evaporator, and freeze-dried (Alpha 1–2 LDplus; Christ, Osterode am Harz, Germany). Finally, the sample was redissolved in double-distilled water and determined by HPLC.

2.4.2. Separation and identification of nucleosides in cultivated C. sinensis

Analysis of the chemical composition of nucleosides was performed on an Agilent 1260 HPLC system equipped with a diode array detector (Agilent Technologies, Palo Alto, CA, USA). Separation was carried out using a Synergi MAX-RP 80A column (4.6 m m × 150 mm, 5 μm; Phenomenex, Torrance, CA, USA), and the injection volume was 10 μL. The column temperature was 25 °C. The mobile phase consisted of solvents A (0.1% formic acid in water, volume percent) and B (methanol) with gradient elution as follows: 0–5 min (5% B); 5–7 min (5%–9% B); 7–10 min (9%–12% B); 10–19 min (12% B); 19–20 min (12%–15% Bt); 20–30 min (15% B). The flow rate was set at 0.4 mL/min and peaks of nucleosides were monitored at 260 nm.

2.5. Detection of sterols in cultivated C. sinensis

2.5.1. Extraction of sterols

The dried cultivated *C. sinensis* powder was mixed with 95% ethanol (1:10 ratio of raw material to ethanol) in a conical flask (500 mL) and ultrasound-assisted (ultrasonic power 100 W) extracted three times for 1 h. Then, the merged suspension was filtrated with vacuum pump and concentrated using a rotary evaporator. After extraction with petroleum ether and ethyl acetate, the merged suspension was filtrated and concentrated again, and then separated by silica gel column. The sterols were eluted with oil ether and ethyl acetate (10:1, volume percent). The flow rate was set at 0.8 mL/min and 10 mL for each tube. Tubes with sterols were collected with ergosterol as the control, the merged elution was concentrated, recrystallized, and vacuum dried.

2.5.2. Qualitative analysis of sterols by GC–MS

Analysis of the chemical composition of sterols was performed on GC–MS system using an Agilent HP-5 quartz capillary chromatographic column (30 m × 0.25 mm × 0.25 μm; Agilent, USA). The programmed temperature condition was as follows: initial temperature at 240 °C for 1 min, and raise to 270 °C at a rate of 5 °C/min, then remained for 30 min. The inlet temperature was set as 300 °C. The injection volume was 1 μL with a spilt rate of 10:1. The mass spectrometry conditions were set as follows: electron bombardment ion source at 70 eV and the quality range was from 50 to 400 amu. Finally, the sterols chromatogram was compared with the NIST 08 standard mass spectrometry library.

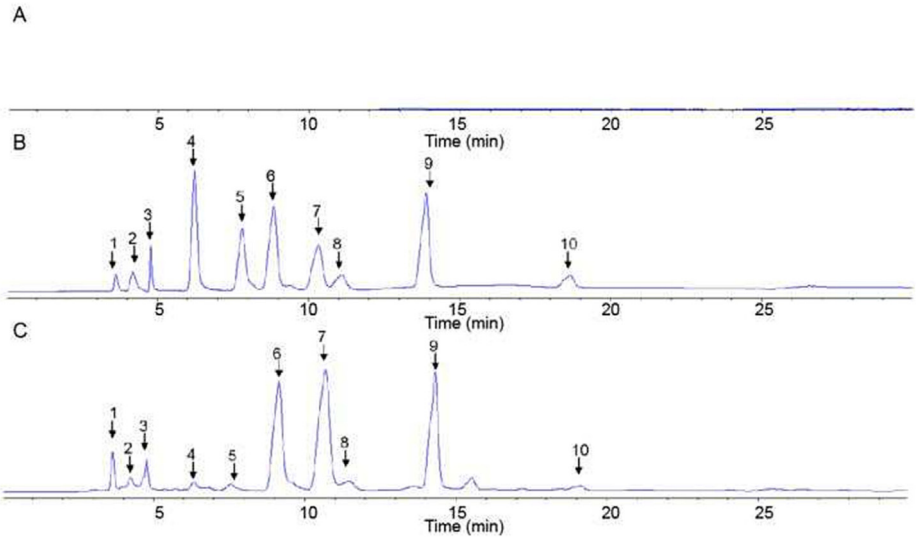

Fig. 1. Typical chromatograms of blank (A), nucleoside standards (B) and unknown peaks in cultivated *C. sinensis* (C) determined by HPLC. 1, cytidine; 2, adenine; 3, guanine; 4, uracil; 5, hypoxanthine; 6, uridine; 7, adenosine; 8, 2′-deoxyadenosine; 9, guanosine; 10, thymidine. Same as below.

2.5.3. Quantitative analysis of sterols by HPLC

The quantitative of the sterols in cultivated *C. sinensis* was performed on an Agilent 1260 HPLC system equipped with a diode array detector (Agilent Technologies, Palo Alto, CA, USA), using a phenomenex C_{18} column (4.6 mm × 250 mm, 5 μm; Phenomenex, Torrance, CA, USA), and the injection volume was 10 μL. The column temperature was 25 °C. The mobile phase consisted of solvents A (methanol) and B (water) at a rate of 98:2 (volume percent), and run for 40 min. The flow rate was set at 1.0 mL/min and the peak of sterols was monitored at 282 nm.

2.6. Detection of polysaccharides in cultivated C. sinensis

2.6.1. Extraction of polysaccharides

The cultivated *C. sinensis* powder which has been extracted with 95% ethanol was further mixed with distilled water (1:10 ratio of raw material to water) in a conical flask (500 mL) and ultrasound-assisted (ultrasonic power 100 W) extracted three times for 1 h. After centrifugation and concentration, Sevage regents (4:1 ratio of chloroform to butyl alcohol, volume percent) were used to remove proteins in the supernatant (2:1 ratio of supernatant to Sevage, volume percent). Repeated for 5–10 times until there were no more flocculent proteins in the lower organic phase, the aqueous phase was acquired with tap funnel. A total of 1/5 vol of 30% H_2O_2 was added at pH 8.8 and 40 °C with agitation for 5 h. Ethanol was added with final concentration of 75% and then left for overnight at 4 °C. Polysaccharides precipitation was collected and diluted to appropriate concentration, the polysaccharides solution was subjected to dialysis with distilled water for 1 d, then, concentrated and freeze-dried.

2.6.2. Content detection of polysaccharides

The sulfuric acid-phenol method was used to determine the content of polysaccharides (Jiang et al., 2016; Rasouli, Ostovar-Ravari, & Shokri-Afra, 2014). In brief, 0.2 mL redissolved polysaccharides was added to 1.8 mL distilled water in test tube with plug. Then, 1 mL 5% fresh phenol was added into the tube, and followed by 5 mL concentrated sulfuric acid, 40 °C for 20 min. After being cold for 5 min, the absorption was monitored at 490 nm. The glucose was used as the standard to draw the standard curve. Finally, the Biuret reaction (Ponomareva, Golovchenko, Patova, Vanchikova, & Ovodov, 2015), Ninhydrin reaction (Xu et al., 2018b), Flynn's regent reaction and Molisch reaction (Liu, Luo, Dai, Ge, & Yang, 2004) were used to detect the impurity of polysaccharides.

2.7. Determination of extracellular matrix expression

RMCs were cultured in 6-well plates for 24 h with DMEM medium containing 5.6 mmol/L glucose and 0.5% FBS before treatment. Cells were treated with high glucose (30 mmol/L glucose) in the absence or presence of water extract (400 μg/mL), purified nucleosides (100 μg/mL), polysaccharides (100 μg/mL), ethanol extract (200 μg/mL) and ergosterol (20 μmol/L).

RMCs were lysed in RIPA lysis buffer to extract proteins. Samples were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk, the blots were incubated with primary antibodies against fibronectin, collagen I and β-actin overnight. The membranes were incubated with secondary antibody and the protein bands were detected with enhanced chemiluminescence (ECL) substrates (Amersham, Little Chalfont, UK).

3. Results and discussion

3.1. Analysis of nucleosides fraction by HPLC

The major compounds in the nucleosides were detected and identified by HPLC with a diode array detector. As shown in Fig. 1, the typical HPLC chromatograms of 10 major compounds were initially identified by comparing the retention time and ultraviolet spectra of the sample peaks with corresponding standards.

Table 1
Retention times of unknown nucleosides and their concentrations in cultivated *C. sinensis*.

Nucleosides	Concentrations (mg/g)	t_R (min)
Cytidine	0.753 ± 0.016	3.76
Adenine	0.247 ± 0.006	4.49
Guanine	6.582 ± 0.082	5.02
Uracil	0.211 ± 0.005	6.64
Hypoxanthine	0.251 ± 0.004	8.71
Uridine	4.028 ± 0.023	9.94
Adenosine	5.909 ± 0.018	12.18
2′-Deoxyadenosine	0.572 ± 0.003	12.94
Guanosine	4.241 ± 0.041	15.39
Thymidine	0.374 ± 0.013	20.28

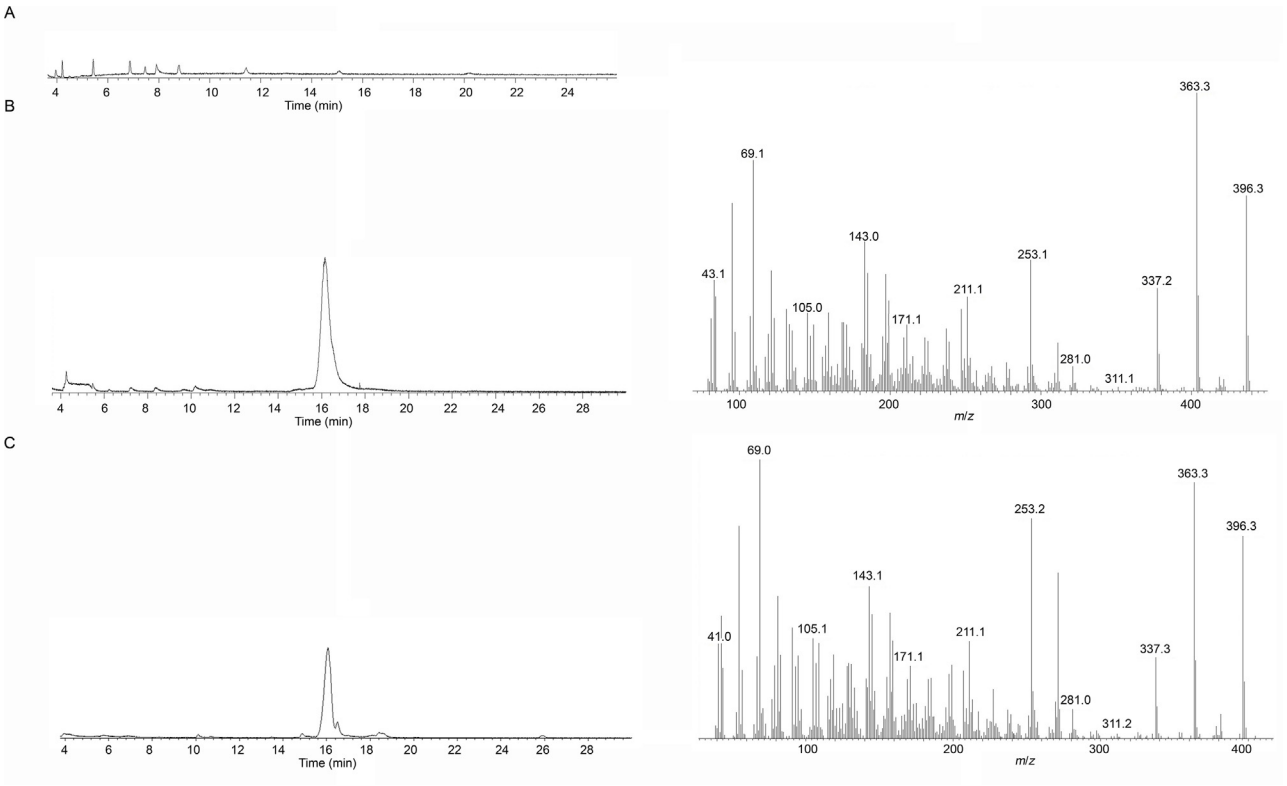

Fig. 2. Typical chromatograms of (A) blank, (B) ergosterol standard and (C) unknown peaks in cultivated *C. sinensis* determined by GC–MS.

The retention times of the 10 nucleosides and their concentrations in cultivated *C. sinensis* were shown in Table 1. Results showed that the total content of nucleosides in cultivated *C. sinensis* was nearly 2%.

3.2. Analysis of sterols fraction by GC–MS and HPLC

The major compounds in the sterols were identified by GC–MS, and compared to the NIST 08 standard mass spectrometry library. As shown in Fig. 2, the typical GC–MS chromatograms of sterols were initially identified, and ergosterol is the main sterol in cultivated *C. sinensis*. The molecular formula of ergosterol is $C_{28}H_{44}O$, with a molecular weight of 396.65 g/mol and a CAS number of 57–87-4. Fig. 3 showed the typical HPLC chromatograms of ergosterol standard and cultivated *C. sinensis* sample. The retention time of the ergosterol was at 21.26 min. Result calculated that the content of ergosterol in cultivated *C. sinensis* was about 5.55 mg/g.

3.3. Detection of polysaccharides fraction by sulfuric acid-phenol method

The content of polysaccharides in cultivated *C. sinensis* powder was determined by sulfuric acid-phenol method. The standard curves were linear over the concentration range 5 to 100 μg/mL (y = 0.0054x - 0.0046, r = 0.9984), and results showed that the content of polysaccharides in *C. sinensis* was 43.71 mg/g. As shown in Table 2, no proteins, peptides, amino acids and reductive monosaccharides existed in the extracted polysaccharides.

3.4. Effects of nucleosides, sterols and polysaccharides on extracellular matrix expression

Collagen I and fibronectin are important compositions of extracellular matrix, making them indicators for fibrosis evaluation (Ge et al., 2019). As is shown in Fig. 4, the expression of collagen I and fibronectin proteins were elevated after high glucose stimulation, indicating that the synthesis and secretion of ECM components increased. However, the treatment of isolated nucleosides, polysaccharides and ergosterol significantly decreased the levels of ECM proteins, which means all of them possess anti-fibrotic activity to some extent. In addition, nucleosides and ergosterol exhibited superior inhibitory effects, suggesting that they may be the main active components in *C. sinensis* for anti-fibrosis.

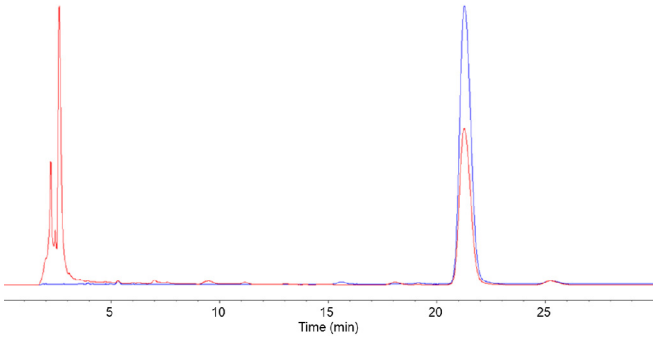

Fig. 3. Typical chromatograms of ergosterol standard (blue line) and ergosterol in cultivated *C. sinensis* (red line) determined by HPLC.

Table 2
Impurity determination of polysaccharides.

Methods	Positive control	Negative control	Polysaccharides sample
Biuret reaction	+	–	–
Ninhydrin reaction	+	–	–
Flynn's regent reaction	+	–	–
Molisch reaction	+	–	+

Notes: Positive control, glucose standard; Negative control, distilled water; + means positive result; — means negative result.

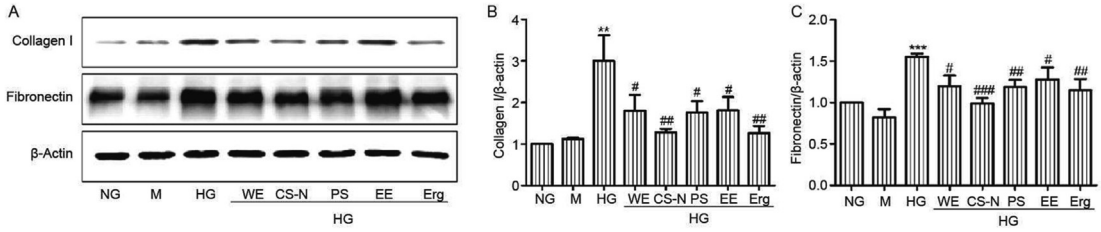

Fig. 4. Effects of various components on expression of ECM in RMC cells stimulated by high glucose. A: Typical images of collagen I, fibronectin and β-actin by Western blot; B and C: Relative expression levels of collagen I and fibronectin. NG: normal glucose, 5.6 mmol/L glucose; M: 5.6 mmol/L glucose + 24.4 mmol/L mannitol; HG: high glucose, 30 mmol/L glucose; WE: water extract; CS-N: nucleoside sample; PS: polysaccharides; EE: ethanol extract; Erg: ergosterol. Data are presented as the mean ± SD (n = 3). ** P < 0.01, *** P < 0.001 *vs.* NG group; $^{\#}$ P < 0.05, $^{\#\#}$ P < 0.01 and $^{\#\#\#}$ P < 0.001 *vs.* HG group.

4. Conclusion

Above all, this study revealed the chemical compounds and content of nucleosides, sterols and polysaccharides in cultivated *C. sinensis* powder. Ten kinds of nucleosides were quantified (cytidine, adenine, guanine, uracil, hypoxanthine, uridine, adenosine, 2′-deoxyadenosine, guanosine, and thymidine), and their total content in cultivated *C. sinensis* was about 2%; Ergosterol was the main sterol in cultivated *C. sinensis* and its content was about 0.55%; The content of polysaccharides in cultivated *C. sinensis* was about 4.4%; All three components exhibited anti-fibrotic activity, among which, nucleosides and sterols/ergosterol showed superior activity and were the main active components responsible for anti-fibrosis. Further investigation of the mechanistic insight into the critical role of nucleosides and sterols as promising anti-fibrotic agents may be useful for the therapy of pulmonary and renal disease. This study provides reference for the separation and determination of nucleosides, sterols/ergosterol and polysaccharides, as well as provides valuable information on the further optimization of more effective remedies in functional foods.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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