Khan *et al. Annals of Microbiology* (2024) 74:9
https://doi.org/10.1186/s13213-024-01752-z

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE

Open Access

Comprehensive analysis of metabolites in the mycelium of *Cordyceps fumosorosea* cultured on *Periplaneta americana*

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Abstract

Background *Cordyceps fumosorosea* is one of the common species within the *Cordyceps* genus, which are cultured on *Periplaneta americana*. This study aimed to determine the composition of bioactive compounds, including β-glucans, polysaccharides, cordycepic acid, flavonoids, ergosterol, and nitrogenous compounds (specifically nucleosides: adenosine, guanosine, adenine, and hypoxanthine), present in the mixture and mycelium at various time incubation.

Methods Different bioactive compounds, including β-1,3-glucan, polysaccharides, cordycepic acid, flavonoids, ergosterol, and nitrogenous compounds (specifically nucleosides: adenosine, guanosine, adenine, and hypoxanthine) are detected from *C. fumosorosea* which cultured on *P. americana* by UV and HPLC.

Results Mycelia of *C. fumosorosea* were cultivated in *P. americana* (medium). The highest total β-1,3-glucan content was observed in the mixture (*C. fumosorosea* + *P. americana*) after 25 days (69.21 ± 0.07 mg/g) and in the mycelium after 25 days (56.32 ± 0.39 mg/g) using different solvents. The highest β-1,3-glucan content was attained at specific time incubation in other solvents. The content of cordycepic acid peaked at 52.28 ± 0.11 mg/g in the mixture after 25 days and at 46.96 ± 0.13 mg/g in the mycelium after 25 days. The polysaccharide content reached its highest level in the mixture after 20 days (16.68 ± 0.38 mg/g) and in the mycelium after 20 days (14.85 ± 0.10 mg/g). The peak flavonoid content was observed in the mixture after 25 days (4.65 ± 0.24 mg/g) and in the mycelium after 25 days (4.04 ± 0.07 mg/g). Nucleosides, including adenine, adenosine, hypoxanthine, and guanosine, exhibited their highest levels after 25 days in the mixture and mycelium. Ergosterol content peaks at 25 days (2.25 ± 0.04 mg/g).

Conclusion *Cordyceps fumosorosea* mixture and time incubation of 20 and 25 days are optimal for detecting a diverse array of bioactive compounds, including β-1,3-glucan, polysaccharides, cordycepic acid, flavonoids, ergosterol, and nucleosides.

Keywords Comprehensive analysis, Metabolites, *Cordyceps fumosorosea*, *Periplaneta americana*

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Introduction

Historical records date the use of *Cordyceps* as a medicinal genus to approximately 2000 BC in ancient China. It is highly valued in traditional Chinese medicine (TCM) owing to its various biological activities and potential health benefits (Li et al. 2001). *Cordyceps* extracts have a long history of use in TCM and have gained attention from the pharmaceutical industry because of their potential health benefits, including immune function, vitality, and respiratory (Gu et al. 2007). An important TCM belonging to the Ascomycota has been widely used in East Asia as a traditional crude extract for various disorders. It contains several beneficial active ingredients such as vitamins, functional proteins, polysaccharides, nucleotides, various oils, and sterols, which are utilized for multiple medicinal purposes (Ng and Wang 2005). *Cordyceps* can enhance the immune system and alleviate metabolic disorders. It possesses anti-inflammatory, antioxidant, anti-aging, anti-cancer, antibacterial, and anti-fatigue activities (Miao et al. 2022). Like other medicinal herbs, *Cordyceps* is considered an important medicinal herb for the treatment of different disorders (Wasser et al. 2002). Alterations in the growth medium profoundly influence the growth and quality of *Cordyceps*. Grains have been extensively employed as substrates for *Cordyceps* cultivation, owing to their convenience and widespread accessibility. Insects, which are the primary natural source of nutrients for *Cordyceps*, have also been incorporated, particularly pupae, to replicate the natural conditions. The growth and quality of *Cordyceps* are profoundly influenced by alterations in the growth medium (Turk et al. 2022). Dried worms and fresh adults of *P. americana* have been used as (TCM) drugs (Yu et al. 2016). *Periplaneta americana* is described as salty and acid that promotes blood circulation, removes blood stasis, and benefits conditions related to poor circulation, such as edema. It is used in traditional medicine to treat infantile malnutrition, tonsillitis, body phlegm, carbuncles, sore throats, and insect bites. Modern research suggests potential anti-tumor effects, enhanced immunity, tissue repair, stabilized blood pressure, and improved microcirculation in the liver (Yu et al. 2016). The active ingredients extracted from *P. americana* have been formulated as clinical drugs in China. These include "Xiaozheng Yigan Tablets," "Kangfuxin Liquid," "Ganlong Capsule," and "Xinmailong Injection." Among these, "Xiaozheng Yigan Tablets" stand out as oral medications that exhibit strong anti-tumor and antibacterial effects. Research has indicated its efficacy in reducing liver inflammation, fostering the recovery of liver function, and lessening the extent of liver fibrosis in individuals afflicted with hepatitis B virus (HBV) infection (Zhao et al. 2017). The optimal conditions for bioactive compound production were pH 5.5, 25 °C, inoculum size 8% v/v, inoculum time 72 h, and incubation time of 24 days. The optimal culture medium included 1.5% dextrose, 0.8% yeast extract, 0.3% dibasic potassium phosphate, 0.1% monobasic potassium phosphate, 0.05% sodium chloride, 0.05% magnesium sulfate, and 0.05% sodium chloride (Tuli et al. 2014). Since the beginning, insect pathogenic fungi have been considered potential agents for the biological control of a variety of insects. Kepler et al. regrouped *I. fumosorosea*, *S. fumosorosea* and *P. fumoroseus* into *C. fumosorosea* (Kepler et al. 2017; Ali et al. 2010a; Khan et al. 2023), *C. fumosorosea* has wide geographic distribution, strong ecological adaptability, easy culture, fast growth rate, quick spore production, and extensive use in biological control. Owing to its wide host range, it is a highly effective and economical insecticide with a broad range of activities, low production costs, and safety for humans and non-target species (Ali et al. 2010b). However, there are complications associated with *C. fumosorosea*, as it takes some time to take effect after practical application, and it is easily affected by its surroundings. Recent findings indicate that nanoparticles of *C. fumosorosea* can effectively control various insect pests (Ali et al. 2017; Banu and Balasubramanian 2014). Active compounds (e.g., cordycepin, polysaccharides, cordycepic acid, cordymin) isolated from the *Cordyceps* exhibit a variety of pharmacological effects, including anti-tumor, antioxidant, anti-inflammatory, immunomodulatory, and anti-microbial activities (Ahn et al. 2000; Zhang et al. 2017; Zhao et al. 2019). *Cordyceps* is a medicinal mushroom known for its various bioactive components, including *Cordyceps* polysaccharides (CP). Polysaccharides (CP) are molecules that have been found to have anti-inflammatory and anti-tumor properties (Li et al. 2001; 2002). The polysaccharides in the form of extracellular polysaccharides, intracellular-polysaccharides (ICP), exopolysaccharides (EPS), heteropolysaccharides (HPS), mannoglucan (MG), and D-glucan are distributed in various species of the genus *Cordyceps* and possess several biological activities, including immunomodulatory, hypolipidemic, hyperglycemic, steroidogenic, anticancer, anti-metastatic, anti-diabetic, anti-inflammatory, and anti-oxidative activities to manage fatal diseases (Shashidhar et al. 2013). β-glucan, a key bioactive compound found in various medicinal mushrooms such as Phellinus and Chaga (Inonotus), enhances the immune system and exhibits anti-tumor properties. This facilitates an increase in the white blood cell population, thereby bolstering cellular immunity (Song et al. 2020). β-Glucans in medicinal mushrooms, such as *C. militaris*, consist of d-glucose units with β-1,3 glycosidic linkages. These polysaccharides have various beneficial effects, including anti-inflammatory, antioxidant, anticancer,

antimetastatic, immunomodulatory, hypoglycemic, and hypolipidemic effects. Therapeutic interventions have successfully eliminated tumor cells by inducing apoptosis in different types of malignancies, including leukemia, breast cancer, liver cancer, stomach cancer, ovarian cancer, urinary bladder cancer, lung cancer, and epithelioma cancer (Oh et al. 2019; Xiao and Zhong 2007). Nitrogenous bases and nucleosides play major roles in biological systems (Zhang and Yuan 1997). Nitrogenous compounds, such as nucleosides (adenosine, cordycepin, guanosine, inosine, thymidine, and uridine), have been found in *Cordyceps*. Cordycepin, derived from adenosine, is an important compound in TCM used for treating diseases, particularly cancer, because of its potent anti-angiogenic, anti-metastatic, and anti-proliferative properties as well as its ability to induce apoptosis (Yang et al. 2011). Ergosterol plays a multifaceted role in the food, feed, and pharmaceutical industries. It serves as a quality indicator for food, dietary supplements in animal feed, precursors for vitamin D2, and a starting material for the production of steroid hormone drugs (Kitchawalit et al. 2014). Nearly 80–85% of all medicinal mushroom products are extracted from their fruiting bodies, whereas only 15% are derived from mycelial cultures (Lindequist et al. 2005). This is the first study to investigate the cultivation of *C. fumosorosea* using *P. americana* as the culture medium. This study focused on characterizing bioactive compounds such as β-1,3-glucan, polysaccharides, cordycepic acid, flavonoids, ergosterol, and nitrogenous compound nucleosides (adenosine, guanosine, adenine, and hypoxanthine) in *C. fumosorosea*. This involved a comparison of the bioactive compound content between the mixture (*C. fumosorosea* + *P. americana*) and the mycelium (*C. fumosorosea* only), as well as determining the optimal time incubation (20 and 25 days).

Material and method

Experimental species

The experimental species are *Cordyceps fumosorosea* and *Periplaneta americana*.

Sample collection and culture preparation

Cordyceps fumosorosea was collected from the Dayao County, Chu Xiong City, Yunnan Province, China was isolated and identified by Prof. Yu Hong and his team members, and was stored in the Yunnan Fungal Culture Collection (YFCC). The related specimens were deposited in the Yunnan Herbal Herbarium (YHH), and the strain number SP24. PDA medium was prepared as follows: 200 g peeled potato, 20 g agar, 20 g uveose, and fixed to 800 mL. Wash and peel the potatoes, cut them into thin slices, add water 1000 mL, and boil for about 20 min; then mix, heat, divide into conical flasks, kill bacteria at 121 °C for 30 min, cool to 50 °C, add 0.1 g streptomycin and 0.05 g tetracycline per liter to inhibit the growth of bacteria. Plates were poured at approximately 10 mL per flask (Dong et al. 2022). Second, the seed liquid was cultured by low-temperature preservation of the tube slope strain inoculated on the PDA solid medium for 10 days at 25 °C, and a large number of colonies covered the surface of the medium. The activated bacteria block was approximately 0.5 cm^2 and inoculated in the base liquid medium, which was sterilized and cooled. After 5 days of constant temperature culture, rotating speed 120/rpm by centrifuge for 2 min, temperature 25 °C, liquid bacteria (bacterial balls) were sucked at a Babbitt tube, and 6% inoculated into a sterilized cold cooling medium of *P. americana*. The *P. americana* culture medium was prepared as follows: 35 g *P. americana* was crushed, soaked for 4 h, added 250 mL conical flask, added 1.35 g glucose, 0.3 g glycerol, 5 g β-1, 3glucan, mixed and killed at 121 °C for 30 min, cooled and reserved.

Morphological observations

The specimens were tested in a laboratory. In order to initiate observations, the cultures grown on *P. americana* (medium) slants were transferred to a medium flask and placed in an incubator at 25 °C for 15, 20, 25, 30, 35, 40, 45, 50, and 55 days at 5 days incubation. The purpose of this incubation was to allow specimens to develop and mature. The colors of fresh specimens and cultures were assessed using a color standard. The macroscopic stature of different stages of *P. americana* was used as the medium. Under solid culture conditions, *C. fumosorosea* produces sporophore bundles. Morphological descriptions of the asexual morphs were conducted following a previously described method (Wang et al. 2020).

Determination of the flavonoid content

Ultraviolet (UV) spectroscopy was used to determine total flavonoid content using the aluminum nitrate-sodium nitrite chromogenic method (Wu et al. 2010). Samples (0.200 g) were collected in tube-shaped bottles, 5 mL of methanol (70%), and the tube-shaped bottle was allowed to stand for 60 min in a water bath at 25 °C. Then sample solution was centrifuged for 3 min, 2 mL of the sample solution was taken test tube, add 1 mL of buffer solution [100 mL distilled water, 2.72 g sodium acetate trihydrate ($C_2H_3NaO_2$) (NaAc)] plus 1.15 mL acetic acid ($C_2H_4O_2$) or (CH_3COOH) (HAC) at pH 5.2, and chemical reagent 2 mL [100 mL methanol plus 1.34 g aluminum chloride hexahydrate ($AlCl_3.6H_2O$)] and shake color become yellow. The absorbance of the reaction solution was measured at 400 nm using a spectrophotometer (UV–Vis Jasco V-730, Jasco, USA). Distilled water was used as the control. A standard curve

($y = 0.0092x + 0.0249$, $R^2 = 0.985$) for flavonoids (Sigma-Aldrich, USA) was used to calculate.

Presence of the polysaccharides content

Crude polysaccharides were prepared using a slightly optimized procedure (Wang et al. 2014). The 0.200-g sample is taken in tube-shaped bottles. Add 5 mL methanol (80%) to each tube-shaped bottle. The tube-shaped bottles were refluxed using an ultrasound machine for 30 min. Refluxing helps to extract polysaccharides from the sample. After refluxing, the tube-shaped bottles were centrifuged for 3 min. Take, 1 mL of the sample solution was transferred to a test tube, and 1 mL of water was added to the test tube containing the sample solution. A mixture of 6 mL 80% sulfuric acid (H_2SO_4) and anthrone ($C_{14}H_{10}O$) (1 g) was added to a test tube containing the sample and water. The solution was then allowed to stand for 10 min in a water bath at 40 °C. After 10 min, the color became green, and the absorbance of the reaction mixture was measured at 625 nm using a spectrophotometer. In order to prepare a control solution, the sample was replaced with distilled water in the same reaction mixture. A standard curve ($y = 0.64688_X + 0.05339$ $R^2 = 0.99942$) for polysaccharides was established using a known polysaccharide standard (such as the one from Sigma-Aldrich, USA). The polysaccharide content in the extract was calculated based on the absorbance values obtained from the sample.

Detection of cordycepic acid content

Cordycepic acid content was detected using a previously described method (Xiao et al. 2009). This technique involves measuring the absorbance of a sample of *Cordyceps* at a specific wavelength of 412 nm, which corresponds to cordycepic acid. The sample (0.200 g) was placed in a tube-shaped bottle, 5 mL water was added, and the mixture was refluxed at 90 °C for 60 min. Add 1 mL of the sample solution in a test tube, 1 mL of potassium periodate (KIO_4) 0.346 g/L plus hydrochloric acid (HCl) 12 mol/L reagent in the test tube, and let stand the test tube for 10 min. Then add 2 mL of 0.1% L-rhamnose solution [(1 g L-rhamnose plus 100 mL water)], 4 mL of the newly prepared 60 mL Nash solution (9 g ammonium acetate [$NH_4CH_3CO_2$] plus 120 µl Acetic acid [CH_3COOH] plus 120µL diacetone), mix well, sample solution heat at 53 °C for 15 min, and then put test-tube into 15 °C cool water for 10 min. The absorbance of the reaction mixture was measured at 412 nm using a spectrophotometer (UV–Vis Jasco V-730, Jasco, USA). In order to prepare a control solution, the sample was replaced with distilled water. A standard curve ($y = 0.7007x - 0.1868$ ($R^2 = 0.999$) for cordycepic acid (Sigma-Aldrich, USA) was used for calculations.

Assessment of β-1,3-glucan content using various solvents

B-1,3-glucan was determined using a colorimetric method (Dávila et al. 2023). This technique involves measuring the absorbance of *cordyceps* samples at a specific wavelength of approximately 523 nm. Assessment of β-1,3-glucan ($C_{18}H_{32}O_{14}$) through 4 solvents, potassium hydroxide (KOH), sodium hydroxide (NaOH), hydrogen chloride (HCl), and dimethyl sulfoxide ($(CH_3)_2SO$ (DMSO). Take a 0.200-g sample in tube-shaped bottles. Four milliliters of potassium KOH was added, and the bottle was refluxed for 30 min in a 60 °C water bath. It was centrifuged for 3 min in order, then 0.5 mL of the sample solution in a test tube, 0.5 mL HCl to regulate the pH level of the sample solution, 1 mL of buffer solution pH 7.5 [sodium phosphate (Na_2HPO_4)] plus sodium phosphate (NaH_2PO_4)•H_2O) and 2 mL of Congo Red, and the color of the sample solution becomes dark red. The test tubes were left to stand for 10 min at 20 °C. The sample solution was analyzed using UV spectroscopy, and β-1,3-glucan was detected. Sodium hydroxide (NaOH) is an important solvent for reducing β-1,3-glucan content. Four milliliters of NaOH was added to the solution and allowed to stand for 60 min in a water bath at 90 °C. Centrifugation was performed for 3 min to rotate the sample solution. Subsequently, 0.45 mL of the sample solution was extracted and transferred to a test tube, where 0.55 mL of HCl was added to regulate the pH level. Following this, 1 mL of buffer solution and 2 mL of Congo Red were added, resulting in a dark red coloration of the sample solution. Test tubes were allowed to stand for 10 min in a 20 °C bath. The sample solution was analyzed using UV spectroscopy. The detection of β-1,3-glucan by HCl solvent was added to 4 mL, and the bottle was refluxed for 60 min in a 90C° water bath. The sample solution was centrifuged for 3 min, then 0.70 mL of the sample solution in a test tube, 0.30 mL KOH to regulate the pH level of the sample solution, 1 mL of buffer solution, and 2 mL of Congo Red, and the color of the sample solution becomes dark red. The sample solution in the test tube was allowed to stand for 10 min at 20 °C water. The sample solution was then analyzed by UV spectroscopy, and β-1,3-glucan was detected using hydrogen chloride (HCl) solution. Four milliliters of dimethyl sulfoxide ($(CH_3)_2SO$ (DMSO) was added to the sample in the test tubes, and the test tubes were refluxed for 60 min in a 70 °C water bath. It was centrifuged for 3 min to rotate the sample solution, then take 0.25 mL of the sample solution in a test tube, 0.75 mL ($(CH_3)_2SO$ (DMSO), adds 1 mL of buffer solution, and 2 mL of Congo Red, and the color of the sample solution becomes red. Test tubes were left to stand for 10 min in a water bath at 20 °C. β-1,3-glucan was detected by UV spectroscopy using a dimethyl sulfoxide ($(CH_3)_2SO$ (DMSO) solvent. A standard

curve ($y = 0.4415 + 0.0006$, $R^2 = 0.9801$) for β-1,3-glucan (Sigma-Aldrich) was used to calculate the β-1,3-glucan content.

Measurement of the ergosterol concentration

Ergosterol content was determined using high-performance liquid chromatography (HPLC) (Chang et al. 2005). First, a standard curve was acquired by accurately weighing 0.200 g of standard ergosterol and obtaining a solution of standard ergosterol (0.04 mg/mL) in pure methanol within a 50-mL volumetric bottle. Subsequently, 1 mL of the sample solution was absorbed using a syringe and filtered manually through a 0.45-μm micro-porous filter membrane into an HPLC™ (Ultimate 3000, Shimadzu, Kyoto, Japan) sample vial. Various sample sizes were used for detection based on the chromatographic conditions. The chromatographic conditions were as follows: Waters C_{18} column, pure methanol as the mobile phase, equal-degree elution as the elution method, column temperature of 30 °C, detection wavelength of 284 nm, flow rate of 1 mL/min, and run time of 20 min. For test sample determination, a liquid culture sample weighing 0.05 g was accurately weighed and placed in a 5-mL centrifuge tube. Subsequently, 2 mL of pure methanol solution was added to the tube. After thorough oscillation and mixing, the samples were subjected to extraction for 3 h, followed by ultrasonic extraction for 1 h. The solution was centrifuged at 4000 r/min for 5 min. One milliliter of the sample liquid was absorbed using a syringe and filtered manually through a 0.45-μm microporous filter membrane into an HPLC sample vial. The sample was subsequently analyzed using the aforementioned chromatographic conditions, with a sample volume of 30μL.

Analysis of nucleosides and their analogs

Nucleosides and their analogs were determined using high-performance liquid chromatography (HPLC) (Zou et al. 2017). Standard curves were generated for adenine, hypoxanthine, adenosine, and guanosine. Each standard product (10 mg) was dissolved in a 10-mL volume bottle containing 20% methanol to achieve a fixed content of the standard product solution. One-milliliter sample was then taken using a syringe and filtered manually through a 0.45-μm micro-porous filter membrane into an HPLC sample. Subsequently, the sample was analyzed using an HPLC™ (Ultimate 3000, Shimadzu, Kyoto, Japan) chromatography under the following conditions: Waters Symmetry C_{18} column (4.6 mm × 250 mm, 5 μm) was employed as the column type, and the mobile phases consisted of methanol (A) and water (B). A gradient elution method was employed with the following proportions:0–10 min, 5–10% A; 10–15 min, 10–40% A; 15–25 min, 40–25% A; 25–27 min, 25–5% A; 27–30 min, 5% A. The column temperature was set at 30 °C, the detection wavelength was 260 nm, the flow rate was maintained at 1 mL/min, and the total running time was 30 min. A standard regression curve was obtained by plotting the mass on the abscissa and the peak area on the ordinate. In the test sample determination, a 0.05-g sample was accurately weighed and soaked in 2 mL of a 20% methanol solution for 1 h. The sample was subjected to ultrasound extraction for 30 min, followed by 5 min of centrifuge at 4000r/minute to collect the supernatant. The extraction process was repeated, and the solvent was added to achieve a constant volume of 5 mL, ensuring thorough mixing. A 1-mL sample solution was then obtained using a syringe and manually filtered through a 0.45-μm micro-porous filter membrane into an HPLC sample vial. The content of each component in the test sample solution was determined under the same chromatographic conditions, with a sample volume of 40μL, was determined using the same chromatographic conditions.

Statistical analysis

The data was analyzed with GraphPad Prism version 5.01 for Windows (GraphPad Software, LanJolla, CA, USA), with error bars representing mean ± SD. Statistical significance analysis was performed using a one-way ANOVA with Tukey's Multiple Comparison Test (Graph-Pad Software, La Jolla, CA) or by (for Western blots) t-test for independent samples in PASW Statistics 18 (PASW Statistics for Windows, Version 18.0. SPSS Inc., Chicago, IL) with a p-value: $p < 0.05$, $p < 0.01$, $p < 0.001$ (Sawadpongpan et al. 2023).

Result

Evaluation of various compounds production by *C. fumosorosea*

The various compounds of *C. fumosorosea* mixtures and mycelia, such as β-1,3-glucan, cordycepic acid, polysaccharides, flavonoids, ergosterol, nucleosides and their analogs (adenine, adenosine, guanosine, and hypoxanthine) were examined over a period of 15 to 25 days as well as 35, 40, 45, 50, and 55 days.

Morphological features

Figure 1 shows macroscopic pictures of *P. americana* used as a medium on different days. In solid culture conditions, *C. fumosorosea* produced sporophore bundles at about 15 days, mycelium thicken at 20 days, and sporophore bundles continued to grow beyond 55 days. At 15, 20, 25, 30, 35, and 55 days, the mycelium bundles and colonies were white at first and gradually became light pink to pink. The growth and development of *C. fumosorosea*

Fig. 1 Different stages of *C. fumosorosea* cultured on *P. americana* medium. Scale bars: A-F=2cm, A: 15 days, B: 20 days, C: 25 days, D: 30 days, E: 35 days, F: 55 days, G: wild type

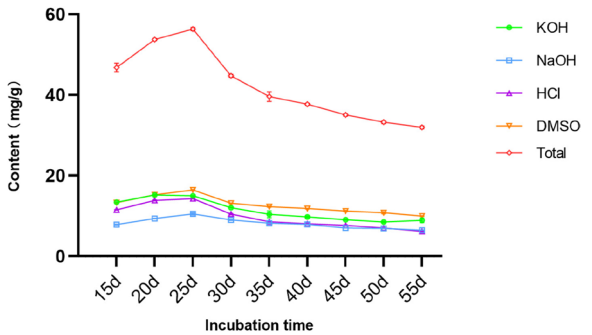

Fig. 2 β-1,3-glucan content of mycelium detected by KOH, NaOH, HCl, DMSO, and total. The error bar indicates the mean ± standard error, which can be seen on the bar graph

were clearly delayed in comparison to liquid culture conditions. The highest biomass content was observed.

Determination of the β-1,3-glucan content

The total β-1,3-glucan content obtained from Figs. 2, 3, and 4 verifies that in the 25-day mixture, the content was 69.21 ± 0.07 mg/g, while the mycelium had a content of 56.32 ± 0.39 mg/g. This indicated that both parts of *C. fumosorosea* had the highest total β-1,3-glucan content. However, the total β-1,3-glucan content ceased after 25 days in the mixture and 25 days in the mycelium. This implies that the response of total β-1,3-glucan content in *C. fumosorosea* depends on both the body part and incubation. Consequently, the total β-1,3-glucan content of the mixture indicated that the total β-1,3-glucan content ceases after 25 days. Similarly, the minimum total β-1,3-glucan content in the body parts of the mixture was observed after exposure for 20 days. The results clearly suggest that on a 25-day period, the mixture body part contains higher total β-1,3-glucan content than the mycelium. The structure of total β-1,3-glucan is shown in Fig. 11 (1). KOH was used as a solvent for β-1,3-glucan content detection, and the mixture showed the highest β-1,3-glucan content at 25 days (19.26 ± 0.07 mg/g), whereas the mycelium bodies showed a low β-1,3-glucan content at 20 days (15.24 ± 0.01 mg/g). β-1,3-glucan content decreased after 25 days in the mixture and 20 days in the mycelium. NaOH solvent showed the highest β-1,3-glucan content in the mixture at 25 days

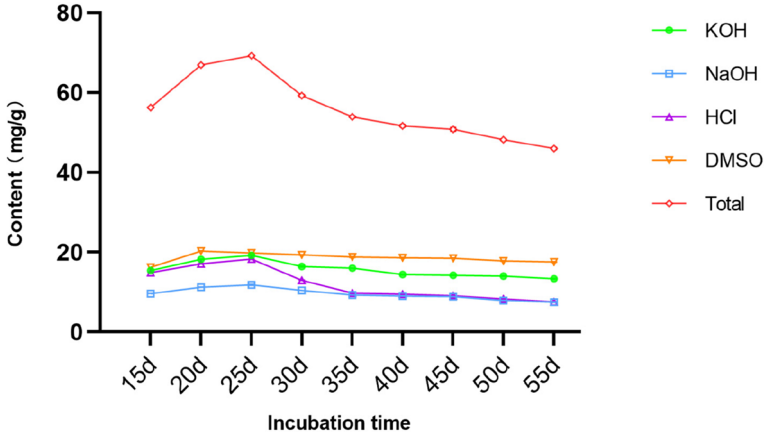

Fig. 3 β-1,3-glucan content of mixture detected by KOH, NaOH, HCl, DMSO, and total. The error bar indicates the mean ± standard error, which can be seen on the bar graph

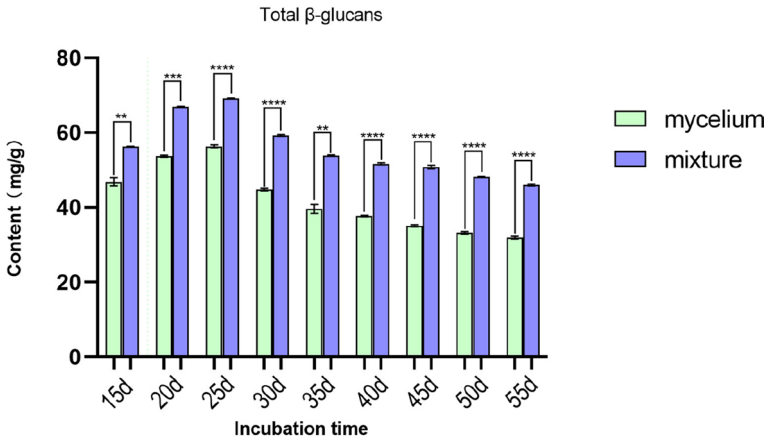

Fig. 4 For total β-1,3-glucan content, the error bars indicate mean ± standard error, which can be seen in the bar graph. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

(11.82 ± 0.14 mg/g), whereas in the mycelium, 25 days (10.52 ± 0.25 mg/g). After 25 days, β-1,3-glucan gradually decreased in both the mixture and mycelium. HCl solvent showed the highest β-1,3-glucan content in the mixture at 25 days (18.30 ± 0.04 mg/g), whereas in the mycelium, it was 25 days (14.34 ± 0.08 mg/g). After 25 days, the contents gradually decreased. DMSO was used as a solvent for β-1,3-glucan content detection, and the mixture showed the highest contents at 20 days (20.33 ± 0.02 mg/g), whereas the mycelium bodies showed a similar content at 25 days (16.45 ± 0.10 mg/g). The β-1,3-glucan content decreased after 20 days in the mixture and 25 days in the mycelium. The results in Fig. 4, $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, suggest that at 25 days (KOH solvent), 25 days (NaOH solvent), 25 days (HCl solvent), and 20 days (DMSO solvent), the body part of the mixture had a higher total contents than the mycelium body part of *C. fumosorosea*.

Determination of the cordycepic acid content

The results obtained from Fig. 5 verify that the 20-day 52.28 ± 0.11 mg/g mixture and the mycelium 25-day 46.96 ± 013 mg/g showed the maximum cordycepic acid content in both parts of *C. fumosorosea*. The cordycepic acid content mixture part is 47.45 ± 0.08, 49.17 ± 0.08, 52.28 ± 0.11, 50.65 ± 0.19, 48.32 ± 0.25, 46.87 ± 0.42, 44.96 ± 0.15, 43.26 ± 0.11, and 41.93 ± 0.22 mg/g and in the mycelium part, 46.44 ± 0.10, 46.96 ± 013, 46.96 ± 013, 45.76 ± 0.10, 43.20 ± 0.09, 42.19 ± 0.13, 41.65 ± 0.08, and 41.03 ± 0.01 mg/g from 15 to 55-day with 5-day incubation. However, the minimum cordycepic acid content in both parts has been reported at 50 days and 55 days, which indicates that the cordycepic acid content decreases with time. This implies that the response to cordycepic acid content depends on the body part and incubation. Consequently, the cordycepic acid content of the mixture indicated that after 20 days, it gradually

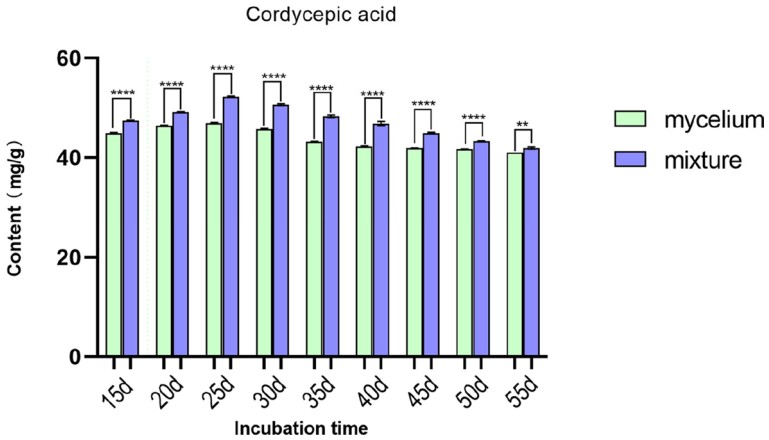

Fig. 5 For cordycepic acid content, the error bars indicate the mean and ± standard error, which can be seen in the bar graph. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

decreased in the mycelium after 25 days. Similarly, the minimum cordycepic acid content in both parts was reported after 25-day induction. The results suggested that ($p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$) the 20-day mixture of body parts had a higher cordycepic acid content than the mycelium body. The structure of cordycepic acid is shown in Fig. 11 (2).

Determination of the polysaccharides content

The results obtained from Fig. 6 verify that the 20-day 16.68 ± 0.38 mg/g mixture and the mycelium 14.85 ± 0.10 mg/g showed the maximum polysaccharide content in both parts of *C. fumosorosea*. The polysaccharide content mixture part is 14.98 ± 0.04, 16.68 ± 0.38, 16.11 ± 0.16, 15.45 ± 0.20, 14.50 ± 0.29, 14.39 ± 0.05, 13.88 ± 0.04, 13.74 ± 0.02 and 13.41 ± 0.25 mg/g and in the mycelium part, 14.08 ± 0.07, 14.85 ± 0.10, 14.23 ± 0.11, 13.63 ± 0.06, 13.50 ± 0.01, 13.39 ± 0.01, 12.86 ± 0.14, 12.22 ± 0.10, and 11.96 ± 0.10 mg/g from 15 to 55-day with 5-day incubation. However, the minimum polysaccharide content in both parts has been reported at 50 days and 55 days, which indicates that the polysaccharide content decreases with time. This implies that the response of polysaccharide content depends on the body part and the incubation time. Consequently, the polysaccharide content of the mixture indicated that after 20 days, the polysaccharide content decreased gradually. Similarly, the minimum polysaccharide content in both body parts was reported after 20 days of induction. The results suggest that ($p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$) at 20 days the mixture of body parts had a higher polysaccharide content than the mycelium body. The structures of the polysaccharides are shown in Fig. 11 (3).

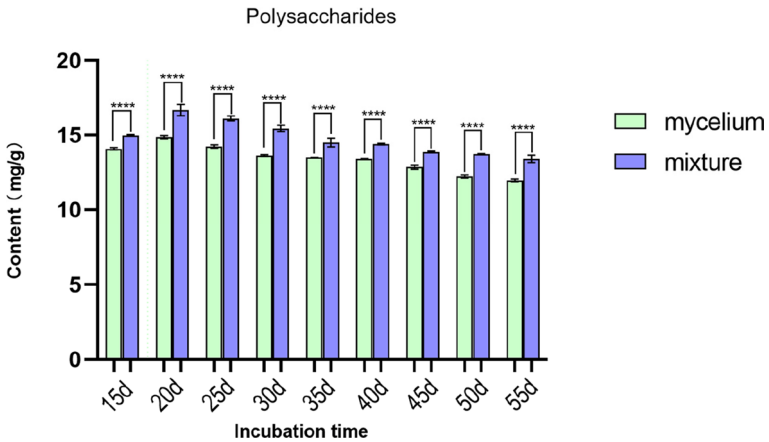

Fig. 6 For polysaccharide content, the error bars indicate the mean and ± standard error, as shown in the bar graph. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

Determination of the flavonoid content

The results obtained from Fig. 7 verify that the 25-day 4.65 ± 0.24 mg/g mixture and the mycelium 4.04 ± 0.07 mg/g showed the maximum flavonoid content in both parts of *C. fumosorosea*. The flavonoid content mixture part is 4.15 ± 0.05, 4.44 ± 0.37, 4.65 ± 0.24, 4.31 ± 0.06, 4.14 ± 0.02, 4.03 ± 0.02, 3.80 ± 0.05, 3.62 ± 0.03, and 3.41 ± 0.15 mg/g, and in the mycelium part, 3.55 ± 0.11, 4.01 ± 0.03, 4.04 ± 0.07, 3.90 ± 0.02, 2.91 ± 0.02, 2.63 ± 0.16, 2.81 ± 0.02, 2.61 ± 0.07, and 2.36 ± 0.21 mg/g from 15 to 55-day with 5-day incubation. However, the minimum flavonoid content in both parts has been reported at 50 days and 55 days, which indicates that the flavonoid content decreases with time. This implies that the response to flavonoid content depends on the body part and the incubation time. Consequently, the flavonoid content of the mixture, indicated that after 25 days, the flavonoid content decreased gradually. Similarly, the minimum flavonoid content in both body parts was reported after 25-day induction. These results suggest that (ns: no statistical difference, $p < 0.05$, $p < 0.01$, 0.001, $p < 0.0001$) at 25 days the mixture of body parts had a higher flavonoid content than the mycelium body. The structures of these flavonoids are shown in Fig. 11 (4).

Determination of the adenine content

The results obtained from Figs. 8 and 9 verify that the 25-day 0.1623 ± 0.0006 mg/g mixture and mycelium 0.1294 ± 0.0005 mg/g showed the maximum adenine content in both parts of *C. fumosorosea*. The adenine content mixture part is 0.1358 ± 0.0033, 0.1590 ± 0.0007, 0.1623 ± 0.0006, 0.1318 ± 0.0013, 0.1287 ± 0.0008, 0.1256 ± 0.0009, 0.1223 ± 0.0007, 0.1204 ± 0.0004 and 0.1152 ± 0.0031 mg/g and in mycelium part, 0.1194 ± 0.0006, 0.1216 ± 0.0011, 0.1294 ± 0.0005, 0.1207 ± 0.0008, 0.1129 ± 0.0013, 0.1100 ± 0.0008, 0.1032 ± 0.0010, 0.0982 ± 0.0044 and 0.0817 ± 0.0014 mg/g from 15 to 55-day with 5-day incubation. However, the minimum adenine content in both parts has been reported under 50 days and 55 days, which indicates that the adenine content decreases with time. This implies that the response of adenine content depends on the body part and incubation time. The adenine content of the mixture is shown in Figs. 8 and 9,

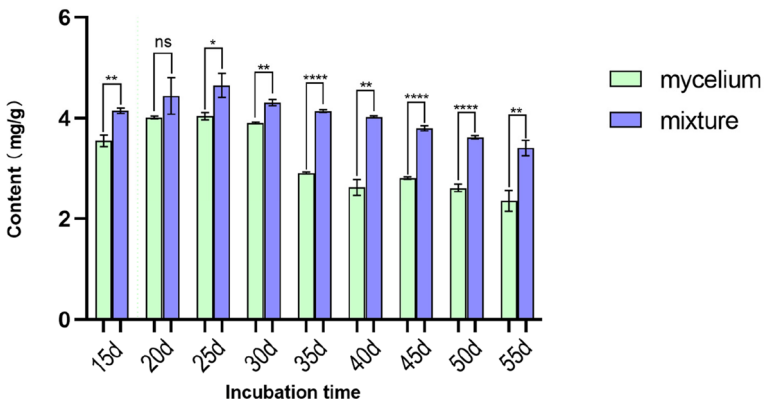

Fig. 7 Flavonoid content of mycelium and mixture, the error bars indicate the mean and ± standard error, as shown in the bar graph, ns: no statistical difference, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

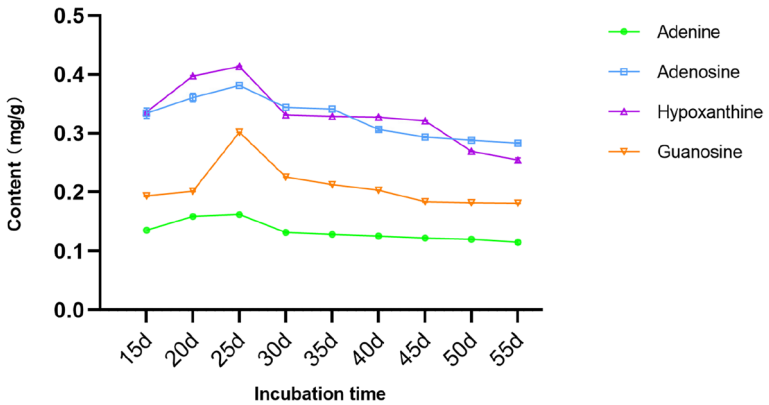

Fig. 8 The variation trend of four nitrogen-containing compounds in a mixture

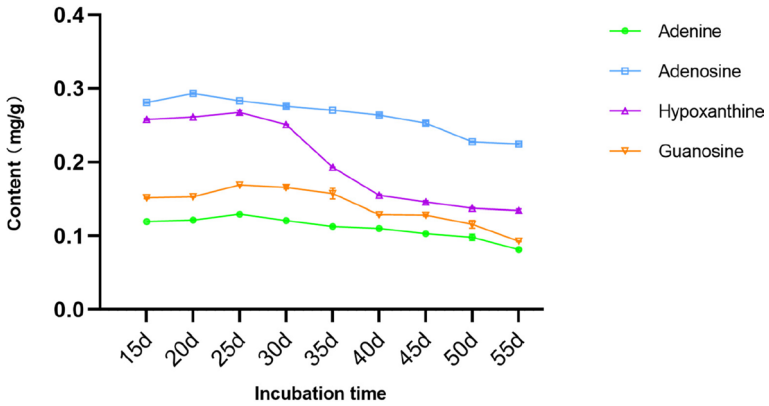

Fig. 9 The variation trend of four nitrogen-containing compounds in mycelium

indicating that after 25 days, the adenine content decreased gradually. Similarly, the minimum adenine content in both body parts was reported after 25-day induction. The results suggested that at 25 days the mixture of body parts had a higher adenine content than the mycelium body. The structure of adenine is shown in Fig. 11 (5).

Determination of the adenosine content

The results obtained from Figs. 8 and 9 verify that the 25-day 0.3811 ± 0.0008 mg/g mixture and mycelium 0.2811 ± 0.0008 mg/g showed the maximum adenosine content in both parts of *C. fumosorosea*. The adenosine content mixture part, 0.3339 ± 0.0087, 0.3605 ± 0.0073, 0.3811 ± 0.0008, 0.3440 ± 0.0005, 0.3411 ± 0.0004, 0.3063 ± 0.0025, 0.3934 ± 0.0012, 0.2883 ± 0.0015 and 0.2832 ± 0.0011 mg/g, and in the mycelium part 0.2808 ± 0.0010, 0.2932 ± 0.0012, 0.2811 ± 0.0008, 0.2757 ± 0.0020, 0.2705 ± 0.0002, 0.2639 ± 0.0017, 0.2528 ± 0.0024, 0.2275 ± 0.0009 and 0.2245 ± 0.0013 mg/g from 15 to 55-day with 5-day incubation. However, the minimum adenosine content in both parts has been reported under 50 days and 55 days, which indicates that the adenosine content decreases with time. This implies that the response to adenosine content depends on body part and incubation. Consequently, the adenosine content of the mixture shown in Figs. 8 and 9 indicates that after 25 days, the adenosine content decreased gradually. Similarly, the minimum adenosine content in both body parts was reported after 25-day induction. The results suggested that after 25 days and mixture, body parts had higher adenosine content than the mycelium body. The structure of adenosine is shown in Fig. 11 (6).

Determination of the hypoxanthine content

As shown in Figs. 8 and 9 verified that the 25-day 0.4135 ± 0.0007 mg/g mixture and mycelium 0.2675 ± 0.0026 mg/g showed the maximum hypoxanthine content in both parts of *C. fumosorosea*. The hypoxanthine content mixture part is 0.3361 ± 0.0023, 0.3973 ± 0.0013, 0.4135 ± 0.0007, 0.3312 ± 0.0004, 0.3287 ± 0.0005, 0.3275 ± 0.0005, 0.3211 ± 0.0006, 0.2692 ± 0.0007 and 0.2545 ± 0.0035 mg/g and in the mycelium part, 0.2577 ± 0.0012, 0.2613 ± 0.0005, 0.2675 ± 0.0026, 0.2508 ± 0.0005, 0.1928 ± 0.0005, 0.1550 ± 0.0011, 0.1457 ± 0.0017, 0.1376 ± 0.0011 and 0.1344 ± 0.0021 mg/g from 15 to 55-day with 5-day incubation. However, the minimum hypoxanthine content in both parts has been reported under 50 days and 55 days, which indicates that the hypoxanthine content decreases with time. This implies that the response to hypoxanthine content depends on body part and incubation. Consequently, the hypoxanthine content of the mixture shown in Figs. 8 and 9 indicates that after 25 days, the hypoxanthine content decreased gradually. Similarly, the minimum hypoxanthine content in both body parts was reported after 25-day induction. The results suggested that the 25-day mixture body parts had a higher hypoxanthine content than the mycelium body part. The hypoxanthine structure is shown in Fig. 11 (7).

Determination of the guanosine content

The results obtained from Figs. 8 and 9 verify that the 25-day 0.3017 ± 0.0004 mg/g mixture and mycelium 0.1688 ± 0.0007 mg/g showed the maximum guanosine content in both parts of *C. fumosorosea*. The various guanosine content in the mixture part are 0.1935 ± 0.0011, 0.2014 ± 0.0006, 0.3017 ± 0.0004, 0.2258 ± 0.0006, 0.2127 ± 0.0006, 0.2032 ± 0.0005, 0.1834 ± 0.0005, 0.1818 ± 0.0004 and 0.1810 ± 0.0005 mg/g, and in the mycelium part, 0.1513 ± 0.0012, 0.1529 ± 0.0007, 0.1688 ± 0.0007, 0.1656 ± 0.0022, 0.1572 ± 0.0072, 0.1288 ± 0.0005, 0.1281 ± 0.0015, 0.1157 ± 0.0052 and 0.0929 ± 0.0008 mg/g from 15 to 55-day with 5-day incubation. However, the minimum guanosine content in both parts was reported at 50 days and 55 days, which indicates that the guanosine content decreases with time. This implies that the response of guanosine content depends on the body part and

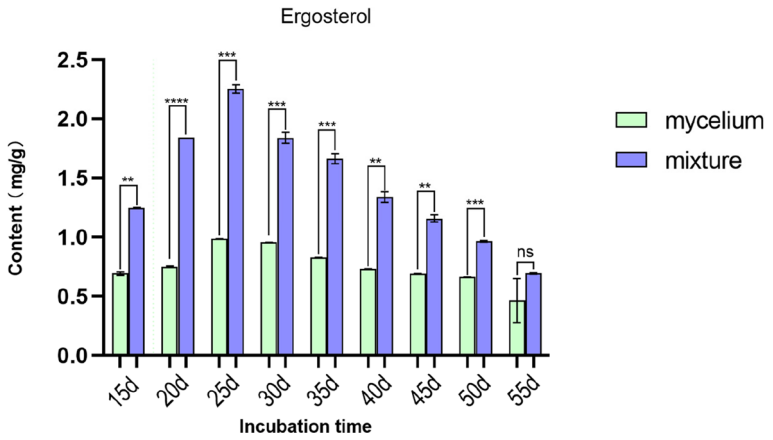

Fig. 10 Ergosterol content of mycelium and mixture, the error bars indicate the mean and ± standard error, as shown in the bar graph, ns: no statistical difference, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

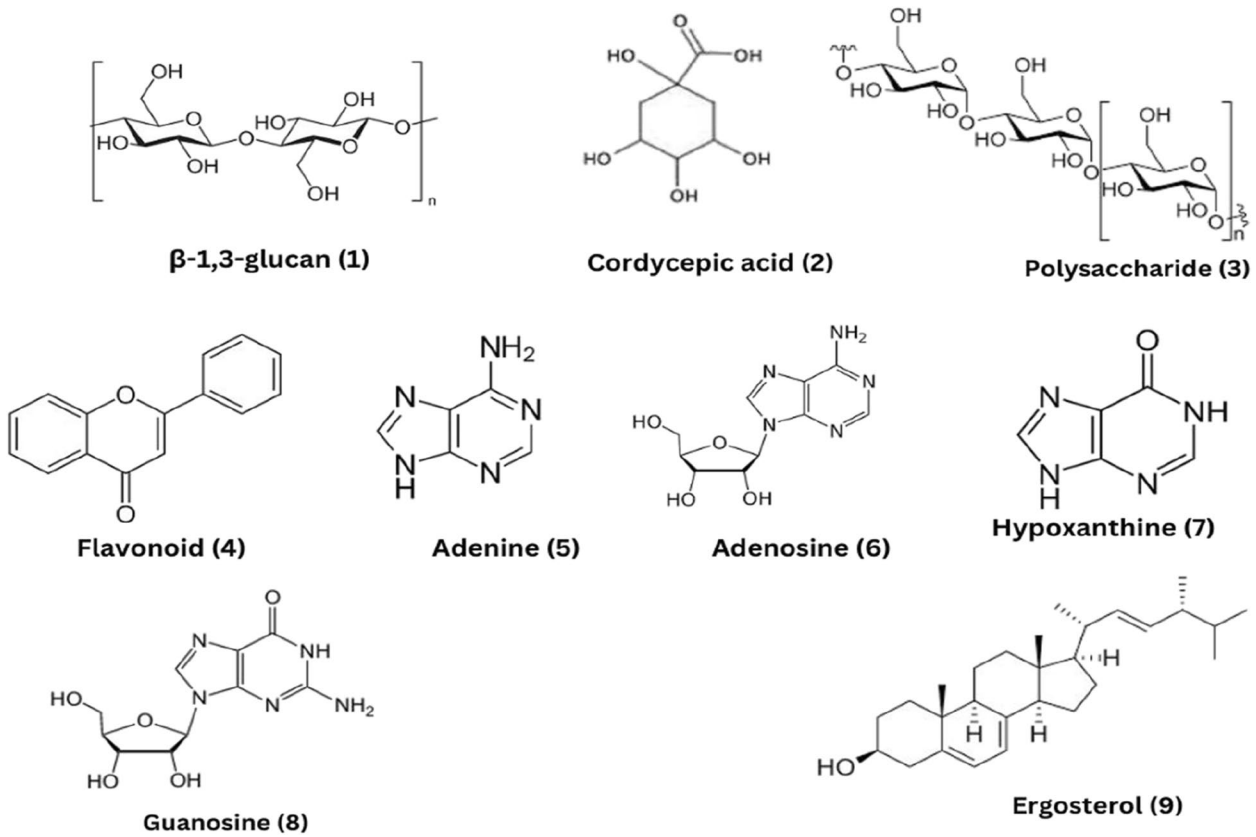

Fig. 11 Chemical structures of compounds 1–9 that were found in *C. fumosorosea* culture on *P. americana medium*

incubation time. Consequently, the guanosine content of the mixture shown in Figs. 8 and 9 indicated that after 25 days, the guanosine content decreased gradually. Similarly, the minimum guanosine content in both body parts was reported after 25-day induction. The results suggest that on 25 days, the mixture of body parts had a higher guanosine content than that in the mycelium body part. The guanosine structure is shown in Fig. 11 (8).

Determination of the ergosterol content

The results verified that the 25-day 2.25 ± 0.04 mg/g mixture and mycelium 0.99 ± 0.04 mg/g showed the maximum

ergosterol content in both parts (Fig. 10). The various content of ergosterol content in the mixture part are 1.25 ± 0.00, 1.84 ± 0.00, 2.25 ± 0.04, 1.84 ± 0.04, 1.66 ± 0.04, 1.34 ± 0.04, 1.16 ± 0.03, 0.96 ± 0.01 and 0.69 ± 0.01 mg/g, and in the mycelium part, 0.69 ± 0.02, 0.75 ± 0.03, 0.99 ± 0.04, 0.95 ± 0.00, 0.83 ± 0.05, 0.73 ± 0.03, 0.69 ± 0.03, 0.66 ± 0.02 and 0.56 ± 0.01 mg/g from 15 to 55-day with 5-day incubation. However, the minimum ergosterol content in both parts has been reported under 50 days and 55 days, which indicates that the content decreases with time. This implies that the response of ergosterol content depends on body part and incubation. Consequently, the ergosterol content of the mixture, indicates that after 25 days, the ergosterol content decreased gradually. Similarly, the minimum ergosterol content in both body parts was reported after 25-day induction. The results suggested that (ns: no statistical difference, $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$) at 25 days, the mixture body parts had a higher ergosterol content than the mycelium body part. The ergosterol structure is shown in Fig. 11 (9).

Discussion

In this study, we investigated the presence and levels of various bioactive compounds, including β-1,3-glucan, polysaccharides, cordycepic acid, flavonoids, nucleosides, and ergosterol in *C. fumosorosea* mixtures and mycelia at different time incubation (days). These results provide valuable insights into the optimal time points for obtaining the maximum content of these compounds. β-1,3-glucan are carbohydrates found in diverse organisms. Figure 11 shows compound structures with medicinal properties, including cancer and diabetes prevention and treatment. They lower cholesterol levels, exhibit antiviral activity against HIV, and enhance the immune system (Rahar et al. 2011). *Cordyceps* species were analyzed for glucan content. The mycelium contained 64.8 g/100 g of glucan, whereas pure *Cordyceps* capsules had 65.5 g/100 g of glucan. *Aspergillus niger* mycelium control contained 51.9 g/100 g glucan (49% β-glucan) (McCleary and Anna 2016). The analysis revealed that the highest β-1,3-glucan content was observed in the *C. fumosorosea* mixture (*C. fumosorosea* + *P. americana*) on 25 days, with a content of 69.21 ± 0.07 mg/g. In contrast, mycelia showed the highest β-1,3-glucan content after 25 days, with a content of 56.32 ± 0.39 mg/g. These findings suggest that the production of β-1,3-glucan in both the mixture and mycelia might follow a temporal pattern, with an increase in content up to a certain point and a subsequent decrease. It is important to note that the extraction solvent used also influenced the β-1,3-glucan, as different solvents yielded different levels of this compound at specific times incubation. Cordycepic acid, another bioactive compound of interest, exhibited a similar trend. To measure β-1,3–1,6-glucan content in the mycelium and synnemata of *C. fumosorosea*, values of 0.41–3.28 g/100 g for mycelia and 1.92–12.91 g/100 g dry mass for synnemata were used. Significant variations were observed between species, with up to 87.5% difference in mycelia and 85% difference in fruiting bodies. *Pleurotus ostreatus* (mushroom) had a higher glucan content than *P. pulmonarius*. Some species from *Marasmiaceae* displayed similar glucan contents in both mycelia and fruiting bodies. Morchella esculenta was present in comparable amounts. Glucan content did not correlate definitively with order, family, or species. Fruiting bodies generally have a higher glucan content than mycelia (Nitschke et al. 2011). *Cordyceps* species contain cordycepic acid, which has various pharmacological activities (Do and Nguyen 2019). The content of cordycepic acid peaked at 52.28 ± 0.11 mg/g in the mixture and at 46.96 ± 0.13 mg/g in the mycelium after 25 days. These results indicate that the optimal time for cordycepic acid production in both the mixture and mycelium was around the 20–25-day mark. The presence of cordycepic acid in *C. fumosorosea* indicated its potential therapeutic and pharmacological properties. *C. jiangxiensis* had the highest cordycepic acid yield, accounting for 11.81% of its dry weight (Do and Nguyen 2019). *Cordyceps* samples had polysaccharide content ranging from 3 to 8% of the mushroom's total weight. *C. militaris* fruiting bodies contained around 104.19 ± 0.57 mg/g polysaccharides, while mycelium had approximately 49.21 ± 0.80 mg/g (Yue et al. 2012; Huang et al. 2015). Polysaccharides, a group of complex carbohydrates with various biological activities, were also analyzed in this study. Polysaccharides derived from mushrooms act as antioxidants, anti-tumor agents, and immunological compounds (Nie et al. 2013). The highest polysaccharide content was found in the mixture after 20 days (16.68 ± 0.38 mg/g) and in the mycelium after 20 days (14.85 ± 0.10 mg/g). These findings highlight the importance of the 20-day time point for polysaccharide production in both the mixture and mycelia of *C. fumosorosea*. Flavonoid content, known for its antioxidant and anti-inflammatory properties, peaked in the mixture after 25 days (4.65 ± 0.24 mg/g) and in the mycelium after 25 days (4.04 ± 0.07 mg/g). It was suggested that a 25-day interval was critical for obtaining high levels of flavonoid in *C. fumosorosea*. The nucleosides adenine, adenosine, hypoxanthine, and guanosine were also analyzed in this study. Adenine constituted 0.71% w/w of the Chinese caterpillar fungus (*O. sinensis*), whereas adenosine was absent. Hypoxanthine content was investigated, with an *O. sinensis* cultured substitute containing 12.6 μg/g and *C. militaris* having undetectable levels (Khan et al. 2015; Huang et al. 2003). The highest levels of these nucleosides were observed after 25 days in both the mixture and the mycelium. Guanosine has the highest content among the

nucleosides and derivatives of *Cordyceps* (Yue et al. 2012). The adenosine content in *O. sinensis* varied between 0.28 and 14.15 mg/g (Li et al. 2008). The guanosine content in *C. cicadae* coremia ranged from 351.44 μg/g to 1,483.06 μg/g, while in the sclerotium (compact mass of hardened fungal mycelium containing food reserves), it ranged from 260.00 μg/g to 978.12 μg/g (Zeng et al. 2014). These findings suggest that the 25-day time point is favorable for nucleoside accumulation in *C. fumosorosea*. Furthermore, ergosterol, a precursor of vitamin D and an important marker of fungal biomass showed the highest content at 25 days. This indicates that the 25-day time point is optimal for ergosterol accumulation in *C. fumosorosea*. Ergosterol content in *Cordyceps* mycelia was reported as 1.44 mg/g, lower than the levels in fruiting bodies (10.68 mg/g) (Yue et al. 2012). The drying methods minimally affected cordycepin content, except for hot drying at 70 °C, which yielded approximately 0.18–0.21 mg/g (Li et al. 2019). *Cordyceps militaris* and *O. sinensis* share nucleoside sequences. Although their chemical compositions are similar, *C. militaris* contains higher levels of certain unique functional components than *O. sinensis*. For instance, the amount of adenosine in *C. militaris* is 34 times greater than that in *O. sinensis*, and the total nucleoside content in *C. militaris* is 5.25 times higher than that in *O. sinensis* (Zhang et al. 2003).

Future direction

Future studies should explore the specific health benefits and potential applications of these bioactive compounds in *C. fumosorosea*. Additionally, investigations should focus on optimizing cultivation conditions and extraction methods to maximize the production of these compounds. Further studies are needed to investigate the mechanisms of action of these compounds and their potential therapeutic effects on various health conditions.

Conclusion

This study concluded that the mixture and mycelia of *C. fumosorosea* harvested after 20 and 25 days was optimal for detecting a variety of bioactive compounds such as β-1,3-glucan, polysaccharides, cordycepic acid, flavonoids, ergosterol, and nucleosides (adenosine, guanosine, adenine, and hypoxanthine). This study found that the highest content of bioactive compounds was observed at specific incubation times (20 and 25 days). The highest β-1,3-glucan content was observed in the mixture after 25 days, while the mycelium had the highest β-1,3-glucan content after 20 days. The cordycepic acid content peaked in the mixture after 20 days and in the mycelium after 25 days. Similarly, other compounds, such as polysaccharides, flavonoids, and nucleosides, reached their highest levels at specific times of incubation. This experiment suggests that the mixture and mycelium of *C. fumosorosea* harvested at the aforementioned time of incubation could provide a rich source of bioactive compounds with potential health benefits.

Acknowledgements
The authors would like to acknowledge the technical support provided by the Yunnan Fungal Culture Collection (YFCC), Yunnan Herbal Laboratory (YHL), and the School of Life Science at Yunnan University.

Authors' contributions
Tahir Khan and Dr. Prof. Hong Yu (authors) contributed to the study's conception and design. The paper was written by Tahir Khan, grammar-checked by Dong-Hai Hou, and reviewed by Jin-Na Zhou and Yin-Long Yang, and supervised by Dr. Prof. Hong Yu. All authors have read and approved the final manuscript.

Funding
This study was supported by the National Natural Science Foundation of China (Grant No.31870017).

Availability of data and materials
All processed data used in this study can be obtained from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The experiments were performed at the Yunnan Herbal Laboratory and were approved by the Experimental Ethics Committee of the School of Life Science, Yunnan University (R-06202032). This article does not contain any studies with human participants performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 14 November 2023 Accepted: 19 January 2024
Published online: 05 February 2024

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